

# 14

## DEGRADATION OF LIGNIN

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### I. INTRODUCTION

Lignin is the predominant renewable source of aromatic carbon on earth. On land, where an estimated 65% of biomass production occurs (1), lignin probably rivals cellulose, the most abundant renewable organic resource, in reduced carbon and photosynthetic energy content. Lignin and cellulose, together with the hemicelluloses, occur as the structural components of the vascular tissues of higher land plants. Quantitatively, biodegradation of such woody tissues (lignocellulosics) is by

far the most important process in the recycling of terrestrial biosynthetic carbon. In intact woody tissues, however, lignin is present as a physical barrier which protects the cellulose and hemicelluloses from the degradative enzymes of microbes. Such lignified tissues are not significantly degraded by many potentially polysaccharolytic microbes—the much-studied *Trichoderma reesei*, for example—which are unable to degrade lignin or fully lignified tissues. For this reason, the biodegradation of lignin has assumed through evolution a disproportionate and central importance in the cycling of terrestrial carbon,

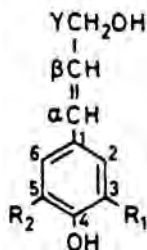
This chapter presents an overview of the process of lignin biodegradation. An attempt has been made to provide a coordinated interpretation based on the current understanding, which is rapidly improving but still quite incomplete. Emphasis is placed on the chemistry, biochemistry, and physiology of lignin degradation by higher fungi. Recent reviews (2-7,164) should be consulted for more comprehensive treatments of other aspects.

## II. DISTRIBUTION AND STRUCTURE OF LIGNIN

Lignin is found in higher plants, including ferns, but not in liverworts, mosses, or plants in lower taxa (8-10). It comprises from less than 1% to over 35% of the dry weight of the plant tissues, being in highest concentration in the compression wood of gymnosperms such as pines, spruces, and firs. Woody tissues generally contain 20-30% lignin.

In wood, and presumably in the lignified vascular tissues of non-woody plant materials, most of the lignin (~80%) is found within the cell walls. There it is intimately interspersed with the hemicelluloses, forming a matrix which surrounds the orderly cellulose microfibrils. About 20% of the lignin is in the intercellular areas—the middle lamella and "cell corner" regions—where it acts as a cement binding cells together. Lignin is the dominant constituent (>50%) in this part of woody tissues. For a more complete description of the distribution of lignin within woody tissues the reader is referred to papers by Goring and co-workers (11,12).

Lignin is derived from phenylalanine, and in grasses such as bamboo and wheat, from both phenylalanine and tyrosine, which are synthesized from sugars via the shikimic acid pathway. Deamination, ring hydroxylation, phenolic methylation, and carboxyl reduction steps lead to the immediate cinnamyl alcohol precursors of lignin, p-coumaryl [1], coniferyl [2], and sinapyl [3] alcohols (13-18). The relative proportions of these precursors incorporated into lignin vary with the plant species (14,15,19,20), with the tissues, and even within tissues (19). However, in general, gymnosperm lignin is made from coniferyl alcohol, and angiosperm lignin from mixtures of coniferyl and sinapyl alcohols [20-60% of the latter (21)]. In most lignins, small proportions (Usually



- [1]  $R_1 = R_2 = H$
- [2]  $R_1 = OCH_3, R_2 = H$
- [3]  $R_1 = R_2 = OCH_3$

less than 10%) of the units derive from *p*-coumaryl alcohol. Understanding of the biodegradation of lignin has not progressed to the point where the significance of variation of lignin composition is known, but it is unlikely that the chemistry and biochemistry involved in angiosperm and gymnosperm lignin biodegradations differ in principle.

Lignin is made by the oxidative polymerization of the cinnamyl alcohol precursors [1] - [3]. Single electron oxidation of the phenolic hydroxyls in these precursors within the lignifying cell wall produces radical species which exist in mesomeric forms ( $[R_a]$  -  $[R_d]$ , Fig. 1). These couple essentially randomly with each other, but primarily with radicals in the growing lignin polymer, which contains phenolic hydroxyls and is itself a substrate for single electron oxidation (14,15,18,20, 21). Figure 2 illustrates formation of a trimeric segment, comprised of the two most frequent types of interunit linkage, the  $\beta$ -Q-4 (6-aryl ether) and  $\beta$ -5 (phenylcoumaran) substructures. (The word "substructure" (22) is convenient for referring to two-unit segments containing specific types of interunit linkage.) All of the mesomeric forms,  $[R_a]$ - $[R_d]$ , are involved in coupling, and all of the possible coupling

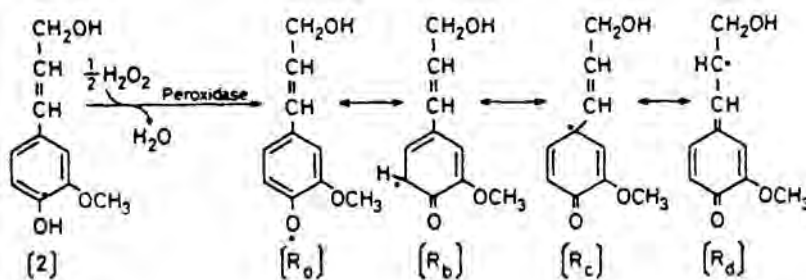


Figure 1 Single electron oxidation (peroxidase/H<sub>2</sub>O<sub>2</sub>) of coniferyl alcohol. (From Ref. 21.)

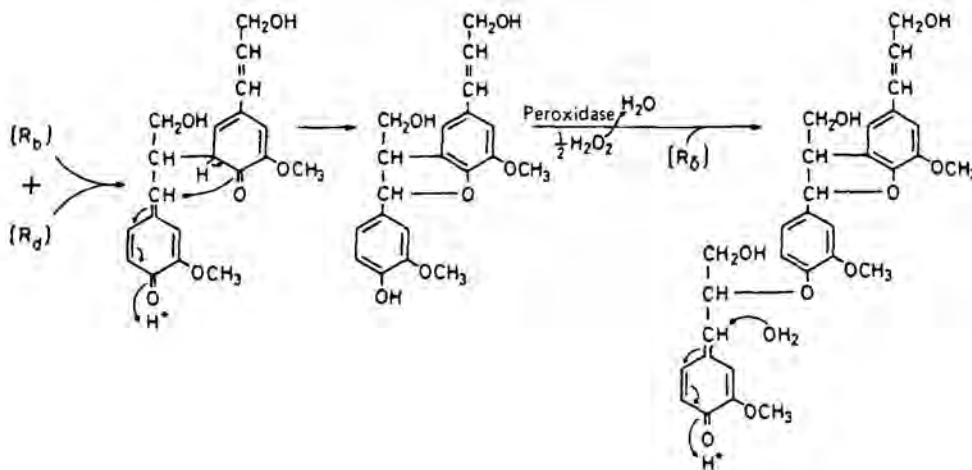


Figure 2 Oxidative coupling of three coniferyl alcohol units to form a trimeric segment of lignin, illustrating the important  $\beta-5$  and  $\beta-Q-4$  linkage types.

modes have been evidence in lignin (except  $[R_a]-[R_a]$  and  $[R_c]-[R_c]$  (21). Because the coupling reactions do not take place on an enzyme surface, lignin and its chemical degradation products are optically inactive. Most of the linkage types are not hydrolyzable.

During the formation of lignin, nucleophilic addition to the important quinone methide intermediates usually involves water (Fig. 2), resulting in formation of benzyl alcohol groups. However, the nucleophile can also be phenolic hydroxyls in lignin, producing " $\alpha$ -aryl ether" linkages. Alcohol or carboxyl groups in hemicelluloses might also serve as nucleophiles, producing ether or ester linkages between lignin and carbohydrates (e.g., ref, 23). Although lignin-hemicellulose linkages are known to exist, their frequency and exact structures remain to be elucidated (21).

Further complexity in lignin is introduced by rearrangement of certain of the structures formed by radical coupling; a portion of the product of  $R_c-R_d$  coupling rearranges to form  $C_5-C\beta$  linkages. Also, in the lignin of grasses and aspen wood, ester linkages occur between the carboxyl groups of *p*-coumaric (grasses) or *p*-hydroxybenzoic (aspen) acids and, primarily, the  $\gamma$ -hydroxyl groups of lignin; formation is probably via specific enzymatic catalysis, and might occur before polymerization (24,25). Similar esters probably occur in other plants.

Figure 3 illustrates various structural features in a schematic formula of aspen lignin. The proportions of the various types of substructure are not quantitatively representative, but Table 1 lists the frequencies of the main substructures in gymnosperm and angiosperm

lignins, as deduced analytically (21,26,27); the substructures are referenced to Fig. 3.

The structural features of lignin dictate some unusual constraints on lignin biodegradative systems, most obviously perhaps a requirement for nonspecificity or unusual complexity of the enzymes system, and the probability that hydrolases can only be of secondary importance. Nonhydrolytic, polymer-degrading enzymes must be extracellular. The system must differ fundamentally from those involved in the biodegradation of all the other important biopolymers, and meaningful analogies cannot readily be drawn with the other systems.

### III. MICROBIOLOGY OF LIGNIN DEGRADATION

Table 2 provides an overview of the groups of microbes shown to degrade (metabolize or significantly alter) lignin.

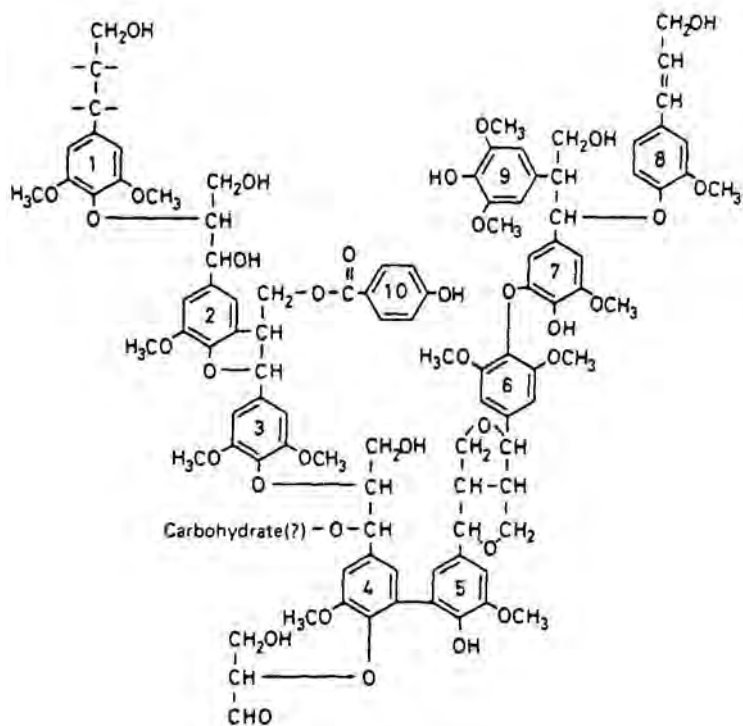


Figure 3 Schematic formula for a portion of aspen lignin, qualitatively illustrating important interunit linkage types.

Table 1 Frequencies of the Major Substructures in Representative Gymnosperm and Angiosperm Lignins

Substructure	Units in Fig. 3	Proportions (% of total C <sub>9</sub> -units)	
		Spruce	Birch
$\beta$ -O-4 ( $\beta$ -aryl ether)	1-2,3-4	48	60
$\beta$ -5 (phenylcoumaran)	2-3	9-12	6
5-5 (biphenyl)	4-5	9.5-11	4.5
$\alpha$ -O-4 ( $\alpha$ -aryl ether)	7-8	6-8	6-8
$\beta$ -1 (1,2-diarylpropane)	7-9	7	7
4-O-5 (diphenyl ether)	6-7	3.5-4	6.5
$\beta$ -O-4 (glyceraldehyde-2-aryl ether)	4	2	2
$\beta$ - $\beta$ (resinol-type)	5-6	2	3
Total		87-94	95-97

Source: Refs. 21, 26, and 27.

Anaerobic metabolism of unaltered lignin apparently does not occur (41), despite early reports that it does. Intact wood is not degraded anaerobically. This resistance of lignin and Lignified tissues under anaerobic conditions might account for coal deposits, and indeed, evidence for a lignin origin of coal has been presented (42). The apparent absence of lignin metabolism in the absence of oxygen is related to molecular size; various low molecular weight aromatic compounds (Chap. 16), and aromatic fragments produced by alkaline degradation of lignin (43), are readily metabolized anaerobically. The initial fragmentation of lignin evidently requires molecular oxygen. Interestingly, degradation of lignin by certain fungi is very sensitive to oxygen concentration, and does not occur at low concentrations which still permit good growth (44,45).

#### A. Lignin in Wood

As pointed out in the introduction, many microbes able to metabolize isolated wood cellulose and hemicelluloses but unable to degrade lignin cannot degrade intact wood, which can be attributed to physical protection of the polysaccharides by lignin (46,47). Evidence

Table 2 Lignin-Degrading Microorganisms

Microbe classification and examples (genera)	Lignin substrate used experimentally	Methods used to determine lignin degradation <sup>a</sup>	Reference(s)
Actinomycetes			
Soil bacteria			
<i>Nocardia</i> , <i>Streptomyces</i>	synthetic lignin; corn stalks; Douglas-fir phloem	A	(28,29)
Fungi Imperfecti			
Soil fungi			
<i>Fusarium</i>	synthetic lignin	A,B	(30,154)
Soft-rot fungi			
<i>Papulospora</i>	wood blocks	C	(31)
Ascomycetes			
Soft-rot fungi			
<i>Chaetomium</i>	wood blocks; synthetic lignin; corn stalks	A,C	(31-33)
Pseudo soft-rot fungi <sup>b</sup>			
<i>Hypoxylon</i> , <i>Xylaria</i>	wood blocks	C	(34,35)
Basidiomycetes			
Litter-degrading			
<i>Collybia</i> , <i>Mycena</i>	forest litter	C	(36)
White-rot			
<i>Coriolus</i> , <i>Phanerochaete</i> , <i>Poria</i>	wood; synthetic lignin	A,C,D	(37-39)
Brown-rot			
<i>Gloeophyllum</i> , <i>Poria</i>	wood; synthetic lignin	A,C,D	(33,36-39)

<sup>a</sup>A = [<sup>14</sup>C]lignin + CO<sub>2</sub>; B = growth on lignin; C = mass balance study based on gravimetric determination of lignin; D = chemical structural studies.

<sup>b</sup>Decay by these and related fungi is slow and exhibits features of both white- and soft-rots (35).

suggests that the polysaccharides do not protect lignin from ligninolytic enzymes (48,49). It follows, therefore, that ability to cause weight loss in intact wood, beyond the few percent in nonstructural "extractive" components, indicates an ability to degrade lignin.

Many different bacteria and fungi can be isolated from rotting wood, and numerous studies utilizing pure cultures have been conducted to assess their abilities to cause weight loss. These investigations have clearly established only three kinds of decay, all caused by fungi (50): (a) white-rot (Basidiomycetes), in which all wood components are metabolized more or less simultaneously; (b) soft-rot (Ascomycetes, Fungi Imperfecti), a slow deterioration in which the polysaccharides and lignin are metabolized, but the lignin at a lower rate than the carbohydrates; and (c) brown-rot (Basidiomycetes), in which the polysaccharides are removed and the lignin altered and left as a brown residue.

Various microscopic studies have revealed localized cell wall erosion and destruction of pit membranes by bacteria. However, bacterial wood decays are not known; the occasional reports of significant weight losses resulting from bacterial attack have not been confirmed by follow-up studies. After extensive studies of bacterial attack of wood, Greaves (51) concluded that there is no direct evidence to implicate any particular species in breakdown of lignin in situ. Thus it appears likely that bacteria do not play a primary role in degrading the mature xylem tissues of woody plants. A similar conclusion was reached by Ander and Eriksson (3).

#### B. Other Lignin

If we turn our attention to tissues having a relatively low lignin content, such as those of herbaceous plants, most grasses, leaves, etc., and to isolated lignins studied experimentally, evidence can be found that members of a broader range of microbial taxa can degrade lignin.

Litter-degrading basidiomycetes, closely related to the white- and brown-rot fungi, were shown by Lindeberg (36) to decompose lignin in pure cultures; this work has recently been confirmed and evidence presented for a dominant role of these microbes in degrading litter in northern forests (52).

The metabolism of lignin by various *Fusarium* spp. (Fungi Imperfecti) has been reported recently (30). They were isolated from soils by enrichment techniques with synthetic lignins (dehydrogenative polymerizates; DHPs) as limiting carbon source, and evidence presented that high- as well as low-molecular-weight components were degraded. Demonstration that [ $^{14}\text{C}$ ]lignin is converted to  $^{14}\text{CO}_2$  would provide needed proof of lignin polymer-degrading ability. A *Fusarium* sp. isolated by Norris (154) caused only slow and limited conversion of synthetic [ $^{14}\text{C}$ ]lignin to  $^{14}\text{CO}_2$  (154). Decomposition of kraft lignin (a

heavily modified pulping by-product lignin), labeled with  $^{14}\text{C}$ , by *Aspergillus fumigatus* (53), and recent evidence for degradation of the lignin in wheat straw by several fungi (54), suggest that a closer look at various common soil fungi might reveal ligninolytic capabilities.

Unequivocal assays for biodegradation based on [ $^{14}\text{C}$ ]lignins are making it possible to assess the true abilities of various microorganisms to metabolize lignin, and should be used for definitive demonstration of ligninolytic activity (for review see Ref. 55).

Reviews by the author in 1971 (39) and 1977 (56) concluded that evidence for the bacterial degradation of lignin was equivocal and, as pointed out above, bacteria probably do not degrade lignin in wood. However, recent studies, employing experimental techniques based on [ $^{14}\text{C}$ ] lignins, point to at least partial degradation of certain other lignins by bacteria in several different genera (28,29,33,57,58; for review see Ref. 4). So far, reported rates and extents are much lower than with ligninolytic fungi. The most extensive bacterial degradation of lignin (15-25% of the lignin supplied) has been reported for the actinomycetes *Nocardia* (28) and *Streptomyces* (29). These studies have employed [ $^{14}\text{C}$ ]lignins in natural tissues labeled by feeding lignin precursors. Odier and Monties (59,60) have reported metabolism of 50% or more of chemically isolated lignins from wheat straw and poplar wood by *Xanthomonas*, *Pseudomonas*, *Acinetobacter*, and other bacteria. Evidence was presented that several strains could also degrade the lignin in thin slices of poplar wood. Experiments failed, however, to demonstrate significant release of  $^{14}\text{CO}_2$  from lignin-labeled poplar wood (Odier and Monties, personal communication).

In conclusion, evidence now indicates that many soil- and litter-inhabiting bacteria and fungi in pure culture can partly decompose lignin in nonwoody plant tissues as well as isolated lignins. In concert these microbes no doubt account for extensive, albeit perhaps slow (61,62), metabolism of lignin in some environments. Biodegradation assays based on [ $^{14}\text{C}$ ]lignins have made it feasible to evaluate microbial populations from various natural environments (53,55,61-63), as well as defined mixed cultures, for ligninolytic activity. No reports of the latter have yet appeared.

#### IV. CHEMISTRY OF THE FUNGAL DEGRADATION OF LIGNIN

The recent advances discussed above in establishing the abilities of members of various microbial taxa to degrade lignin should lead to studies of the chemistry, physiology, and biochemistry involved. At the present time, only the white-rot and brown-rot wood decay fungi have been examined. Consequently, the remainder of this chapter deals almost exclusively with them. The story of how lignin is degraded is far from complete, but considerable insight has been gained in recent years.

## A. The Lignin Polymer

### 1. *Brown-rot fungi*

As pointed out above, lignin is only slightly depleted during brown-rot of wood. (Circumvention of the lignin barrier probably involves an unusual nonenzymatic oxidation of the cellulose (see 50,64,155).) The residual lignin in brown-rotted wood has been termed "enzymatically liberated lignin," and was once advocated (65) as a source of lignin for research into lignin chemistry and reactions. Investigations since have shown, however, that brown-rotted lignin, while probably not significantly depolymerized, is enriched in oxygen and deficient in methoxyl, and that it has undergone changes in various functional groups (Table 3). Chemical characterization studies (40,66,69) revealed that demethylation of aryl methoxyl groups and aromatic hydroxylation are major reactions (Fig. 4). In these studies care was taken to purify the lignin and also to assure that only polymeric material was being studied. The fungal reactions, therefore, occurred in *in vivo* *in situ*. Demethylation as illustrated in Fig. 4A (40) occurs in both phenolic and nonphenolic units. Aromatic hydroxylation occurs specifically in the 2-position, as illustrated in Fig. 4B (69), but it has not been established whether the methoxyl at C-3 is demethylated prior to hydroxylation at C-2. These demethylation and hydroxylation reactions account for most of the increase in phenolic content and some of the increased oxygen content of brown-rotted lignin (Table 3). It is possible that the increased carboxyl and carbonyl contents (66) are the result of secondary, nonenzymatic reactions caused by autooxidation of the *o*-diphenols resulting from demethylation of units bearing 4-hydroxyl groups (Fig. 4A), or those resulting from hydroxylation at C-2 and demethylation at C-3. Such autooxidations probably cause the brown color of the lignin, and perhaps contribute to the elevated oxygen content. Limited conversion of side chain and aromatic carbon in [<sup>14</sup>C] labeled lignins to <sup>14</sup>CO<sub>2</sub> (33,70) shows that brown-rot fungi have some degradative capacities in addition to demethylation and aryl hydroxylation.

### 2. *White-rot fungi*

Although white-rot and brown-rot fungi are closely related taxonomically (156), and both groups decay wood, their effects on lignin, as well as on cellulose, differ (37,38,46,50,155). It is possible that both groups of fungi degrade lignin via the same reactions, but that brown-rot fungi are blocked in some reactions and cannot, like white-rot fungi, completely decompose the polymer.

Deciphering the effects of white-rot fungi on lignin is not proving to be as straightforward as with the brown-rot fungi, simply because their degradation is complete. It was originally thought, by analogy with other biopolymer-degrading systems, that lignin must be depolymerized to oligomers and eventually to monomers which are taken into

Table 3 Analytical Properties of Lignin Isolated and Purified from Spruce Wood Before and After Decay by Brown-Rot and White-Rot Fungi

Lignin	Formula for average C <sub>9</sub> -unit	Functional groups (moles /C <sub>9</sub> -unit)				
		Conjugated carbonyl	Total carboxyl	Hydroxyl		
				Phenolic	Aliphatic	Total
Sound	C <sub>9</sub> H <sub>8.66</sub> O <sub>2.75</sub> OCH <sub>3</sub> 0.92	0.07	0.10	0.24	0.92	1.16
Brown-rotted <sup>a</sup>	C <sub>9</sub> H <sub>8.44</sub> O <sub>3.75</sub> OCH <sub>3</sub> 0.61	0.14	0.23	0.58	0.78	1.36
White-rotted <sup>b</sup>	C <sub>9</sub> H <sub>7.70</sub> O <sub>3.80</sub> OCH <sub>3</sub> 0.72	0.16	0.58	0.10	0.77	0.87

<sup>a</sup>Wood decayed by *Gloeophyllum trabeum* (= *Lenzites trabea*) (66).

<sup>b</sup>Wood decayed by *Polyporus anceps* (67,68).

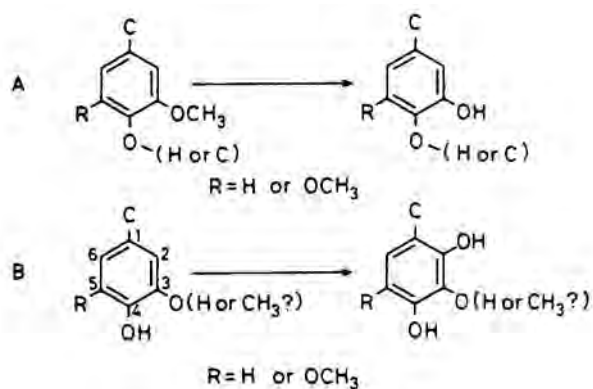


Figure 4 Demethylation of methoxyl groups (A) and hydroxylation of aromatic nuclei (B) are major reactions during degradation of lignin by the brown-rot fungus *Gloeophyllum trabeum* (from Refs. 40, 66, and 69.)

the fungal cells and metabolized. The picture, however, is not that simple.

Much of what is known about the chemistry of lignin metabolism by white-rot fungi has come from studies of white-rotted wood, of lignins isolated and purified from such wood, and of isolated lignins after incubation with the fungi (67,68,71-77). Results with various fungi and lignins are in good agreement. Analytical comparisons of sound and white-rotted *polymeric* lignins (Table 3) have shown that the degradative process is heavily oxidative. More detailed studies have shown that low molecular weight products of oxidative and hydrolytic chemical degradations are obtained in much lower yields from white-rotted than from sound lignins (68,70-72,75). Despite a considerably reduced methoxyl content, white-rotted lignin does not contain methoxyl-deficient units as seen in brown-rotted lignin (68,77). Detailed interpretation of the comparative analytical data for sound and white-rotted lignins from spruce led to the hypothesis (68) that degradation of the polymer occurs both in the side chains, which are oxidized with formation of  $\alpha$ -carbonyl and  $\alpha$ -carboxyl groups; and in the aromatic nuclei, which are oxidatively cleaved following demethylation in phenolic units to form 3,4-dihydroxyphenyl moieties. Further "erosion" of the ring-cleavage fragments presumably releases aliphatic products and frees new phenolic hydroxyl-bearing units for sequential degradation. Investigation of white-rotted lignin provided direct evidence for the side-chain oxidations (68,73), and provided indirect but compelling evidence for demethylation followed by cleavage of aromatic nuclei in the polymer (68).

Direct evidence for aromatic cleavage in the polymer has recently been obtained through the use of  $^{13}\text{C-NMR}$  spectroscopy. Ellwardt et al. (78) prepared synthetic lignins enriched with  $^{13}\text{C}$  at C-4 in the aromatic nuclei. The  $^{13}\text{C-NMR}$  spectrum exhibited several strong signals in the aromatic-C region (143-150 ppm). After incubation with the white-rot fungus *Pleurotus ostreatus*, the lignin was reisolated and the diethyl ether-insoluble product (presumed to be polymeric) was found to exhibit aliphatic signals from carbons derived from C-4 (e.g., a prominent peak at 92 ppm). Chua et al. (79), also using  $^{13}\text{C-NMR}$  spectroscopy, have identified alkoxyacetic acid moieties in polymeric degraded lignin isolated from spruce wood decayed by *Phanerochaete chrysosporium*. These moieties were almost certainly derived via aromatic ring cleavage.

This picture of degradation suggests that part of the aromatic carbon of lignin is released from the polymer in aliphatic products. However, it is not yet clear how important ring cleavage in the polymer and subsequent release of aliphatic products really are. Chua et al. (80) have shown recently that synthetic lignin is rapidly depolymerized when added to ligninolytic cultures of *P. chrysosporium*. It is unlikely that such depolymerization is the result primarily of aromatic ring cleavage. As discussed below, studies with nonphenolic substructure model compounds point to a predominance of initial side-chain cleavages. Such reactions probably account for the observed rapid depolymerization of Lignin in cultures. The fact that polymeric lignin containing ring-cleavage fragments is found in white-rotted wood suggests that accessibility within the woody matrix might play a role in determining whether attack is in the aromatic portions as well as in side chains. Lignin apparently is degraded only at the surfaces accessible to fungal hyphae within wood (81).

Release of low molecular weight aromatic products is observed during lignin degradation. Investigations several years ago showed that small amounts of vanillin and vanillic acid and several other aromatics are produced from lignin during degradation (72,82,83). Recent research has examined more closely the low molecular weight aromatics extractable from spruce wood partially decayed by the white-rot fungus *Phanerochaete chrysosporium*. Only the acidic component's have been investigated, although neutral products of low molecular weight as well as acidic and neutral oligomeric products have also been found (75,84). Several aromatic acids have been identified (e.g., [4]-[8], Fig. 5), vanillic acid [4] being by far the most abundant. Reference to Fig. 3 illustrates the substructural origins of these products from lignin (the extra methoxyl groups in the lignin structure are disregarded): [4] could arise from units 1, 3, or 6; [5] from unit 2 and perhaps units 4 or 5; [6] from unit 9; [7] from units 6-7; and [8] from units 4-5. Formation of these acids involved  $\text{C}_\alpha\text{-C}_\beta$  cleavages, with this either being oxidative, or being followed by  $\text{C}_\alpha$ -oxidation. These cleavages probably occurred in the polymer; see Sec. V.B.

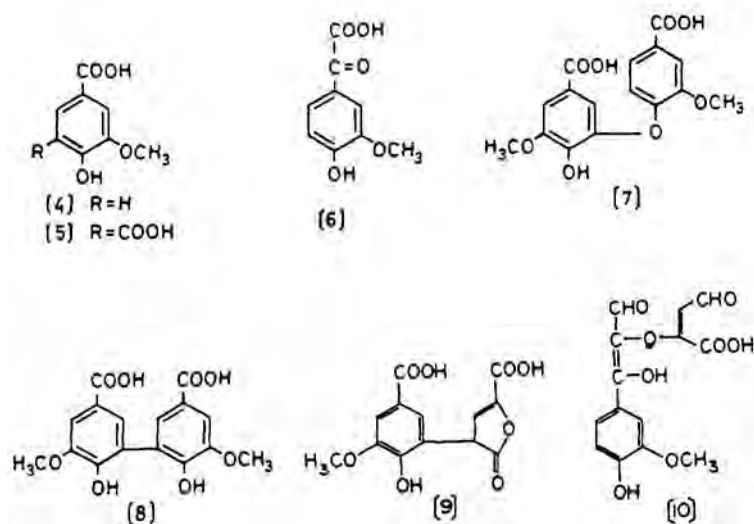


Figure 5 Carboxylic acids identified in extracts of white-rotted spruce wood. (From Refs. 84 and 159.)

More interesting than the aromatic acids in Fig. 5 were trace amounts of a variety of compounds consisting of aliphatic residues attached to aromatic nuclei (157). Structural assignments of 14 such compounds, based on high resolution mass spectrometry, cannot be considered final; however, the compounds are clearly lignin-derived aromatics with attached aliphatic moieties. Two tentatively identified structures, [9] and [10], are included in Fig. 5; these obviously arose from 5-5 (biphenyl) and  $\beta$ -O-4 ( $\beta$ -aryl ether) substructures. Whether the ring cleavages occurred in the polymer or in low molecular weight aromatic fragments cannot be deduced from the structures. It is probable, however, that at least some did occur in the polymer, because, as already discussed, demethylation and aromatic hydroxylations in the polymer have been proven with brown-rot fungi (66,69,70), and ring cleavage in the polymer by white-rot fungi almost certainly occurs, as discussed above.

Based on this reasoning, and on past work discussed above, it seems plausible to suggest that lignin is degraded by white-rot fungi primarily via a few key reactions: (a)  $C_{\alpha}$ - $C_{\beta}$  cleavage, which is perhaps a major reaction releasing both aliphatic and aromatic products, (b) demethylation of methoxyl groups, (c) hydroxylation at C-2 in aromatic nuclei, and (d) aromatic ring cleavage (intradiol 2,3- and 3,4-), with subsequent release of aliphatic products.  $\alpha$ -Carbon oxidation, detected in white-rotted lignin (68), and  $\gamma$ -carbon oxidation, indicated by the structures of compounds obtained from white-rotted wood and

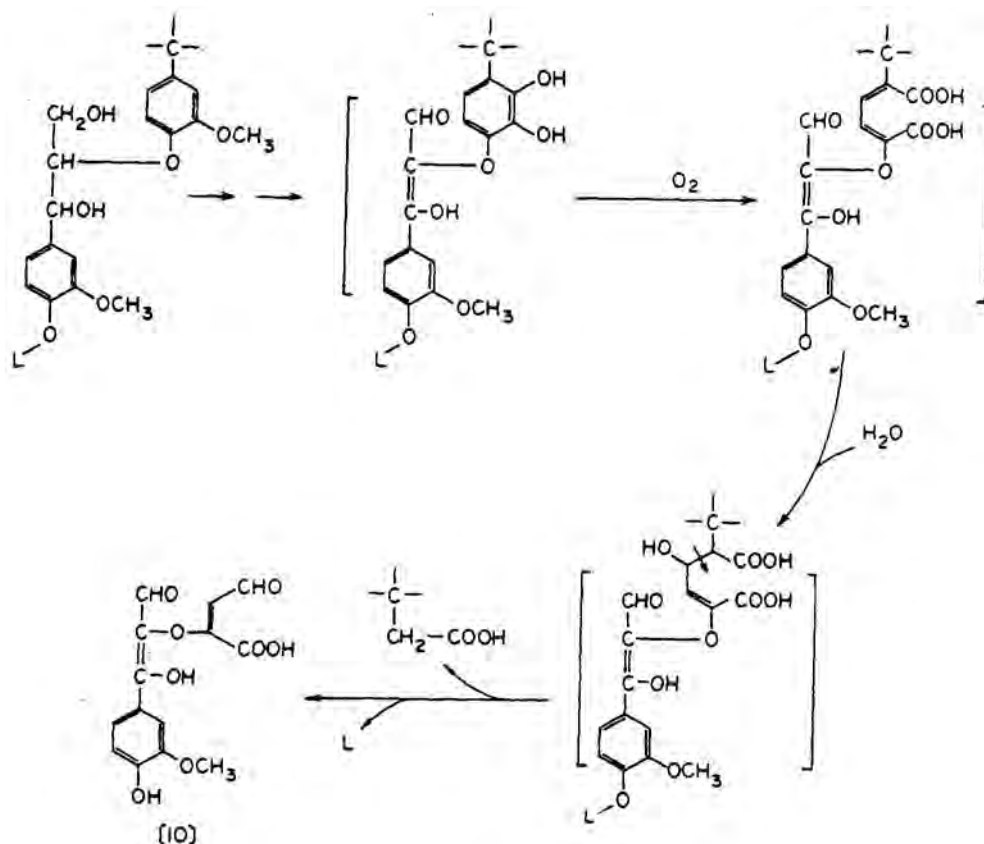


Figure 6 Hypothetical pathway for the formation of compound [10] from  $\beta$ -O-4 substructures in spruce lignin during degradation by *Phanerochaete chrysosporium*. (From Ref. 157.)

tentatively identified by mass spectrometry (84), might also be important. This picture suggests that each of the various lignin substructures is not degraded via a set pathway, but via many intermediate products along several different "pathways." Figure 6 outlines a plausible pathway for formation of compound 10 from a  $\beta$ -O-4 substructure in lignin. The correct view of lignin biodegradation probably is one of "principles" rather than "pathways"—just as it is principles of polymerization of lignin precursors rather than a pathway of polymerization that are involved in formation of the lignin polymer. Only further study will establish whether this view is correct, and if so, the detailed chemistry and biochemistry involved.

## B. Low Molecular Weight Compounds Related to Lignin

Working out the details of the specific reactions of lignin biodegradation, and of the biochemistry involved, will probably rely heavily on the use of low molecular weight compounds representing specific substructures, rather than on the complex and heterogeneous lignin polymer. Through the years lignin chemists have used "model compounds" to elucidate much of what is known about lignin chemistry and reactions. New and improved methods for the synthesis of lignin substructure models, including trimeric compounds, have been described by Nakatsubo and Higuchi (85-87).

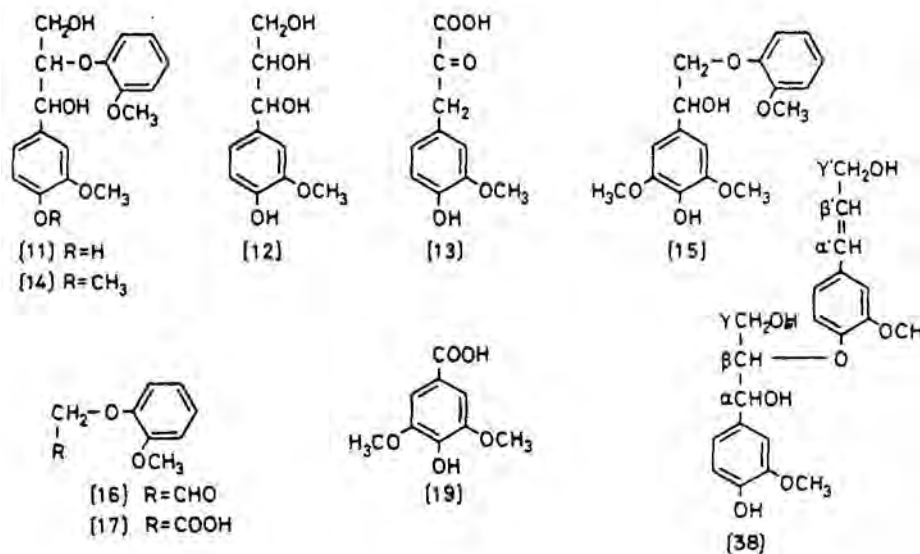
Despite its potential, study of the metabolism of lignin-related aromatics by known ligninolytic microbes had until recently received relatively little research attention. The scant literature was reviewed in 1971 (39) and in 1978 (3). Microbial metabolism of phenylpropanoids was reviewed by Subba Rao et al. (88), and more recently Cain (89) reviewed the microbial degradation of lignin-related aromatics. The following is an overview of transformations of lignin-related aromatics by known lignin-degrading fungi. Metabolism of lignin-related aromatics by the bacteria that have been reported to degrade lignin has not yet been studied, although metabolism of lignin-related compounds by other bacteria has been reported by many authors (see 3,89,90).

### 1. Substructure model compounds ("dimers")

Ishikawa et al. (91) reported vanillin, and vanillic acid [4] as products of metabolism of  $\beta$ -5 (phenylcoumaran),  $\beta$ - $\beta$  (resinol), and  $\beta$ -O-4 ( $\beta$ -aryl ether) substructure models by the white-rot fungi *Fomes fomentarius* and *Coriolus versicolor*. Intermediates of degradation, reported only for the  $\beta$ -Q-4 model, guaiacylglycerol- $\beta$ -(*q*-methoxyphenyl) ether [11], were guaiacol (*q*-methoxyphenol) and guaiacylglycerol [12]; the latter was reportedly further degraded via guaiacylpyruvic acid [13] to vanillin and vanillic acid [4].

Fukuzumi et al. (92) also studied degradation of a  $\beta$ -Q-4 model, veratrylglycerol- $\beta$ -(*q*-methoxyphenyl) ether [14], using a crude enzyme preparation from wood being decayed by the white-rot fungus *Poria subacida*. Again guaiacylglycerol [12] and guaiacol were reported products, the former compound indicating 4-O-demethylation as well as cleavage of the glycerol-aryl ether linkage. Neither Ishikawa and co-workers (91) nor Fukuzumi and co-workers (92) suggested a possible mechanism for the proposed, energetically unfavorable, hydrolysis of the glycerol ether linkage.

The fate of [11] and [14] in cultures of *Coriolus versicolor* (= *Polyporus versicolor*) and *Stereum frustulatum* was also studied by the author and co-workers (93). The only transformations detected resulted from the activity of phenol-oxidizing enzymes (POs), even



though *S. frustulatum* was chosen for its atypically low PO activity. These same two fungi degraded a third  $\beta$ -O-4 model, 4-hydroxy-3,5-dimethoxyphenyl-glycol- $\beta$ -(*q*-methoxyphenyl) ether [15] to 2,6-dimethoxybenzoquinone, 2-(*o*-methoxyphenoxy)-acetaldehyde [16] and the corresponding acid [17] (94). This degradation proceeded via PO-catalyzed oxidations and subsequent disproportionation reactions (94). Guaiacol was not detected in these investigations (93,94). Recent work by Goldsby et al. (95) has confirmed and extended these findings.

The metabolism of various alkyl ethers of vanillic acid [4], of 3-ethoxy-4-hydroxybenzoic acid [18], and of syringic acid [19] in cultures of *Polyporus dichrous* was examined (96) in attempts to learn more about aryl-alkyl ether cleavages in white-rot fungi. A variety of 4-*Q*-alkyl ethers were degraded via hydroxylation of the 4-*Q*-alkyl moieties, but no evidence was found for hydroxylation of the 3-*Q*-alkyl groups. Both formation of alkyl-hydroxylated products and 4-*O*-dealkylations were observed (e.g., Fig. 7), the latter presumably the result of hydroxylations of the aryl ether-linked carbons. Unexpectedly, the 6-glyceryl ether of [18], and various alkyl ethers of syringic acid [19], were not metabolized. A probably reason for this failure is that the culture conditions employed in that study, as in most previous investigations with model compounds, were unfavorable for lignin metabolism (see Sec. V.A). The actual significance of the early results to lignin degradation can only be determined after further research.

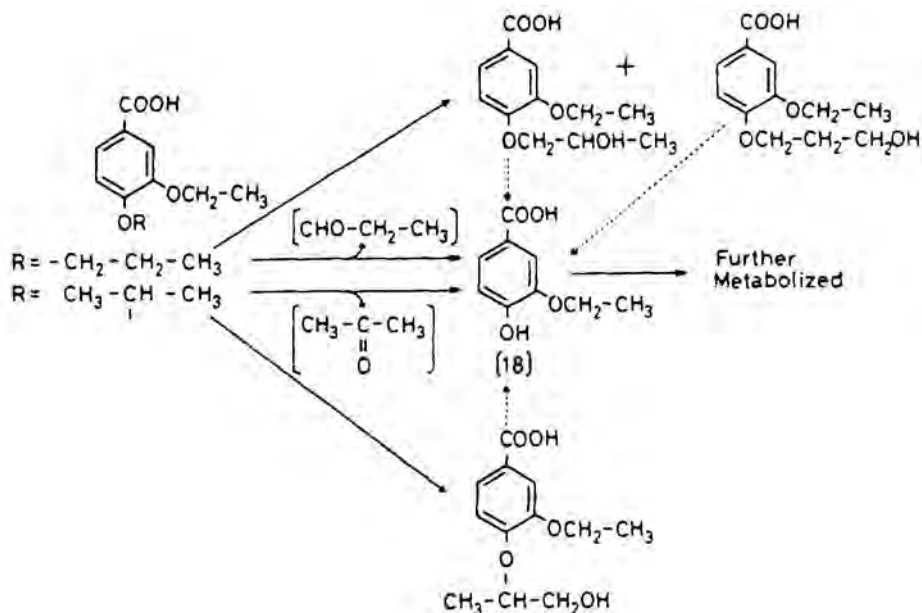


Figure 7 Degradation of alkyl ethers of 4-hydroxy-3-ethoxybenzoic acid [18] by *Polyporus dichrous*; compounds in brackets are suspected only. Dashed lines indicate probable pathways for further metabolism. (From Ref. 96.)

Recent studies of substructure model compound degradation have been conducted with cultures of the white-rot fungus *Phanerochaete chrysosporium* containing the complete enzyme system for degrading [ $^{14}\text{C}$ ]lignins to  $^{14}\text{CO}_2$ . Establishment of such "ligninolytic cultures" is not straightforward, and is described in Sec. V below. Figures 8, 9, and 10 summarize the initial reactions in the degradation of  $\beta$ -Q-4,  $\beta$ -5, and  $\beta$ -1 substructure model compounds, respectively, in ligninolytic cultures.

Gold and co-workers have published a series of papers describing metabolism of  $\beta$ -Q-4 substructure models in ligninolytic cultures of *P. chrysosporium* (97-100). The key products from nonphenolic models, illustrated with 1-(4-ethoxy-3-methoxyphenyl)-2-(*q*-methoxyphenoxy) propane-1,3-diol [20], (Fig. 8), indicate that the  $\beta$ -Q-4 ether linkage is simply cleaved, as reported earlier by Ishikawa et al. (91) and Fukuzumi et al. (92). The phenylglycerol product [21] was rapidly cleaved between  $\text{C}_\alpha$  and  $\text{C}_\beta$ , presumably to yield the  $\text{C}_\alpha$ -aldehyde and a 2-carbon fragment. The aldehyde is known to be rapidly reduced, and was not detected; the alcohol [22] was the major product.  $\beta$ -Q-4 models containing  $\alpha$ - $\text{CH}_2$  or  $\gamma$ - $\text{CH}_3$  also underwent  $\beta$ -Q-4 ether cleavage (100),

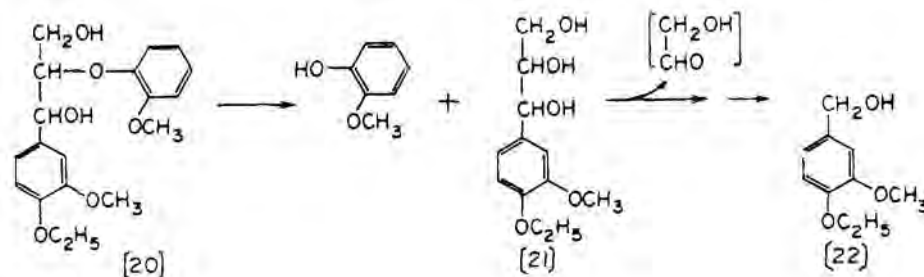


Figure 8 Partial degradative pathway for 1-(4-ethoxy-3-methoxyphenyl)-2-(*o*-methoxyphenoxy)propane-1,3-diol [20] in ligninolytic cultures of *Phanerochaete chrysosporium*. (From Refs. 97-100.)

indicating that neither the C<sub>α</sub>- nor the C<sub>γ</sub>-hydroxyl is essential for ether cleavage. Degradation of the nonphenolic β-*Q*-4 models was not rapid, and product yields were generally low; thus, it is possible that β-*Q*-4 ether cleavage was actually preceded by cleavage of one or the other aromatic nucleus. Products of 4-*O*-dealkylation were not detected.

As shown in Fig. 9, the fate of a β-5 (phenylcoumaran) substructure model, the 4-*O*-methyl ether of dehydridiconiferyl alcohol [23] (101), is not as apparently straightforward as that of the β-*Q*-4 model. On addition to ligninolytic cultures, compound [23] was degraded via compound [24], which contains a novel glycerol side chain formed from the cinnamyl alcohol moiety of [23]. Subsequent loss of the two terminal side-chain carbons produced the compound [25], the α'-C of which was interconverted in cultures between the alcohol, aldehyde, and acid. Compound [25], added to cultures as the α'-COOH compound, was further oxidized to the phenylcoumarone [26], containing a γ-COOH group. Further degradation produced veratric acid [27] (Fig. 9) and unidentified polar products, probably derived by cleavage of the C<sub>5</sub>-linked aromatic ring. As with the β-*Q*-4 models, products of 4-*O*-dealkylation were not observed.

Most recently studied are β-1 models, represented by 1,2-bis(3-methoxy-4-alkoxyphenyl) propane-1,3-diol [28]. As with the β-*Q*-4 and β-5 models, C<sub>α</sub>-C<sub>β</sub> cleavage occurs (Fig. 10). The initial products of this cleavage were diol [29] and aldehyde [31]. The diol was cleaved to yield a second mole of aldehyde [31], probably with release of formaldehyde, or it was oxidized to yield keto1 [30]. Aldehyde [31] was rapidly reduced to alcohol [22], the major product detected, or oxidized to acid [32] (102). Although compound [28] was a mixture of four stereoisomers, it was degraded nonstereoselectively. Studies with <sup>18</sup>O<sub>2</sub> showed that cleavage between C<sub>α</sub> and C<sub>β</sub> involves insertion of an

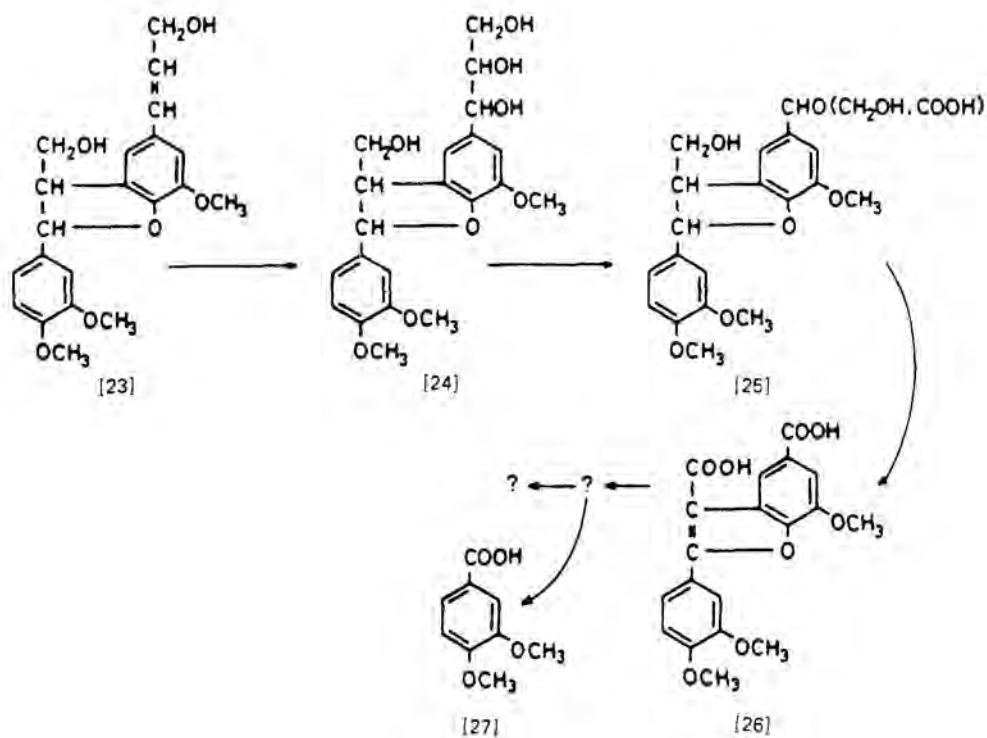


Figure 9 Partial degradative pathway for the 4-O-methyl ether of dehydrodiconiferyl alcohol [23] in ligninolytic cultures of *Phanerochaete chrysosporium*. (From Ref. 101.)

$\text{O}_2$ -derived hydroxyl group at  $\text{C}_\beta$  (102). Experiments with related  $\beta$ -1 models labeled at  $\text{C}_\alpha$  or  $\text{C}_\beta$  with deuterium revealed that the deuterium remains in the products (158). Thus,  $\text{C}_\beta$ -hydroxylation and  $\text{C}_\alpha$ - $\text{C}_\beta$  cleavage occur simultaneously. Product diol [29], although chiral, was a mixture of the d and l enantiomers, which establishes nonstereospecificity in product formation. Approximately one-third of the diol [29] formed during degradation in cultures under  $^{18}\text{O}_2$  did not contain  $^{18}\text{O}$ , suggesting simultaneous operation of two cleavage reactions, or existence of a mechanism for exchange between the  $^{18}\text{O}$  species and  $\text{H}_2\text{O}$ .

Assuming that the view of lignin metabolism by white-rot fungi described above in Sec. IV. A. 2 is correct, many degradation products—e. g., those containing ring-cleavage fragments—are not detected in studies with model compounds. Only the most stable and/or quantitatively most important products are seen, and only one of several possible pathways identified.

Degradation of dimeric lignin substructure model compounds by *Fusarium solani*, isolated by enrichment cultures with synthetic lignin as carbon source (30), has also been studied recently. Figure 11 shows the transformations delineated for the  $\beta$ -5 (phenylcoumaran) model compound dehydrodiconiferyl alcohol [33], which UV spectroscopic studies showed to be completely degraded (103). Identification of intermediates revealed that oxidation of the cinnamyl alcohol side chain proceeded through the  $\gamma'$ -aldehydic compound and the  $\gamma'$ -carboxylic compound [34] to the  $\alpha'$ -aldehydic compound [35]. The next identified product, [36], was the result of  $C_\alpha$ - $C_\beta$  cleavage in the coumaran ring and reduction of the  $\alpha'$ -aldehyde group; vanillic acid [4] was presumed to be the other aromatic product. Further oxidation of [36] produced 5-carboxyvanillyl alcohol [37]. Metabolism of [37] was not investigated. The initial oxidation of the  $\gamma'$ -alcohol group in [33] is catalyzed by an extracellular, oxygen-utilizing enzyme [104].

Comparison of the schemes in Figs. 9 and 11 shows that *Fusarium* and *Phanerochaete* degrade the phenylcoumaran substructure in different ways, although both fungi degrade the Compounds via  $C_\alpha$ - $C_\beta$  cleavages. An extracellular enzyme that catalyzes  $C_\alpha$ - $C_\beta$  cleavage has recently been isolated from *Phanerochaete* (Sec. V. B).

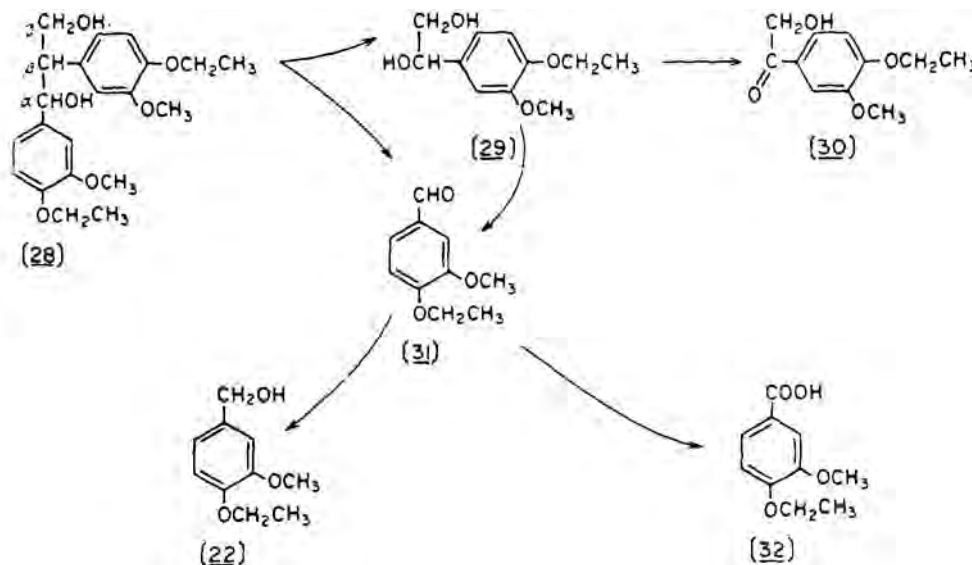


Figure 10 Partial degradative pathway for 1,2-bis(3-methoxy-4-ethoxyphenyl) propane-1,3-diol [28] in ligninolytic cultures of *Phanerochaete chrysosporium*. Initial cleavage is between  $C_\alpha$  and  $C_\beta$ . The new hydroxyl group in the phenylglycol product [29] comes from  $O_2$  (29,102).

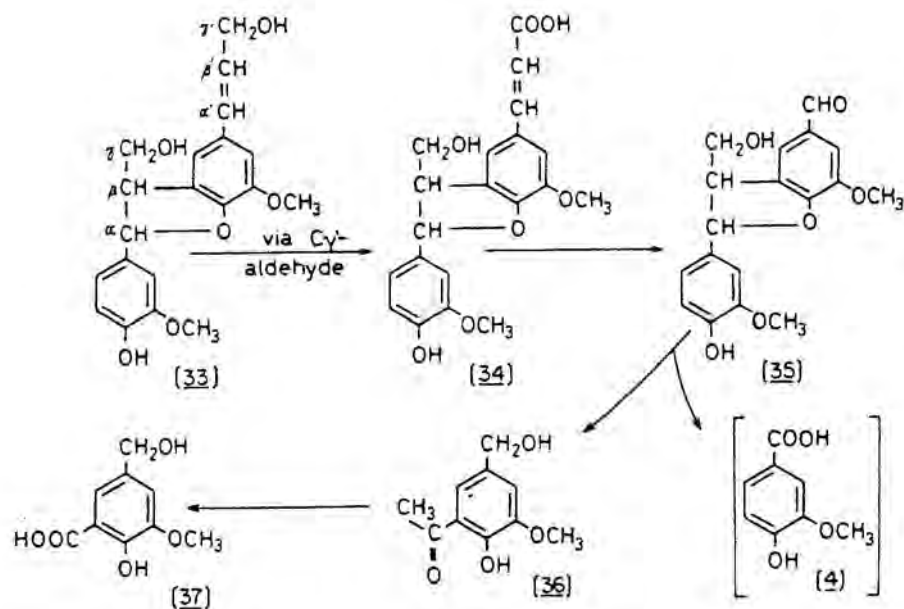


Figure 11 Partial metabolism of dehydrodiconiferyl alcohol [33] in cultures of *Fusarium solani*. (From Ref. 103.)

Oxidation of the cinnamyl alcohol side chain in the E-0-4 substructure model guaiacylglycerol- $\beta$ -coniferyl ether by *F. solani* produced a series of intermediates analogous to the initial products, [34, 35], from the phenylcoumaran model [33] (105). The  $\alpha$ -aldehyde group, however, was further oxidized to yield the  $\alpha$ -carboxylic compound, which was metabolized via unidentified products. In addition to the products described, a tetrameric 5,5'-biphenyl dehydrodimer was formed in a PO(?) catalyzed coupling reaction between two molecules of the  $\beta$ -O-4 model.

Importantly, the *Fusarium* was unable to metabolize compounds that contained no phenolic hydroxyl group (101,103). This would limit the attack of lignin to end groups containing phenolic hydroxyl groups.

## 2. "Monomeric" compounds

The fact that metabolism of single-ring compounds structurally related to lignin has been studied to only a limited extent in lignin-degrading microbes reflects in part uncertainties concerning what structures are relevant to lignin biodegradation. Until quite recently, very few low molecular weight degradation products of lignin or of oligomeric models had been identified.

A consideration of the structure of lignin suggests that substituted hydroxycinnamyl alcohols and aldehydes would be appropriate for

elucidating the fate of these "end groups" in lignin (e.g., unit 8 in Fig. 3). Similarly, the aromatic acids esterified to some lignins (unit 10 in Fig. 3) are presumably released by hydrolysis during biodegradation. These kinds of compounds, however, do not represent the backbone lignin structure, and can yield little information about the true degradative reactions in the polymer.

Vanillic acid [4] has been the most consistently reported single-ring product of lignin biodegradation (73,82-84); by analogy syringic acid [19] and *p*-hydroxybenzoic acid would also be expected products, and the former has been identified in extracts of beech wood undergoing white-rot (71,82). Several single-ring, phenylpropanoid products have also been reported (72,74,83; see 88) as trace products of lignin degradation by ligninolytic fungi, but these isolated reports, of unclear quantitative significance, provide a somewhat shaky foundation for further study. Even so, the few studies of metabolism of such compounds by ligninolytic fungi (88) indicate degradation via corresponding benzaldehydes and benzoic acids. Consequently, vanillic, syringic, and *p*-hydroxybenzoic acids can be studied profitably, and indeed, more is known about the fate of vanillic acid than about any other lignin-related compound in ligninolytic fungi.

Evidence was presented by Flaig and Haider (106) and by Cain et al. (107) that vanillic acid is degraded via demethylation to protocatechuic acid, which is then oxidized by protocatechuate oxygenase in *Coriolus versicolor* (= *Polystictus versicolor*). Similarly, Crowden (108) reported that degradation of tyrosine via 4-hydroxyphenylpyruvic acid probably proceeds with formation of protocatechuic acid in young cultures of the white-rot (?) fungus *Polyporus tumulosus*. Attempts to demonstrate an involvement of protocatechuic acid in vanillic acid metabolism in the white-rot fungus *Polyporus dichrous* failed, however, and further study showed that methoxyhydroquinone [39] is the immediate product in both *P. dichrous* and in the brown-rot fungus *Gloeophyllum trabeum* (= *Lenzites trabea*) (Fig. 12A) (109). Methoxyhydroquinone was degraded rapidly by the former, but only slowly by the latter fungus. Buswell et al. (110) and Yajima et al. (111) subsequently demonstrated that metabolism of vanillic acid by the white-rot fungus *Phanerochaete chrysosporium* (= *Sprotrichum pulverulentum*) also proceeds via methoxyhydroquinone, and Ander et al. (112) have demonstrated this reaction in cultures optimized for lignin metabolism. Both Buswell et al. (110) and Yajima et al. (111) have partially purified an oxidative decarboxylase that catalyzes the conversion, requiring one equivalent each of O<sub>2</sub> and NAD(P)H. Activity is present in cells grown without vanillic acid, but is increased greatly when the substrate is present. The enzyme is intracellular, indicating that a role in decarboxylation of aromatic acid residues in the lignin polymer is unlikely. Cell-free activity against methoxyhydroquinone has not been found (109,110). However, an intracellular oxygenase that cleaves hydroxyquinol [40] with formation of maleylacetate [41] (Fig. 12B) is

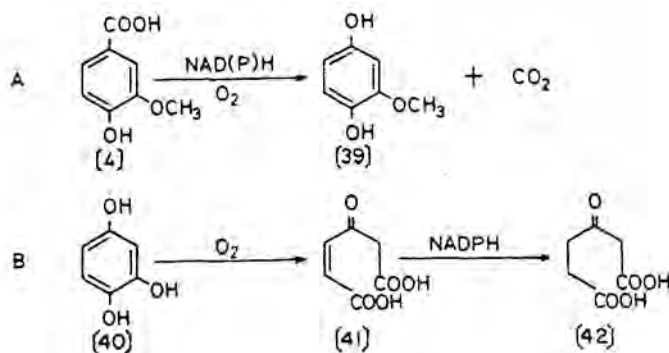


Figure 12 Vanillic acid [4] metabolism by white-rot fungi involves oxidative decarboxylation to methoxyhydroquinone [39], followed by ring cleavage (A). The immediate substrate for ring cleavage is not yet known, but hydroxyquinol [40] is cleaved to maleylacetate [41] by an oxygenase present in cultures of *Phanerochaete chrysosporium* grown with vanillic acid; maleylacetate is reduced to  $\beta$ -ketoadipic acid [42] by another enzyme present in the cultures (B). (A from Refs. 109-111, B from Ref. 113.)

elaborated by cells grown with vanillic acid (113), indicating that either demethylation or ring hydroxylation of methoxyhydroquinone precedes ring cleavage. The late release of  $^{14}\text{CO}_2$  from  $[-\text{O}^{14}\text{CH}_3]$ methoxyhydroquinone as compared to the ring-labeled compound (112) suggests that ring hydroxylation is the actual step. These metabolic reactions of vanillic acid are summarized in Fig. 12; as shown, maleylacetate is reduced to  $\beta$ -ketoadipic acid [42]. Free vanillic acid, therefore, is degraded via methoxyhydroquinone in white-rot fungi. However, as recognized by Ander et al. (112), the fact that the methoxyl carbon in lignin is converted to  $\text{CO}_2$  faster than ring carbons (70,114), whereas the reverse is seen with vanillic acid (112), suggests that most of the units in lignin may not be degraded via vanillic acid, methoxyhydroquinone, and related compounds. Perhaps many of the rings in lignin are cleaved while still in the polymer, with preceding demethylation, as discussed in Sec. IVA2.

Vanillic acid [4] also undergoes other reactions in ligninolytic cultures, the most prominent being single electron oxidation (phenol oxidation). Several products, including some of higher molecular weight, result from the reactions of the free radicals produced (149). Vanillic acid may also be 4-*O*-methylated to form veratric acid (84), a reaction which perhaps serves to protect the phenolic hydroxyl from oxidation. Still another fate of vanillic acid is reduction to the aldehyde and alcohol, common reactions which are discussed below.

In cultures of ligninolytic fungi, both aliphatic and aromatic carboxyl groups are commonly reduced via the aldehydes to the corresponding alcohols (e.g., 91,92,94,96,112,115-119), which apparently are more slowly metabolized than the parent acids (96,112). However, oxidation of primary alcohol and aldehyde groups also occurs (101,102, 117), suggesting that the oxidation state may not be critical for susceptibility to biodegradation. Whether the apparently nonspecific reductions have any significance to lignin degradation, or whether they occur in the polymer, are not yet known.  $^{13}\text{C}$ -Spectroscopic examinations of white-rotted lignins indicate that reduced structures are formed during degradation (78,79). Zenk and Gross (116) showed that reduction of veratric acid by *Coriolus versicolor* proceeded to the aldehyde in the presence of ATP, NADPH, and  $\text{Mg}^{2+}$ . Veratraldehyde in turn was reduced to veratryl alcohol by an alcohol dehydrogenase in the presence of NADH or NADPH. Substrate specificity was not studied.

Two curious reactions, also of unknown significance to lignin degradation, are reduction of the double bond in ferulic (4-hydroxy-3-methoxycinnamic) acid by *P. chrysosporium* (118,119), and nonoxidative decarboxylation of 4-hydroxy- and 3,4-dihydroxycinnamic acids to the corresponding hydroxystyrenes by another white-rot fungus, *Polyporus circinatus* (120). The latter reaction has also been seen in other fungi (88).

Most of the earlier studies with low molecular weight aromatics related to lignin were done without any real knowledge of the culture and physiological parameters that influence lignin metabolism. Indeed, recent findings, described in the following section, suggest that in most early studies, and in some recent ones, the conditions used with white-rot fungi will not support good lignin metabolism. This fact might explain why some substructure model compounds were not degraded (93,96,121), and it also questions the relevance of other observed reactions. Fungi are known to use alternative pathways in aromatic metabolism depending on physiological conditions (108,122).

## V. PHYSIOLOGY AND BIOCHEMISTRY OF LIGNIN DEGRADATION BY WHITE-ROT FUNGI

Most natural products, including biopolymers, can be used in microbial cultures as the source of some limiting nutrient (usually carbon) for primary growth, which leads to induction of the enzymes involved in degradation. Metabolism of the compounds is apparent if growth occurs, and such cultures can be studied with confidence that the relevant degradative enzymes are present. This classical approach does not work with white-rot fungi and lignin; lignin apparently cannot serve as the source of any limiting nutrient, and its presence does not increase ligninolytic activity. Establishing whether cultures contain ligninolytic

activity cannot be based on growth. These facts have become clear only recently when research defined the culture conditions necessary for lignin metabolism, and some of the associated physiology.

#### A. Physiology

Until 1975, studies of lignin degradation suffered from the lack of a specific, sensitive, and rapid assay for ligninolytic activity. Descriptions of the preparation and use of [<sup>14</sup>C]lignins in microbiological studies in 1975 (55,70,114) permitted rapid progress in microbiological and ecological aspects. Subsequent progress has also been rapid in defining the optimum culture conditions for lignin degradation, in defining the pathways of lignin model compound degradation, and in studying the physiology and biochemistry.

Most of the latter investigations have been conducted with *Phanerochaete chrysosporium*, chosen for its rapid growth and relatively rapid metabolism of lignin, its prolific conidiation, its unusually high temperature optimum of 40°C, and its low phenol-oxidizing enzyme activity. Finding all these traits in a single fungus is unusual, and *P. chrysosporium* [= *Spotatrichum pulverulentum* (123)] has become the most widely chosen organism for the study of lignin degradation. Gold and co-workers (124-126) have described techniques for mutation, colony restriction, and replica plating, as well as the methodology for reproducibly obtaining sexual fruiting in plate cultures of *P. chrysosporium*, contributions which have laid the groundwork for genetic approaches to the study of lignin degradation.

The culture parameters optimal for lignin degradation by *P. chrysosporium* in defined media are unusual. Initial studies (2) revealed that only in nitrogen-limited cultures was good lignin degradation observed. Subsequent study revealed that lignin is an inadequate source of carbon and energy for its own metabolism. Elevated O<sub>2</sub> levels were seen to greatly stimulate lignin degradation, whereas culture agitation with mycelial pellet formation had the opposite effect. Control of pH and choice of buffer were found to be critical. These findings have been summarized (2).

Thus, the important factors in medium formulation (44) are excess carbon source, (e.g., glucose, cellulose), limiting amount of a readily used nitrogen source (ammonium salts and some amino acids have been used), and trace elements, minerals, and thiamine. Shallow stationary cultures (10 ml/125-ml Erlenmeyer flask) at 37-39°C, under an atmosphere of approximately 50% O<sub>2</sub> in N<sub>2</sub>, and buffered at approximately pH 4.5 with a noninhibitory buffer such as 2,2-dimethylsuccinate, have given maximum rates of lignin degradation. Rates of >4 μg of synthetic lignin to CO<sub>2</sub> per mg of protein per hr are obtained in optimized cultures (80). These optimized conditions allow the investigation of model

compound metabolism with some assurance of relevance to lignin, as discussed in Sec. IVB.

Preliminary results with the taxonomically distinct fungus *Coriolus versicolor* indicated that it has requirements similar to those of *P. chrysosporium* for lignin degradation. Ellwardt et al. (78), however, working with *Pleurotus ostreatus*, reported degradation of [<sup>13</sup>C]lignins in a high nitrogen medium and with culture agitation. Thus the conditions critical for *P. chrysosporium* and *C. versicolor* are not critical for all fungi. We have recently confirmed that this is the case (159). The following discussion, therefore, concerns *P. chrysosporium*.

Figure 13 illustrates the development of ligninolytic activity in nitrogen-limited cultures of *P. chrysosporium* optimized for lignin degradation (127). Rapid depletion of the nitrogen (NH<sub>4</sub><sup>+</sup>) stopped primary growth, measured as DNA increase. After this depletion the carbon source, glucose, continued to be utilized; and the mycelial weight continued to increase due to synthesis of an extracellular polysaccharide. Ligninolytic activity appeared after a considerable delay following nitrogen starvation; this activity continued until exhaustion of the carbon source. Recent work has shown that the ligninolytic system can also be made to appear by carbohydrate- or sulfur-, but not

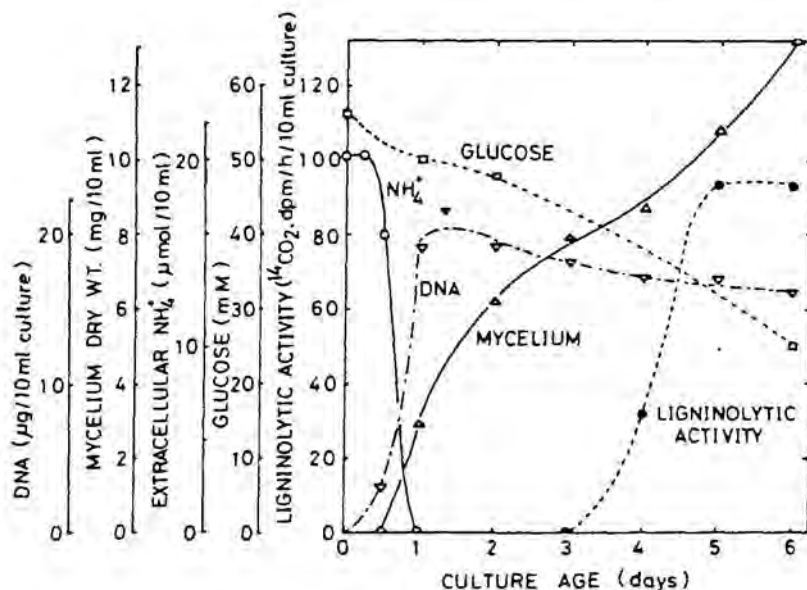


Figure 13 Relationship between culture parameters and ligninolytic activity in *Phanerochaete chrysosporium* ME 446. Details of culture conditions, determination of ligninolytic activity, and other methodology have been described (44,127). (From Ref. 127.)

phosphorus, starvation in the presence of excess nitrogen and other nutrients; highest sustained rates, however, are obtained in nitrogen-limited cultures (128). When the limiting nutrient in carbohydrate- or nitrogen-limited cultures is added back to cultures, ligninolytic activity is temporarily suppressed (127- 129). Using [<sup>14</sup>C]lignin-labeled aspen wood, Reid (130) has recently confirmed the influence of nutrient nitrogen in triggering ligninolytic activity in *P. chrysosporium*; his results indicated that neither sulfur nor phosphorus limitation could trigger activity, although a sulfur-nitrogen interaction was seen.

These results indicate that lignin degradation is a "secondary metabolic" or "idiophasic" event in *P. chrysosporium*, differing completely in this respect and in its apparent lack of inducibility by its substrate from other common bipolymer degradations. All studied secondary metabolic events are anabolic manifestations, involving syntheses of antibiotics, phenolics, and a multitude of other compounds, although these syntheses presumably are preceded by production of degradative enzymes such as proteases involved in redistribution of C, S, and N, and obviously by production of the synthetase systems (131).

It was discovered that *P. chrysosporium* also synthesizes de novo a secondary metabolite, veratryl (3,4-dimethoxybenzyl) alcohol (132). Subsequent studies have shown that ligninolytic activity and veratryl alcohol appear simultaneously in cultures, and that both can be repressed by adding nutrient nitrogen to cultures containing excess carbon (129,133). The metabolite is synthesized from phenylalanine via 3,4-dimethoxycinnamyl alcohol and veratrylglycerol; addition of nitrogen (glutamate) apparently represses the entire biosynthetic system (133). Addition of veratryl alcohol to nonligninolytic cultures prior to normal appearance of activity does not induce early appearance. The important thing is that these findings strongly support the view that lignin metabolism is a manifestation of secondary metabolism in *P. chrysosporium*.

Further study of suppression by NH<sub>4</sub><sup>+</sup> and glutamate indicated that the nitrogen additions cause a biochemical repression of secondary metabolism, including both ligninolytic activity and veratryl alcohol synthesis, as mentioned earlier, and that intracellular levels of L-glutamate might play a regulatory role (134). Glutamate stops not only the oxidation of lignin to CO<sub>2</sub> but also its depolymerization; apparently the entire ligninolytic system is regulated as a part of secondary metabolism (80). Gold et al. (160) have recently isolated a nonligninolytic mutant of *P. chrysosporium* and have shown that it is also deficient in other secondary metabolic functions.

## B. Biochemistry

Logic and the experimental evidence indicate that the lignin polymer-degrading system is oxidative and nonspecific. An oxidative mode of degradation is logically expected from the virtual nonhydrolyzability of lignin. That degradation is in fact primarily oxidative is apparent from the reactions that occur in the lignin polymer, and in substructure model compounds in ligninolytic cultures. Direct participation of molecular oxygen in degradation is indicated by the results with  $^{18}\text{O}_2$  and the  $\beta$ -1 model compound [28], discussed in Sec. IVB. Nonspecificity is logically expected in a system that degrades a heterogeneous and nonstereoregular polymer. Nonspecificity is evidenced directly by the fact that lignin is degraded in ligninolytic cultures after substantial modification in chemical pulping and bleaching reactions (135-137). Also, Crawford (138) has shown recently that "polyguaiacol," a polymer formed by the oxidative polymerization of guaiacol, and bearing only slight resemblance to lignin is metabolized in ligninolytic cultures of *P. chrysosporium*. As described in Sec. IV.B, studies with the  $\beta$ -1 model compound [28] have shown that the ligninolytic system exhibits no stereoselectivity for substrate [28], or in product formation. That the ligninolytic system is not induced by lignin is consistent with there being only a loose connection between the structure of Lignin and the activity of the system that degrades it. Thus the lignin polymer-degrading system is oxidative, nonspecific, and, of course, extracellular.

It is difficult to rationalize these characteristics in terms of specific degradative enzymes. Indeed, it has been suggested that key oxidative reactions in lignin polymer degradation might not be directly enzyme catalyzed. Hall (139) speculated that "active oxygen species" such as superoxide anion might be involved instead, and Zeikus (140) has speculated, too, that "chemical species" might be involved. Koenigs (141) suggested several years ago that  $\text{H}_2\text{O}_2$ , which he detected in cultures of white-rot fungi, might play a role in lignin degradation.

Active oxygen species, including superoxide anion radical ( $\text{O}_2^{\cdot-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hydroxyl radical ( $\cdot\text{OH}$ ) and singlet oxygen ( $^1\text{O}_2$ ), have been implicated or shown to be involved in a variety of biochemical reactions. Only the latter two are strong enough oxidants under physiological conditions to oxidize lignin. The first two species,  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$ , are thought to be precursors of the latter two in biochemical systems (142,143). We have found recently that both catalase and superoxide dismutase, which destroy  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$ , respectively, inhibit lignin degradation when added to cultures of *P. chrysosporium* (166). Similarly, the  $^1\text{O}_2$ -trapping agent, anthracene-9,10-bisethanesulfonic acid (AES, 144), strongly suppresses ligninolytic activity in

*P. chrysosporium* (145). Finally, Forney et al. (146) have reported very recently that the  $\cdot\text{OH}$  scavengers mannitol, benzoate, and butylated hydroxytoluene inhibit lignin degradation by *P. chrysosporium*. Thus, all four of these active oxygen species have been indirectly implicated in lignin degradation.

Evidence that  $\text{O}_2^{\cdot-}$ ,  $\text{H}_2\text{O}_2$ ,  $\cdot\text{OH}$ , and  $^1\text{O}_2$ , are produced by fungi during lignin degradation has been reported. Amer and Drew (147) reported that whole cells of *Coriolus versicolor* produced  $\text{O}_2^{\cdot-}$ , based on their reduction of cytochrome *c*.  $\text{H}_2\text{O}_2$  production by several lignin-degrading fungi was reported by Koenigs (141). Forney et al. (146) demonstrated a correlation between  $\text{H}_2\text{O}_2$  production, using the catalase/aminotriazole assay, and ligninolytic activity in *P. chrysosporium*. These workers (146) also presented evidence, based on three different assays, for  $\cdot\text{OH}$  production by ligninolytic cultures of *P. chrysosporium*. These assays were (a) ethylene formation from  $\alpha$ -keto- $\gamma$ -methiolbutyric acid, (b) hydroxylation of *p*-hydroxybenzoic acid to protocatechuic acid, and (c) ESR spectrometric identification of the adduct of  $\cdot\text{OH}$  with 5,5-dimethyl-1-pyrroline-*N*-oxide. They concluded that  $\cdot\text{OH}$  is involved in lignin degradation, based on these results, and on the inhibition of the ligninolytic system by  $\cdot\text{OH}$ -trapping agents, mentioned above. More recently, Kutsuki and Gold (148) reached the same conclusion in a similar study. Nakatsubo et al. (145) presented evidence for an involvement of  $^1\text{O}_2$  in lignin degradation: (a) the  $^1\text{O}_2$  trapping agent AES strongly inhibited lignin but not glucose oxidation by *P. chrysosporium*; (b) ligninolytic cultures rapidly bleached AES, in accord with  $^1\text{O}_2$  production; and (c) a photochemical  $\text{O}_2$ -generating system produced the same degradation products [29-32] from the  $\beta$ -1 model compound [28] as cultures. Subsequent study, however, has discredited the possible role of  $^1\text{O}_2$  by showing that a chemical system for generating  $^1\text{O}_2$  does not degrade [28] to the same products as cultures (161) and that  $^1\text{O}_2$  is not the active species in the photochemical system (161,162).

It can be concluded from these recent studies of activated oxygen species that  $\text{H}_2\text{O}_2$  and perhaps  $\text{O}_2^{\cdot-}$  are involved in lignin degradation by *P. chrysosporium*. Hydroxyl radical is expected to be found where transition metals and  $\text{H}_2\text{O}_2$  are present, as in ligninolytic cultures, but evidence that  $\cdot\text{OH}$  is a component of the ligninolytic system must be considered to be equivocal.

Knowledge that  $\text{H}_2\text{O}_2$  is involved in lignin degradation by *P. chrysosporium* has led very recently to the first discovery of a lignin-degrading enzyme (163). The enzyme requires  $\text{H}_2\text{O}_2$  for activity. It was first detected in the extracellular culture fluid of ligninolytic cultures as the agent responsible—in the presence of added  $\text{H}_2\text{O}_2$ —for the initial  $\text{C}_\alpha\text{-C}_\beta$  cleavage reaction in  $\beta$ -1 substructure model compounds such as [28] (Fig. 10.). Further study established that the ligninase catalyzes the same cleavage in  $\beta$ -O-4 models such as [20], and in spruce and birch lignins. Its action on the lignins results in their

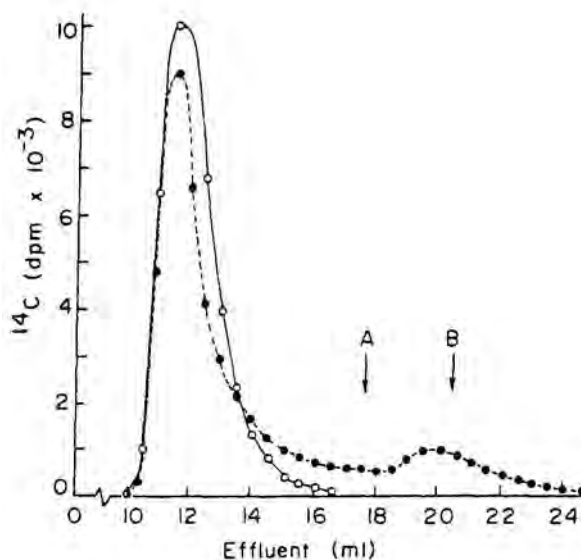


Figure 14 Molecular size profiles showing partial depolymerization of  $^{14}\text{C}$ -methylated spruce lignin by an enzyme from *Phanerochaete chrysosporium*: Active enzyme +  $\text{H}_2\text{O}_2$  (- - • - -); boiled enzyme +  $\text{H}_2\text{O}_2$  or active enzyme -  $\text{H}_2\text{O}_2$  (—o—). Gel filtration employed Sephadex LH-20 in *N,N*-dimethylformamide. A, MW = 348; B, MW = 166. (From Ref. 163.)

partial depolymerization (Fig. 14). Activity resides in a single protein of MW = 42,000, as evidenced by both gel permeation chromatography and polyacrylamide gel electrophoresis. It is inhibited by azide, suggesting metal involvement. Further characterization of the enzyme is under way. Of particular importance is elucidation of the role of  $\text{H}_2\text{O}_2$ . Interestingly, the enzyme activity is absent in growth-stage cultures (see Sec. V.A). It appears to be the major extracellular protein in glucose-grown cultures (163).

Discovery of this relatively nonspecific enzyme brings into question the concept that non-enzyme-bound, highly reactive species of activated oxygen are involved in lignin degradation. However, as described in Sec. IV.A.2 above,  $\text{C}_\alpha\text{—C}_\beta$  cleavage is only one of several reactions thought to be of primary importance in degradation of the Lignin polymer. Further studies of the specific reactions of degradation of model compounds in ligninolytic cultures of *P. chrysosporium* will clarify the biochemical basis for the other reactions.

Previous investigations on the biochemistry of lignin degradation focused primarily on phenol-oxidizing enzymes (laccase, peroxidase, tyrosinase), which catalyze the single electron oxidation of phenols.

Such enzymes can cause limited structural changes in lignin (149), and extensive changes in certain low molecular weight compounds related to lignin (Sec. IV.C.1). These changes include  $C_{\alpha}$ -oxidation in phenolic units, leading to the suggestion (150) that this might be the role for these ubiquitous enzymes. A direct role in extensive structural degradation of lignin seems most unlikely (39,56,151). An indirect but necessary role, possibly regulatory, has been suggested (152) on the basis of studies with a mutant lacking phenol-oxidizing enzyme activity.

The enzyme cellobiose:quinone oxidoreductase, also produced by white-rot fungi (153), catalyzes oxidation of cellobiose to cellobionone- $\delta$ -lactone, with transfer of the two electrons to any of various quinones or phenoxy radicals, including those produced in lignin by phenol-oxidizing enzymes. This allows a cyclic mechanism, involving cellobiose:quinone oxidoreductase and a phenol-oxidizing enzyme, to be envisaged for transferring electrons from cellobiose, the principal extracellular product of cellulose breakdown by white-rot fungi, to  $O_2$  or  $H_2O_2$ , with phenols, including lignin, serving as electron "carriers" (3,153). Whether this mechanism is involved in lignin degradation, however, is unclear.

## VI. CONCLUSIONS

Fungi and filamentous bacteria are primarily responsible for lignin biodegradation. Among the former, the white-rot, wood-destroying basidiomycetes and closely related litter-decomposing basidiomycetes apparently play the dominant role. The bacteria probably are of lesser importance.

The firm experimental foundation necessary for meaningful study of the biochemical mechanism of lignin degradation by white-rot fungi has now been established. That foundation includes elucidation of the major features of the chemistry of degradation of the lignin polymer; development of an assay for ligninolytic activity; selection of fungi suitable for detailed study; description of the culture parameters that influence development of ligninolytic activity, and of their physiological bases; and finally, description of individual reactions of the degradation of substructure model compounds. These developments have led quite recently to the discovery of the first lignin-degrading enzyme, and provide the tools for discovering additional enzymes and for evaluating the possible role of non-enzyme oxidations. Rapid further advances are assured.

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