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The Involvement of Hydroxyl Radicals Derived From Hydrogen Peroxide in Lignin Degradation By The White-Rot Fungus Phanerochaete chrysosporium

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THE INVOLVEMENT OF HYDROXYL RADICALS DERIVED FROM HYDROGEN PEROXIDE IN LIGNIN DEGRADATION BY THE WHITE-ROT FUNGUS PHANEROCHAETE CHRYSOSPORIUM

By

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ABSTRACT

THE INVOLVEMENT OF HYDROXYL RADICALS DERIVED FROM HYDROGEN PEROXIDE IN LIGNIN DEGRADATION BY THE WHITE-ROT FUNGUS PHANEROCHAETE CHRYSOSPORIUM

By

Larry J. Forney

Accumulated data on the chemistry of lignin and its degradation by white-rot fungi has recently led to the postulation that oxygen radicals are involved in the extracellular transformations of the polymer effected by these organisms. In this study we investigated the possibility that the hydroxyl radical (·OH) derived from hydrogen peroxide (H_2O_2) is involved in the degradation of lignin by Phanerochaete chrysosporium, a typical white-rot fungus. When P. chrysosporium was grown in low N medium (2.4 mM N) an increase in the specific activity for H_2O_2 production in cell extracts was observed to coincide with the appearance of ligninolytic activity and both activities appeared after the culture entered the stationary growth phase. Using cytochemical staining techniques and 3,3'-diaminobenzidine (DAB) the H_2O_2 production activity in these cells was shown to be localized in the periplasmic space of cells from ligninolytic cultures. The intensity of the staining reaction (oxidized DAB deposits) in cells of various ages was qualitatively related to the levels of H_2O_2 production activity and in turn to the ligninolytic capacity of the cells. Similar oxidized DAB deposits were not observed in cells from cultures grown in low N medium which had not entered the stationary growth phase and therefore were not ligninolytic. The production of ·OH in ligninolytic cultures was demonstrated by

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α-keto-γ-methiolbutyric acid dependent formation of ethylene. Hydrogen peroxide dependent ·OH formation was also shown in cell extracts. The radical species produced was demonstrated to be the ·OH by the ·OH mediated hydroxylation of p-hydroxybenzoic acid to form protocatechuic acid and by using 5,5-dimethylpyrroline-N-oxide (DMPO) and electron paramagnetic resonance spectrometry to detect the production of the nitroxide radical of DMPO. These reactions were inhibited by ·OH scavenging agents and were stimulated when azide was added to inhibit the endogenous catalase. Lignin degradation was markedly suppressed in the presence of the .OH scavenging agents mannitol, benzoate and the nonspecific radical scavenging agent butylated-hydroxytoluene. The above results clearly indicate that the ·OH derived from H_2O_2 plays an integral role in lignin degradation by P. chrysosporium.

To Sue, Kelly and my parents

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LITERATURE REVIEW

Biosynthesis, Distribution and Importance of Lignin

Introduction

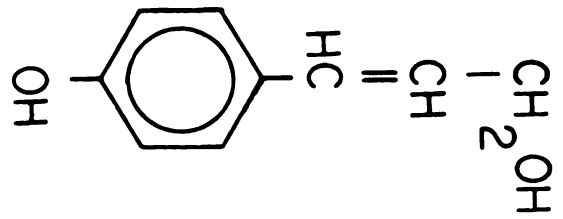
Lignin is one of the most abundant and widely distributed organic polymers in nature. It occurs as an integral cell wall component and in the middle lamellae of all vascular plants (104). Within plants, lignin imparts structural rigidity, resistance to impact, compression and bending (105), decreases water permeation across cell walls of xylem tissue and protects plant tissues from invasion from pathogenic microorganisms (47, 111). Interest in the mechanism of lignin degradation by microorganisms stems from its importance in the carbon cycle and because lignocellulosic materials represent a vast renewable resource for the production of fuels and chemicals (6).

Biosynthesis of lignin

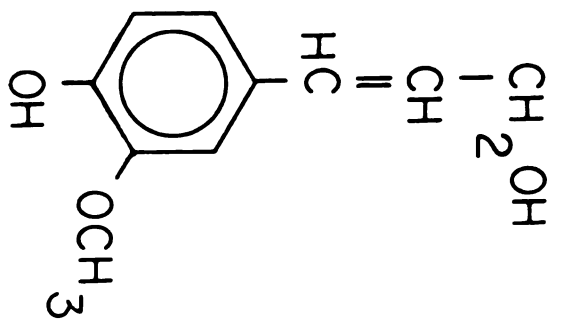
Lignins are formed through the dehydrogenative polymerization of primarily three cinnamyl alcohol derivatives: trans-p-coumaryl alcohol, trans-coniferyl alcohol and trans-sinapyl alcohol (Figure 1). The ratio of the three percursors varies in lignins of different plant species (1) and within different tissues of the same plant species (111). On average, gymnosperm lignin (softwood lignin) contains approximately 94% coniferyl alcohol, 5% p-coumaryl alcohol and 1% sinapyl alcohol, whereas angiosperm lignin (hard wood lignin) contains roughly equal proportions of coniferyl and sinapyl alcohols and a minor amount of p-coumaryl alcohol.

Figure 1. The structures of (a) trans-p-coumaryl alcohol, (b) trans-coniferyl alcohol, (c) trans-sinapyl alcohol, the immediate precursors used in the biosynthesis of lignin.

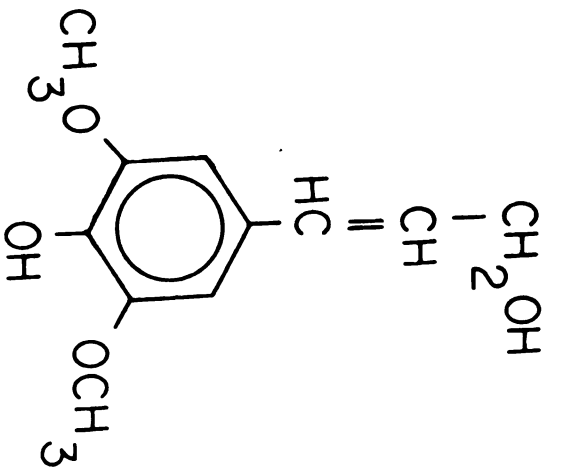
(a)



(b)



(c)



During lignification in young plant tissues a higher proportion of guaiacyl units (derived from coniferyl alcohol) are incorporated, whereas during maturation, the proportion of syringyl units (derived from sinapyl alcohol) increases (36, 47, 111).

At the site of lignification, the phenolic hydroxyl groups of the cinnamyl alcohol precursors are oxidized by a phenol oxidase enzyme, usually peroxidase (EC 1.11.1.7; donor: hydrogen peroxide oxidoreductase), to produce phenoxy radicals (47). Due to the extended π electron systems of the precursors the free radicals are stabilized through equilibrium with several mesomeric forms (57). The resonance structures of the radical produced from coniferyl alcohol are shown in Figure 2. The stability of each mesomer determines its relative concentration and therefore, its availability to participate in coupling reactions to produce covalent bonds. The phenolic hydroxyl groups of oligomeric products are also oxidized and the nonenzymatic polymerization proceeds primarily through addition to the growing polymer (40). As a result of the mechanism of polymerization, all asymmetric carbons in the polymer (C-α and C-β of the monomer side chain) exist in both R and S stereoisomeric forms (106).

The structural and chemical complexity of the polymer is clearly evident in the schematic representation of gymnosperm lignin shown in Figure 3 (39, 57). More than 24 different intermonomer bonding types have been found to occur in lignin (1). The major intermonomer bonding types and their frequencies in representative gymnosperm (spruce) and angiosperm (birch) lignins are shown in Figure 4 (1, 86). The aryl glycerol-β-aryl ether linkage (Figure 4, substructure A) occurs most frequently, with 48% and 60% of the monomers participating in this

Figure 2. Structures of the mesomeric forms of the free radical derived from coniferyl alcohol. (a) phenoxyl radical, (b) o-methylene quinonoid radical, (c) p-methylene quinonoid radical, (d) p-quinone methide radical.

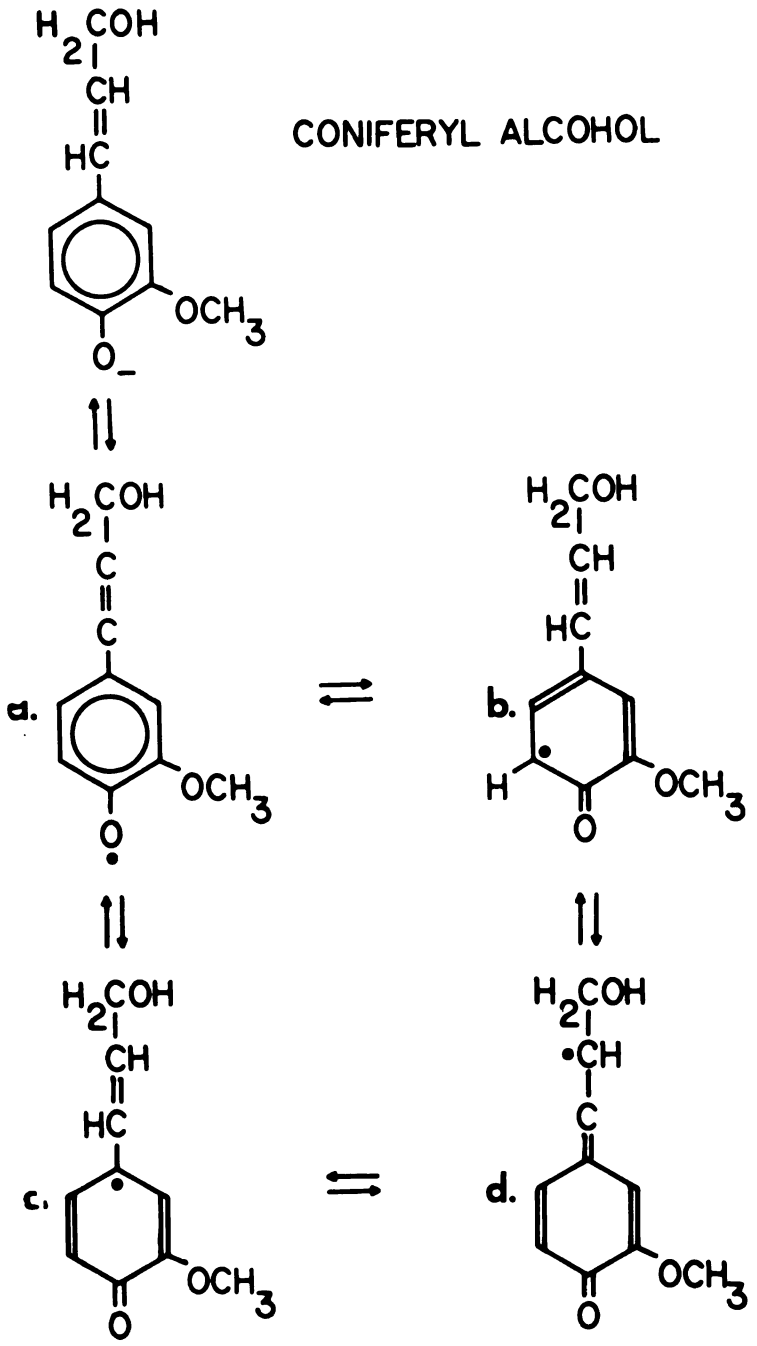

CONIFERYL ALCOHOL
a.
b.
c.
d.

Figure 3. Schematic representation of gymnosperm lignin (from reference 57).

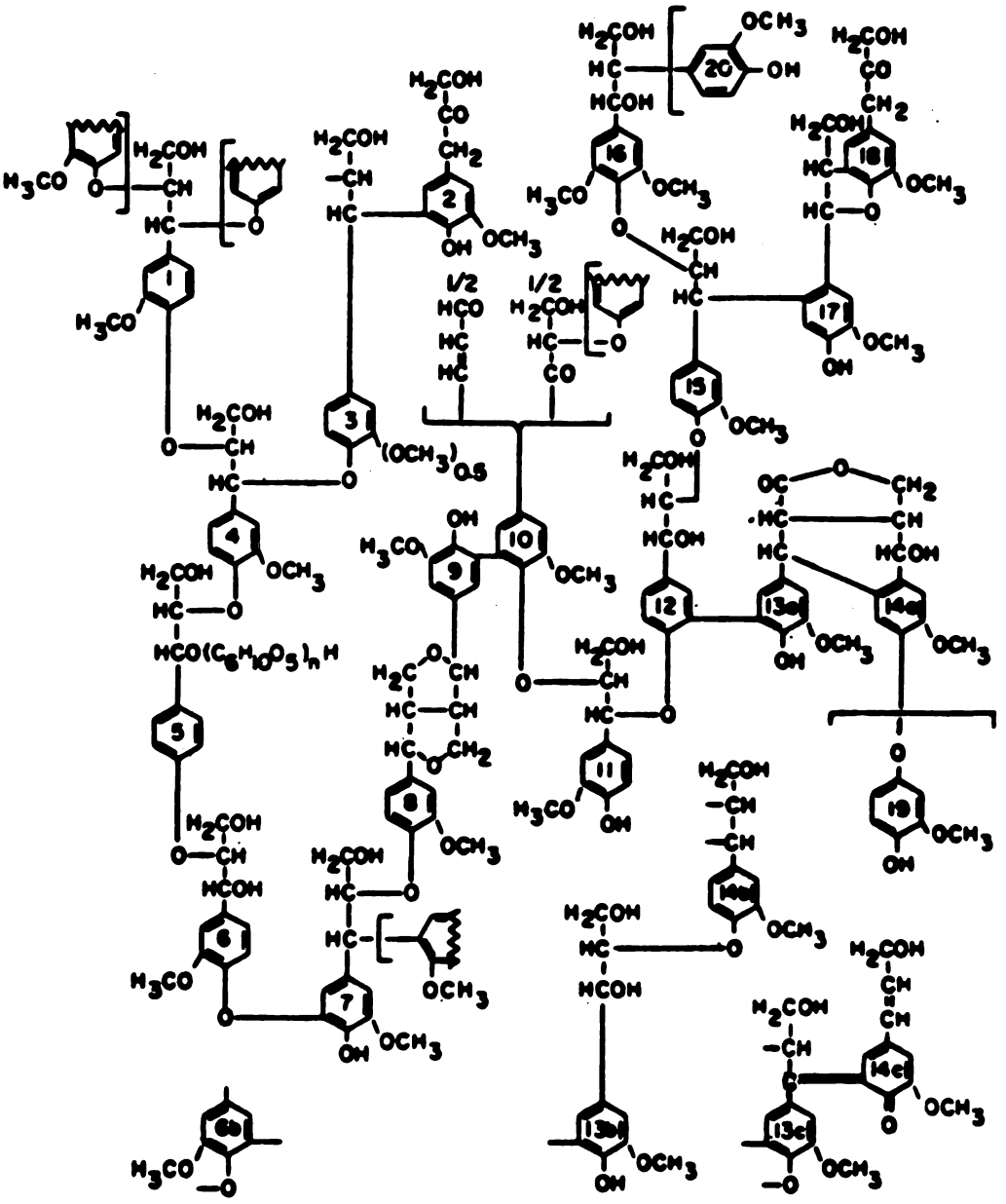
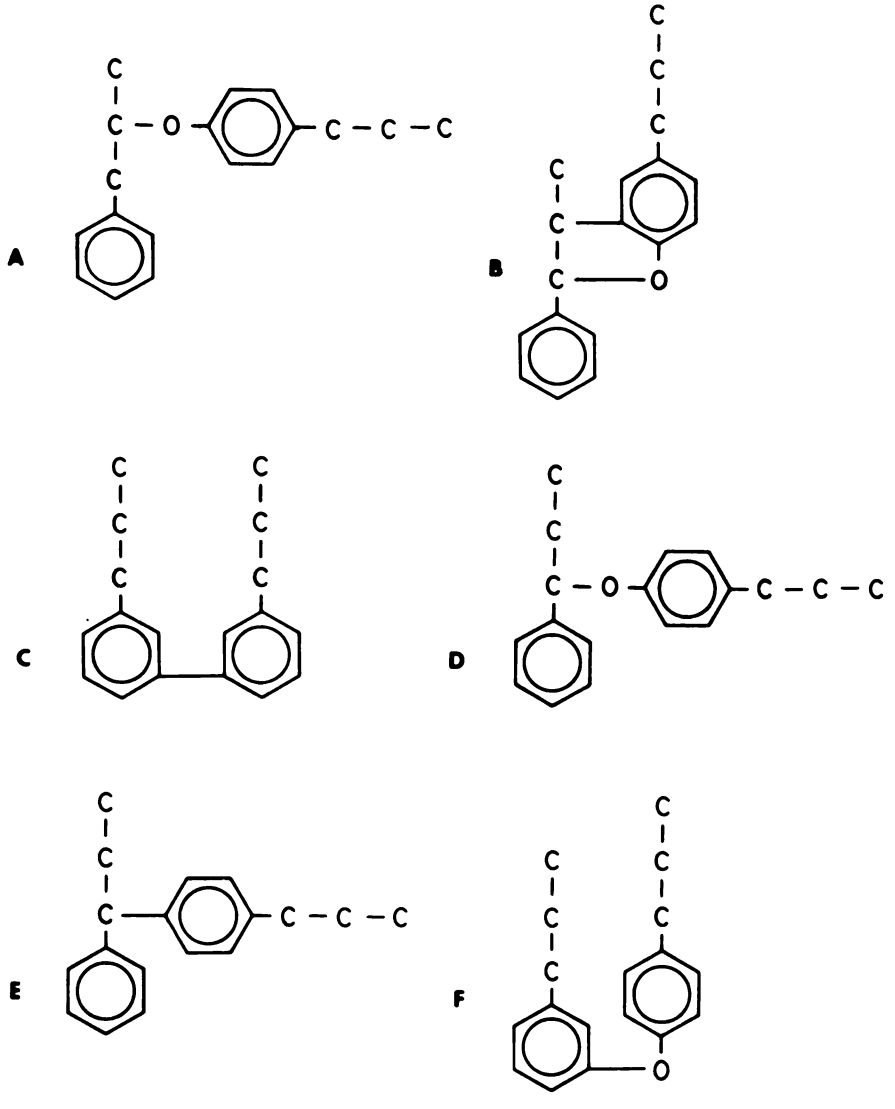


Figure 4. Frequencies of major interunit linkages in lignins from spruce wood and birch wood (from reference 86).



Substructure type	of C_9 units participating	
	spruce	birch
A	48	60
B	9-12	6
C	9.5-11	4.5
D	6-8	6-8
E	7	8
F	3.5-4	6.5

type of linkage in gymnosperm and angiosperm lignins, respectively. Most other intermonomer bonding types involve less than 10% of the lignin monomers. The molecular weight of purified lignin varies from a few thousand to more than 1 X 10^6 depending on the method used for isolation (46). The elemental composition for lignin from spruce wood has been shown to be $C_9H_{7.49}O_{2.53}(OCH_3)_{1.39}$ (40).

Distribution of lignin in plant tissues

Lignin is deposited within cell walls to form an interpenetrating network with hemicelluloses (111). This matrix surrounds the cellulose fibrils in a sheath-like manner to form a physically impenetrable barrier such that polysaccharide degrading enzymes cannot gain access to a large portion of the cellulose and hemicelluloses in fully lignified tissue. Ultraviolet microscopy of thin sections of plant tissues revealed that 72-81% of the total lignin in black spruce early wood is located within the secondary cell wall layers, with the balance present in the middle lamellae (35). The middle lamellae consists largely of lignin (approximately 72%). Since 45-50% of the secondary cell wall is polysaccharide, the largest portion of lignin in plant tissues occurs in close physical association with and is chemically bonded to cellulose and hemicelluloses (112).

Importance of lignin in the carbon cycle

Due to their abundance in nature, lignins play an important role in the earth's carbon cycle. An estimated 5 X 10^8 metric tons of lignin are biosynthesized annually (114) and 1-3 X 10^{12} metric tons of carbon has been calculated to be present in the organic matter of soil

(114), primarily as peat and humus which in a large part are derived from lignins (65).

In soil, plant tissues are subject to degradation by microorganisms. While the sugars and polysaccharide fractions are degraded comparatively rapidly, the lignin fraction is metabolized rather slowly (94). The oxidized, partially condensed lignin polymer, in conjunction with various carbohydrates, proteinaceous material and other plant and microbially produced phenolic compounds, form humus (59, 93, 65). The humus found in the upper layers of soils performs several important functions (93). It influences the structure of soil increasing its aeration and moisture holding capacity. It also serves as an ion exchange resin and is able to sequester and release various ionically charged organic molecules and inorganic ions, in general adding to soil fertility. Humus is degraded very slowly with an estimated half-life of 76 to 326 y depending on the soil type investigated (52). The recalcitrance of humus is reflected in the observed occurrence of lignin derived phenolic residues in lignite (61), bituminous coal (61) and in silicified wood which was 2.8×10^8 y old (107).

Role of lignin in the utilization of lignocellulosic materials

In recent years, numerous investigators have been exploring the potential of using "biomass" for the production of various fuels and chemical feedstocks (6). The term biomass generally refers to the polysaccharides present in lignocellulosic materials, including agricultural residues and crops grown expressly for use in such processes. It has been estimated that approximately 8×10^8 tons of lignocellulosic by-products and residues are generated annually (6). Obviously, these

materials represent a renewable resource of considerable magnitude.

Lignin, due to its close physical and chemical association with plant polysaccharides, has been shown to present both a chemical and physical barrier to enzymes capable of hydrolyzing cellulose and hemicelluloses (15, 78). Thus, lignin is an important factor which impedes the utilization of these polysaccharides in various industrial processes. Therefore, any treatment which depolymerizes, solubilizes, or otherwise removes lignin increases the availability and susceptibility of cellulose and hemicelluloses to enzymatic or chemical attack (14, 15, 78). Various physical and chemical pretreatments individually and in various combinations have been shown to be effective (23, 25). However, most suffer from one or more of the following disadvantages (25, 101): (a) are energy intensive and are therefore not economical; (b) generate large quantities of chemical waste which must be efficiently recycled or disposed of, or (c) are not effective when used on an industrial scale. The use of microorganisms to partially delignify lignocellulosic materials, in conjunction with the use of other chemical or physical pretreatments, could potentially decrease the energy and chemicals required to disrupt the lignin-polysaccharide association and therefore decrease the cost of substrate pretreatment in processes which utilize lignocellulosic materials (101) as sources of fuels, chemicals and feeds.

Lignin also has potential as a source of industrially useful polymers and as a source of aromatic chemical feedstocks (6). Although chemical procedures for the conversion of lignin into commercially valuable products have been developed, none are widely used and many suffer from low product yield (6). A process using microorganisms may offer greater specificity and therefore be more cost effective. Perhaps the

most efficacious approach would be to produce modified lignin polymers for use as dispersants, emulsion stabilizers, complexing agents, coagulants and antioxidants, controlled desorption agents, ion exchange resins, or in thermosetting resins and rubber reinforcement (6). Furthermore, it may be possible to use biological processes to generate aromatic chemical feedstocks such as phenols, benzene, cresols and cresolyic acids from lignin in yields that would be economically attractive (6).

Substrates used in the Study of the Biological Degradation of Lignin

Introduction

A survey of the literature reveals that lignin is transformed by many organisms and in many environments (10, 17, 49, 93, 94). However, study of the biological decomposition of lignin has been hindered due to unavailability of sensitive assays. By far the most common assay used in the past in studies on the biological degradation of lignocellulosic materials has been the Klason assay (26). In this procedure, the carbohydrates are solubilized by hydrolysis with 72% H_2SO_4 and by refluxing in dilute acid. The lignin remains insoluble and is quantified gravimetrically. Using this assay, underestimation of the lignin present in a sample may result from the increased solubility of lignin through oxidation by microorganisms. Conversely, the lignin may be overestimated if condensation reactions occur between lignin and proteinaceous material in the sample during the assay procedure (90). This assay is usually indicative only of the amount of lignin which remains polymeric and acid-insoluble, and no information is obtained as to the extent to which the lignin is depolymerized or respired as CO_2. Furthermore, specific transformations, such as demethylation of methoxyl groups, cannot be assessed. Obviously, this assay

is of very limited use to biologists. In recent years, procedures for the preparation of (^{14}C-lignin) lignocelluloses and ^{14}C-synthetic lignin have been developed and are widely used in studies on the degradation of lignin by microorganisms (20). These lignins have all the advantages of other ^{14}C-labelled substrates used in the study of microbial metabolism including high sensitivity and the ability to specifically label portions of the polymer (i.e., 2'-^{14}C side chain labelled, ^{14}C-ring labelled or methoxyl group (O$^{14}CH_3$) labelled). However, these substrates are primarily used to monitor $^{14}CO_2$ production which does not adequately reflect the complex process of lignin degradation.

(^{14}C-Lignin) lignocelluloses

Crawford and Crawford (16) and Haider and Trojanowski (51) have developed procedures for the preparation of ^{14}C-lignin labelled plant tissue for use in studies of the microbial degradation of lignin. These procedures employ radioactively labelled biosynthetic precursors of lignin such as phenylalanine, cinnamic acid or ferulic acid which are either injected into the cambial layer of the plant or a cut twig or stem is placed in a solution containing the precursor (16, 51, 108). The precursor is then incorporated by the plant into lignin (63). The ^{14}C-lignocellulose is isolated by scraping the cambial layer from the wood or the entire tissue is used. After milling to reduce the particle size, the unincorporated ^{14}C-precursor, waxes, resins and oils are extracted and the lignocellulose is dried.

The (^{14}C-lignin) lignocellulose produced should be fully characterized to determine the distribution of label within the tissue. The analyses should include a Klason lignin assay and quantification of

the label incorporated into carbohydrates and protein, following hydrolysis of the wood polysaccharides and digestion with a protease, respectively (20). When the Klason assay is performed on (^{14}C-lignin) lignocelluloses, the label present in the acid soluble and acid insoluble fractions are determined by total combustion analysis and by liquid scintillation counting. However, it has been shown that a certain fraction of the lignin remains "acid-soluble". As a result, the amount of label incorporated into non-lignin fractions cannot be accurately determined. The amount of acid-soluble label found varies, depending on the plant species examined, from approximately 50% (e.g. Spartina alterniflora, Picea excelas) to 2% (Typha latifola) with most species being 25-35% (20). It can probably be assumed that if a large percentage of the label is found in the "acid-soluble" fraction, that plant components other than lignin have been labelled (e.g. phenolic compounds, flavonoids, etc.).

If (^{14}C)-phenylalanine is used as the precursor the determination of ^{14}C-protein present in ^{14}C-lignocellulose is especially important. However, experimentation has shown that in the plant species examined, little (less than 3%) of the label is incorporated into protein. This problem is easily circumvented by using ^{14}C-ferulic acid or ^{14}C-cinnamic acid where upon incorporation into protein has been found to be less than 1%.

The incorporation of label into non-lignin fractions and less condensed, i.e., recently synthesized, lignin (which is probably degraded more readily), constitutes the major disadvantage to the use of (^{14}C-lignin) lignocellulose. This is especially true if low levels of degradation are observed.

For certain investigations, the use of (^{14}C-lignin) lignocellulose is desirable since the lignins are degraded while in their "natural" states. Thus, if the question under study is whether or not an organism can degrade lignin in plant tissue, the use of (^{14}C-lignin) lignocellulose has obvious advantages.

(^{14}C) Synthetic lignins

(^{14}C)-synthetic lignins or unlabelled synthetic lignins are prepared by the in vitro dehydrogenative polymerization of specifically labelled lignin precursors, using a horseradish peroxidase and H_2O_2 (40). Such model lignin polymers were used extensively by lignin chemists to elucidate the structure of lignin (40, 57) and have been shown to exhibit many of the structural and chemical features of naturally occuring lignin (81, 103). Several significantly different protocols for the synthesis of synthetic lignins have been published (40, 81). Despite careful attention to experimental detail, these lignins frequently differ in their average molecular weight (12, 31), susceptibility to degradation by microorganisms and solubility in various organic solvents (31, L.J. Forney and C.A. Reddy, unpublished data). These differences occur when the same procedure is used on different occasions. This obviously leads to difficulty in reproducibility of results in biodegradation studies and comparison of results between different laboratories. Clearly a thorough examination of the factors affecting the preparation of these lignins is needed.

^{14}C-Synthetic lignins are not commercially available and the ^{14}C-labelled precursors of synthetic lignin, although available are

rather expensive. As a result, most investigators must undertake the synthesis of the precursors themselves. The synthesis of the precursors is somewhat involved and this has discouraged the use of synthetic lignins in studies on the fate of lignin in pure culture and in the environment.

For some investigations, the use of (^{14}C)-synthetic lignin has several advantages as compared to ^{14}C-lignocelluloses. Synthetic lignins can be recovered from microbial cultures without further (artificial) modifications for use in various analytical procedures. In contrast, lignin in plant tissue is modified during the ball milling and extraction or solvolysis which is required to recover it (90). Synthetic lignin is readily fractionated into various molecular weight ranges (12, 31) and the average molecular weight of the lignin can be determined. Therefore, changes in the molecular weight profile of synthetic lignin which occur during degradation can be determined. Such determinations can not be made using lignocelluloses. Furthermore, since synthetic lignin does not contain carbohydrates or other extraneous materials, it can be used to determine if an organism is capable of using lignin as a sole carbon source or for examining the ability of various compounds to serve as cosubstrates for lignin degradation. However, synthetic lignins are not associated with polysaccharides in plant tissue and the extrapolation of conclusions made from experiments using such an "isolated" lignin to lignin in plant tissue may not be possible or accurate. For example, organisms capable of degrading isolated lignin may not attack lignin in plant tissue and vice versa. Robinson and Crawford (102) found that Bacillus sp. could degrade

approximately 12% of (^{14}C-lignin) lignocellulose but only 0.3% of the ^{14}C-synthetic lignin to $^{14}CO_2$ in 20 d. Conversely, Odier et. al (99) isolated numerous strains of bacteria which could degrade more than 50% of acidolysis wheat straw lignin (an "isolated" lignin, albeit not synthetic lignin) but were unable to degrade (^{14}C-lignin) poplar wood.

Polyguaiacol

Recently, Crawford et al (21) described a procedure for the synthesis of (^{14}C)-polyguaiacol which they proposed may serve as a lignin model polymer. The (^{14}C)-guaiacol is polymerized using a procedure analogous to that used for the preparation of synthetic lignins. The polymer is composed of o-o and p-p linked guaiacol moieties and infrequently o-p biphenyl bonds and p-diphenoquinone structures. The polymer had an approximate molecular formula of $C_6H_{2.3}O_{0.3}$ Carbonyl $(OH)_{0.7}(OCH_3)_{1.0}$ and an average molecular weight between 5,000 and 15,000. Experiments with Phanerochaete chrysosporium suggested that the biodegradation of the polymer was affected by the same culture parameters as lignin degradation (see Lignin degradation by Phanerochaete chrysosporium). For example, (^{14}C)-polyguaiacol was degraded in low N cultures (2.4 mM N) but not in the presence of high N (24 mM N). Furthermore, the rate of degradation in an atmosphere of 80% O_2 - 20% N_2 was significantly greater than in an atmosphere of 21% O_2. Since the structure of polyguaiacol is less complicated and more easily defined than that of synthetic lignin, it may be easier to identify the events which occur during its degradation by white-rot fungi. However, the potential and limitations of this model substrate have not yet been fully explored.

Biological Degradation of Lignin

Introduction

The range of microorganisms conclusively shown to degrade lignin includes a wide variety of fungi and bacteria (4, 19). In addition, preliminary evidence suggests that certain yeasts can degrade lignin (70). Wood-decomposing fungi have been classified into three main groups (77) according to the effect they have on wood tissue: (a) white-rot fungi, (b) brown-rot fungi, and (c) soft-rot fungi. The characteristics of wood decomposition by these organisms will be discussed below.

Degradation of lignin by bacteria

Certain strains of Rhodococcus, Nocardia, Pseudomonas, Flavobacterium, Aeromonas and Streptomyces have been shown to degrade ^{14}C-synthetic lignin, ^{14}C-lignocellulose, milled wood lignin, dioxane-lignin, kraft lignin and various other industrial lignins (18, 28, 53, 72, 88, 110). The degradation of lignin by bacteria has recently been reviewed by Crawford and Crawford (19).

Degradation of lignin by white-rot fungi

Several hundred species of Hymenomycetes and a few species of Ascomycetes, collectively known as white-rot fungi, are capable of simultaneously effecting extensive degradation of all major components of wood (4, 77, 85, 86). During the degradation of wood by white-rot fungi, the lignin is concurrently depolymerized and condensed so that a portion of the lignin becomes higher molecular weight and a portion becomes lower molecular weight (48, 66, 84). Chemical and spectroscopic

analyses of the residual white-rot decayed lignin suggest that, in general, the degraded polymer is structurally more complex than the undegraded lignin (79, 80).

Previous investigators have shown lignin degradation by white-rot fungi to be highly oxidative in nature. Several investigators (60, 68, 80) have compared the empirical formula, total hydroxyl, carboxyl and α-carbonyl content for spruce wood lignin degraded by pure cultures of white-rot fungi to that of undegraded lignin (Table 1). In general, a progressive increase in oxygen content and a decrease in hydrogen content was observed which was reflected in an increase in oxygen containing functional groups following white-rot decay. For example, the carboxylic acid group content of spruce wood degraded by P. versicolor increased from 0.02 per C_9 to 0.106 per C_9 after 180 d of incubation (60). The oxidative nature of lignin degradation was also reflected in the observation that white-rot fungi did not degrade lignin in an atmosphere which contained 5% O_2 despite the fact that growth was normal (84). In addition, experiments conducted using ^{14}C-labelled synthetic lignins have shown that lignin is not degraded in many anaerobic environments 49).

Kirk and Chang (80) have shown that white-rot fungi were able to degrade the aromatic rings of the polymer while it is still in the extracellular environment. This was based upon the increase in α,β-unsaturated carboxylic acid moieties which were not derived from the cinnamic acid residues (in the side-chains) of the polymer. Analogous α,β-unsaturated carboxylic acids are known to be produced during the oxidation of low molecular weight aromatic compounds by microorganisms (11). A decrease in the absorbance at 1515 cm^{-1} in the

Table 1. Elemental and functional group analyses of lignin following decay by white-rot fungi.

Organism	Days Incubated	%C	%H	%O	$\%OCH_3$	per C_9 unit -OH	per C_9 unit -COOH	per C_9 unit C=O
Polyporus versicolor[a]	90	60.44	5.67	33.89	12.82	1.42	0.053	0.21
Polyporus versicolor[a]	180	58.54	5.23	36.23	11.75	1.67	0.106	0.28
	0	62.81	5.88	31.31	15.24	1.18	0.02	0.14
Polyporus versicolor[b]	168	57.97	4.70	37.23	11.33	-	0.55	0.17
Polyporus anceps[b]	105	58.71	4.99	36.30	11.21	0.87	0.56	0.16
	0[d]	62.85	6.08	31.07	15.11	1.16	0.12	0.07
Polyporus versicolor[c]	28	62.60	5.86	31.54	12.7	1.48	0.098	-
Fomes fomentarius	28	61.85	5.53	32.62	10.9	1.45	0.081	-
	0	63.45	6.21	30.34	15.9	1.20	0.030	-

[a] Analyses of degraded lignin extracted from wood meal with acetone-water (9:1) (from ref. 60)

[b] Analyses of degraded lignin extracted from decayed wood with dioxane-water (96:4) (from ref. 80)

[c] Analyses of degraded lignin which had been extracted from ball-milled wood prior to adding to liquid cultures. (from ref. 68)

[d] Milled spruce wood lignin.

infrared spectrum relative to other absorption bands and a decreased proportion of aromatic protons in the proton nuclear magnetic resonance spectrum of the degraded lignin provided further evidence for cleavage of the aromatic ring within the intact polymer.

In addition to aromatic ring cleavage, these fungi are apparently able to oxidatively shorten the aliphatic side chains of the intact polymer and to demethylate methoxyl groups in lignin (60, 68, 80, 109). Hata (60) hydrolyzed spruce wood lignin which had been degraded by Poria subacida and intact milled wood lignin and determined the yield of C_6-C_1 compounds as compared to C_6-C_3 compounds. After incubation with the fungus for six months, the yield of vanillic acid increased 5-fold whereas the yield of coniferyl alcohol decreased 6-fold. These results suggest that the terminal two carbons of the side chains of some substructures in lignin are removed to produce C_6-C_1 units which can be liberated by hydrolysis. Furthermore, there was approximately a 25% decrease in the methoxyl group content of the residual lignin decayed by P. versicolor (Table 1). However, extensive demethylation of methoxyl groups in lignin prior to its metabolism does not seem to occur since the methoxyl group content of the residual lignin was similar after 28 and 180 d of incubation (60, 80).

Summary of transformations effected in lignin by white-rot fungi

The following conclusions can be made regarding the extracellular transformation effected while lignin is still polymerized (60, 68, 79, 80): (1) The reactions are primarily oxidative. The degraded lignins show significant increases in oxygen containing functional groups such as carboxyl and carbonyl groups and a decreased

hydrogen content; (b) the C-α carbon of the propyl side chains are oxidized to carbonyl groups; (c) the aromatic rings are oxidatively cleaved; and (d) methoxyl groups are demethylated to yield hydroxyl groups. Identification and characterization of the low molecular weight products formed during lignin degradation has provided data to support the above conclusions and to suggest that hydroxylation at C-2 of some rings occurs (86). This presumably allows for intradiol ring cleavage following demethylation of the adjacent methoxyl groups. Thus, many of the structural elements of the polymer appear to be subject to oxidation during the course of lignin degradation by white-rot fungi.

Evidence for the involvement of enzymes in the initial steps in lignin degradation

Since the reactions discussed above occur in the extracellular environment, one would expect the agents responsible for these transformations to be found in the medium of ligninolytic cultures of white-rot fungi. However, surprisingly low levels of extracellular enzymes have been observed in these cultures (54). It has been suggested that two extracellular enzymes, phenol oxidase (3, 4, 67, 74, 75, 98) and aromatic alcohol oxidase (32, 64), are involved in the degradation of the lignin polymer. Aromatic alcohol oxidase, shown to be elicited by Coriolus versicolor and Fusarium solani, oxidizes a wide range of compounds that possess α,β-unsaturated primary alcohols, including those within the lignin polymer, to the corresponding aldehyde and hydrogen peroxide (32, 64). Phenol oxidases, which are produced by many, but not all lignin decomposing fungi (58) oxidize phenols to the corresponding quinones (8). These enzymes are incapable of effecting all of

the structural changes which occur during lignin degradation (8, 37) and their roles, if any, in lignin degradation are unclear. It is important to note that cell-free culture filtrates do not decompose lignin (41, 42, 54, 69, 109) and conclusive evidence for the involvement of any enzyme in lignin degradation is lacking.

Low specificity of ligninolytic system

Recent work with model compounds (see Model compound metabolism by P. chrysosporium) has provided evidence that some agents involved in the decomposition of lignin are relatively nonstereospecific (96). Methyldehydroconiferyl alcohol is a dilignol which contains a phenylcoumaran linkage and also has an α,β-unsaturated propyl side chain. When metabolized by P. chrysosporium the α,β-unsaturated side chain is converted to a glycerol moiety by nonsteroselective epoxidation followed by nonenzymatic hydrolysis of the epoxide to yield both erythro and threo isomers of the product. It was thought that an enzyme-mediated nonstereoselective epoxidation was unlikely.

Other, more general, observations suggest that at least certain components of the ligninolytic system have relatively low specificity. Lignin has been shown to be extensively degraded (>90%) upon prolonged incubation in pure cultures of white-rot fungi (13). This occurs despite the variety of intermonomer linkages that are known to occur and the racemic carbons in the polymer. Furthermore, lignin decomposing fungi are capable of degrading structurally different lignins including lignins in various species of plants (e.g. guaiacyl, syringyl and grass lignins) within different tissues of the same plant (e.g. early, late and compression wood) as well as industrially modified lignins

(e.g. kraft lignins and lignosulfonates) (7, 29, 77, 92). Thus, the ligninolytic system is capable of recognizing structurally different polymers as substrates. Furthermore, Keyser et. al. (73) suggested that the relatively low rates of lignin degradation by basidiomycetes, and the apparent inability of lignin to serve as a growth substrate for fungi, may indicate low activity of the ligninolytic system and, indirectly, that the system possesses low specificity. The data discussed above suggest that the agents responsible for lignin degradation in the extracellular environment are relatively nonspecific in their mode of attack.

Hydrogen peroxide production by white-rot fungi

Koenigs (87) found that 29 of 32 strains (representing 23 species) of white-rot fungi examined produced hydrogen peroxide (H_2O_2) which could be detected extracellularly. Koenigs (87) suggested the following role for H_2O_2 in the decomposition of wood by white-rot fungi:

> The H_2O_2 molecule, because of its small size and the accompanying lack of restricted substrate range inherent to an enzyme system would be much freer than is polyphenol oxidase, for example, to attack a polymer such as lignin. Formation by H_2O_2 of organic peroxides or free radical in lignin might lead to its subsequent depolymerization or furnish a "substrate" on which oxidation of lignin can occur.

However, later studies on the production of H_2O_2 by wood-decomposing fungi have been concerned with the involvement of oxygen radicals derived from H_2O_2 in cellulose decomposition by brown-rot fungi (88; also see Lignin degradation by brown-rot fungi). To my knowledge, studies on the role of H_2O_2 in cellulose or lignin degradation by white-rot fungi have heretofore not been conducted.

Degradation of lignin by brown-rot fungi

During wood decomposition by brown-rot fungi, the carbohydrates are extensively depleted but the lignin residue is only partially degraded (4, 13, 77). The decay is characterized by extensive depolymerization of the cellulose prior to its metabolism by the fungus. This is somewhat surprising since these fungi apparently do not produce β-1,4-endoglucanase (62, 97). These fungi have been shown to produce and excrete hydrogen peroxide (87) and it has been postulated that hydroxyl radicals derived from hydrogen peroxide in a Fenton type reaction may serve to depolymerize the cellulose in lignocellulose during wood decomposition by brown-rot fungi (87, 88, 89). Although Halliwell has shown that hydroxyl radicals effectively disrupt the crystalline structure of cellulose and reduce the degree of polymerization (56). Conclusive data to support this proposed mechanism of cellulose decomposition by brown-rot fungi is lacking.

Kirk and Adler (76) found that Poria monticola and Lenzites trabea extensively demethylate methoxyl groups in lignin. The demethylation of methoxyl groups in either units with free phenolic hydroxyl groups or in units with etherified phenolic groups, coupled with the hydroxylation of the aromatic ring at C-2, was found to result in the formation of o-diphenolic moieties (catechol) in lignin during brown-rot decay (76, 82). These moieties were thought to undergo autooxidation to produce quinone-type chromophores which give brown-rotted wood its characteristic color. Aromatic ring cleavage was minimal however and some o-diphenolic substructures were found to persist. There were twice as many α-carbonyl and carboxyl groups in lignin decayed by L. trabea as compared to sound lignin. In contrast, to white-rot fungi, brown-rot

fungi appear unable to oxidatively shorten the alkyl side chains of lignin.

In many respects, the structural alteration and decomposition of lignin by brown-rot fungi resembles that of white-rot fungi. Kirk (82) has suggested that the differing abilities of these two taxonomically related groups may be due to the inability of brown-rot fungi to metabolize aromatic rings or the aliphatic products of aromatic ring cleavage.

Degradation of lignin by soft-rot fungi

The ability of soft-rot fungi to degrade lignin has not been extensively investigated. These fungi, which include certain ascomycetes and fungi imperfecti, primarily metabolize the hemicelluloses and celluloses in wood (77). However, they have also been shown to demethylate methoxyl groups within lignin and to metabolize the side chains and aromatic ring elements within the polymer (51). Analyses on wood decayed by soft-rot fungi indicated the lignin component was partially degraded and modified by these fungi in such a way that a larger portion becomes acid soluble (50, 91). This is probably due to partial oxidation and a reduction in the molecular weight of the polymer effected by the fungi. Elsyn (30) found that four of six species studied depleted the carbohydrates in wood faster than lignin whereas the other two species depleted lignin faster than the carbohydrates.

Degradation of lignin by other fungi

Several fungi, which are not classified into the groups discussed above, have also been shown to decompose lignin. Strains of Fusarium oxysporus, Fusarium moniliforme, Fusarium solani and Fusarium sp. have been shown to decompose synthetic lignin (64). Size exclusion chromatography of lignins degraded by these fungi and degradation experiments conducted using synthetic lignins of various molecular weight ranges showed that the low molecular weight fractions were preferentially metabolized.

Drew and Kadam (24) found that when 1% soluble starch was provided as a cosubstrate, more than 50% of the added ^{14}C-labelled kraft lignin was converted to $^{14}CO_2$ by Aspergillus fumigatus in 16 d. This was roughly seven times greater than the rate observed for the white-rot fungus C. versicolor when grown under similar conditions. The ability of cell-free culture filtrates to degrade or modify lignin were rather limited although cellobiose-quinone oxidoreductase and phenol oxidase activities were demonstrated in these filtrates. Based on these studies, it would appear that certain strains of A. fumigatus and possibly other saprophytic fungi are able to rapidly and extensively degrade lignin.

Lignin Degradation by Phanerochaete chrysosporium

Introduction

Phanerochaete chrysosporium Burds, causes a typical white-rot type of decay and was isolated from beech (Fagus grandifolia) wood chips collected in Waterville, Maine in 1964 by W. E. Elsyn (strain ME 446, Center for Forest Mycology Research, Forest Products Laboratory, USDA, Madison, WI or ATCC 34541, American Type Culture Collection, Rockville, MD). The morphology and taxonomic criteria have been described by

Burdsall and Elsyn (9). The fungus is readily cultivated on either complex or chemically defined media, produces conidia in culture, has an optimum pH for growth of 4.5-5.0 and a temperature optimum of 40°C. The low level of phenol oxidase activity expressed by the fungus (73) minimizes the undesirable oxidation and polymerization of phenols during studies of lignin degradation. P. chrysosporium (originally called Peniophora "G") is synonomous with Sporotrichum pulverulentum, Chrysosporium lignorum and C. pruinosum and has been the subject of numerous investigations on the biological degradation of lignin (85, 86). Many of the cultural and nutritional parameters which affect lignin degradation have been established (5, 71, 73, 83, 84, 100) and methodologies for replica plating and mutant isolation have been described (43, 44).

Effect of nitrogen, carbon and sulfur

Lignin degradation by P. chrysosporium has been shown to be derepressed following the cessation of primary growth due to either N, C or S starvation (71, 73, 84, 100). The effect of N concentration on lignin degradation has been investigated in detail. In cultures of P. chrysosporium which contained 56 mM glucose and 2.4 mM N, the extracellular N was depleted after 2 d of incubation (73). The culture appeared to enter stationary phase on day 3 and ligninolytic activity first appeared after day 4. P. chrysosporium was shown to sequester N intracellularly as arginine during the first three days of incubation, during which time it accounted for 27-35% of the total amino acid N (33). During the transition period between the depletion of N in the medium and the onset of ligninolytic activity, the arginine pool decreased 5-fold. Concurrently, there was a rapid increase in

intercellular protein turnover which occurred at maximal rates of 5-7% per hour.

Further studies showed that ligninolytic activity was repressed by nitrogen (73). If 24 μmoles NH_4^+ ml^{-1} was added to the cultures on day three, the appearance of ligninolytic activity was delayed (73). Furthermore, when 2.4 μmoles $NH_4^+ml^{-1}$ was added to 6 day old, actively ligninolytic cultures, lignin degradation was not affected for 16 h, but then was transiently repressed for 40-50 h (73). All of the fourteen nitrogenous compounds examined repressed ligninolytic activity in a manner similar to NH_4^+ when added at equimolar N concentrations, but with varying degrees of effectiveness (33). Glutamate, glutamine and histidine were the most effective and repressed ligninolytic activity by 83, 76, and 76% respectively, as compared to cultures to which no N was added. Ammonium chloride repressed ligninolytic activity approximately 50%. Protein synthesis increased when these various N sources were added. However, the stimulation was not correlated to the relative abilities to serve as N sources for growth or to repress ligninolytic activity. Therefore, the repressive effects of theses compounds does not seem to be due to a resumption of primary growth of the fungus.

The intracellular pools of arginine and glutamine increased sharply following the addition of NH_4^+, but not glutamate, to nitrogen starved cultures (34). Thus high levels of these amino acids did not appear to be essential for the initiation or maintainence of repression. Glutamate pools increased immediately following the addition of either NH_4^+ or glutamate to ligninolytic cultures. However, the pool size returned to that found in control cells by 18 h after glutamate addition, but

the repression of ligninolytic activity persisted. Thus it would appear that neither arginine, glutamate or glutamine is directly involved in the repression of ligninolytic activity but rather the effect of N appears to be a more general one. This conclusion is further supported by the inhibition of the synthesis of the secondary metabolite (3,4-dimethoxy) benzyl alcohol (veratryl alcohol) observed upon the addition of N to nitrogen starved cultures.

Cycloheximide, a protein synthesis inhibitor, prevented the appearance of ligninolytic activity when added to nitrogen limited cultures 3-5 d after inoculation (73). These results indicate that the ligninolytic system, or at least an essential component, is formed de novo following the depletion of N in the culture. When added to actively ligninolytic cultures, cycloheximide suppressed ligninolytic activity in a manner similar to NH_4^+ and glutamate. It would appear that certain nitrogenous compounds repress key enzyme(s) involved in lignin degradation by P. chrysosporium. Repression of ligninolytic activity, as opposed to inhibition or competition, by N is supported by the following: (a) Repression but not inhibition or competition would be expected to take several hours for maximum development, (b) the kinetics of repression by cycloheximide, NH_4^+ and glutamate were found to be nearly identical, and (c) ligninolytic activity was found to be derepressed in response to carbon limitation in the presence of nitrogen at concentrations which would be suppressive in nitrogen starved cultures (discussed below). The repression does not appear to be mediated by suppression of central carbon metabolism, since both glutamate and NH_4^+ stimulated the oxidation of glucose and succinate but repressed the production of $^{14}CO_2$ from ^{14}C-lignin (34).

Kirk et al (83) have clearly demonstrated the inability of lignin to serve as a sole carbon source for growth of P. chrysosporium. Therefore, it is somewhat surprising that ligninolytic activity is derepressed in carbohydrate limited medium following the depletion of carbohydrate (71). The extent of (^{14}C)-synthetic lignin degradation observed in carbohydrate limited cultures was positively correlated to the amount of carbohydrate added and therefore to the amount of mycelium formed in the culture. Mycelial dry weight was found to decrease immediately after the carbohydrate was exhausted and the appearance of ligninolytic activity was associated with this event. The mycelial dry weight decreased throughout the period in which lignin was degraded and no further decrease was observed when lignin degradation stopped (73, 100). P. chrysosporium has been shown to produce copious amounts of extracellular polysaccharide and to have intracellular carbon reserves (L. Forney, S. Pankratz and C. A. Reddy, unpublished). Therefore, it seems likely that upon exhaustion of glucose, the necessary energy to support lignin degradation is provided by extracellular or intracellular reserves. This is consistent with the observation that lignin degradation does not occur in the absence of a cosubstrate and that the extent of degradation observed is dependent on the amount of substrate available (71, 83).

The derepression of ligninolytic activity in carbohydrate limited cultures following the depletion of carbohydrate, despite the presence of high levels of nitrogen in the medium, is somewhat surprising in view of the established ability of N to inhibit lignin degradation. In an attempt to clarify this, Jeffries et al (71) examined the effect of NH_4^+, glutamate and α-ketoglutarate addition on lignin degradation in

carbohydrate limited cultures. When glutamate was added to carbohydrate limited cultures, lignin degradation decreased approximately 50% as compared to control cultures. The inhibition was not due to the presence of additional nitrogen in the medium since the addition of an equimolar amount of NH_4^+ had no effect on lignin degradation. Nor was the effect due to added carbon as an equimolar addition of α-ketoglutarate stimulated lignin degradation by approximately 30%. The effect of adding glutamate and α-ketoglutarate was the same as adding glutamate only. α-Ketoglutarate and NH_4^+ stimulated lignin degradation to a degree equal to that observed upon addition of α-ketoglutarate only. Therefore, in carbohydrate limited cultures, glutamate per se inhibits lignin degradation, however, the mechanistic basis for this has not been established. The repressive species produced upon the addition of N to nitrogen starved ligninolytic cultures of P. chrysosporium is apparently not produced by the fungus under carbohydrate limited conditions, despite the presence of high levels of N.

Lignin degradation was also found to be derepressed in cultures which initially contained limiting levels of $SO_4^=$ (20 μM) and excess carbohydrate and nitrogen after 7 d of incubation (71). The sulfur concentration in the medium at that point was not determined. When the initial $SO_4^=$ concentration in this medium was 200 μM, ligninolytic activity did not appear. Dual limitation of $SO_4^=$ and nitrogen did not result in more rapid or more extensive degradation than when only nitrogen was limiting. Unfortunately, the effect of these culture conditions on mycelial dry weight was not examined. The basis of the observed effect of sulfur on lignin degradation by P. chrysosporium is not known.

In summary, ligninolytic activity in cultures of P. chrysosporium was derepressed following the cessation of growth due to N, C or S starvation and developed as a part of idiophasic growth. The

complex regulation of the process is not well understood, however, it would appear to be associated with the shift to secondary metabolism in the cells.

Effects of oxygen partial pressure

Oxygen partial pressure has been shown to have a profound effect on the rate and extent of lignin degradation by P. chrysosporium (73). When cultures limited in nitrogen were incubated in 0.2 atm of O_2, lignin degradation appeared on day 4 followed by a linear rate of degradation from day 8 through day 28, at which time roughly 40% of the lignin had been degraded. When the cultures were incubated in 1 atm of O_2, the onset of lignin degradation was unaffected, however, the rate of degradation was significantly greater. There was a very rapid evolution of $^{14}CO_2$ from day 4 to day 12 during which 40% of the lignin was degraded. Lignin was not degraded during a 35 d incubation period in 0.05 atm of O_2. The effects of O_2 partial pressure on lignin degradation were also reflected in the molecular weight profile of the residual lignins from these cultures. Lignin from cultures grown in 0.8 atm and 1 atm O_2 was extensively depolymerized whereas little depolymerization of lignin was seen in cultures grown in 0.05 atm of O_2. Thus, lignin degradation by P. chrysosporium in 1 atm of O_2 is roughly 2.5 times greater than in 0.2 atm of O_2, and is not degraded at all by the fungus under low pO_2 (0.05 atm).

The oxygen partial pressure does not appear to affect fungal growth, or the level of glucose metabolic enzymes. Bar-lev and Kirk (5) grew P. chrysosporium in 0.2 atm of O_2 for 2.5 days and then shifted to either 0.2 atm or 0.8 atm of O_2 for 5 days and determined the rate at

which glucose and lignin were metabolized. The rate of ^{14}C-synthetic lignin oxidation to $^{14}CO_2$ was 5-fold greater in cultures which were incubated in 0.8 atm of O_2, whereas the rate of glucose metabolism was the same under both atmospheres. Based on these results, the authors suggested that immediately preceding the appearance of the lignin degrading system the partial pressure of O_2 determines the amount of ligninolytic activity that develops in cultures. To determine the effect of O_2 partial pressure on glucose metabolism and the activity of the ligninolytic system after its appearance, cultures were grown in 0.2 atm O_2 for 2.5 days and then maintained under 0.8 atm of O_2 for an additional 4 days at which time the cultures were actively ligninolytic. The rates of glucose and lignin oxidation in these 6.5 day old cultures were then determined under different O_2 atmospheres. Oxidation of both substrates was enhanced by increasing O_2 partial pressure. The oxidation of both glucose and lignin was maximal at 0.4 atm O_2 but was not significantly lower at 0.8 atm O_2. These results indicate that both glucose and lignin metabolism were stimulated by high partial pressure of O_2; however, a satisfactory explanation for these observations is lacking.

Effect of culture agitation

Culture agitation has also been shown to have a marked yet rather anomalous effect on lignin degradation by P. chrysosporium (84). Lignin degradation was rapid and extensive in stationary cultures incubated under 1 atm O_2, but not degraded in cultures which were shaken from the time of inoculation. Similarly, only 4% of the added lignin was degraded in agitated cultures incubated under 0.2 atm O_2 for 34 d as compared to the 16% degraded in stationary cultures

during the same period. Kirk et al (84) suggested that the "pellet" form of growth that results upon agitation entraps the lignin within the pellet wherein the effective O_2 concentration was too low to allow for the efficient degradation of lignin. This explanation was supported by the fact that pelleted cultures, when incubated without agitation, formed mycelial mats and decomposed lignin at a rate and extent proportional to the amount of lignin which had not previously associated with the pellets. In other experiments, cultures with different partial pressures of O_2 were incubated without agitation for 9 d to allow for the formation of mycelial mats and then agitated under 0.2 atm O_2. Lignin degradation was unaffected by agitation in these cultures. However, in contrast to those results, lignin degradation was dramatically suppressed in cultures under 1.0 atm O_2 upon agitation of the culture. The basis for the differential effect of agitation on these cultures is not clear at this time.

Metabolism of Lignin Model Compound by P. chrysosporium

β-Guaiacyl ether linked dimeric lignin model compounds.

The metabolism of dimeric lignin model compounds with β-ether linkages by P. chrysosporium has been extensively investigated (27, 28, 45, 96, 113). The β-ether linkage constitutes a major intermonomer linkage in lignin and hence the study of the metabolism of these compounds may be useful to elucidate the metabolism of these substructures in the lignin polymer.

Regulation of metabolism of β-guaiacyl ether linked model compounds.

The metabolism of some of these model compounds has been found to be regulated in a manner similar to that of the lignin polymer.

Weinstein et al (113) examined the effect of culture parameters on the metabolism of ^{14}C-labelled guaiacylglycerol-β-guaiacylether (I) and veratrylglycerol-β-guaiacyl ether (II) (Figure 5) by P. chrysosporium. In nitrogen limited (1.2 mM N) cultures these compounds were found to be metabolized only after the culture entered the stationary phase of growth and in response to N starvation. In cultures grown with 12.0 mM N, the $^{14}CO_2$ released from I and II was roughly 10-15% of that observed in nitrogen limited cultures. Furthermore, the evolution of $^{14}CO_2$ from I and II in nitrogen limited cultures was repressed upon the addition of NH_4^+ to the culture and was stimulated 2-3 fold in cultures incubated under 1.0 atm O_2 as compared to those incubated in an atmosphere of air (0.2 atm O_2). After correcting for differences in cell yield the metabolism of I and II in agitated cultures was 12.5% and 7.5%, respectively, of that observed in stationary cultures. Thus, the effect of nitrogen concentration, O_2 partial pressure and culture agitation on the metabolism of these model compounds is analogous to their effect on ligninolytic activity (5, 73, 84) and it may be possible to extrapolate conclusions from these studies to lignin degradation by P. chrysosporium.

Metabolism of model compounds containing phenolic ethers.

It is generally thought that lignin model compounds containing phenolic ethers are useful models of "internal" substructures in lignin. Whereas compounds with free phenolic hydroxyl groups more closely reflect peripheral substructures in lignin.

Goldsby et al (45) studied the metabolsim by P. chrysosporium of guaiacylglycerol-β-guaiacyl ether (I), which has a free phenolic hydroxyl group. 3-Hydroxy-2-(o-methoxyphenoxy)propionic acid (III) and

2-(o-methoxyphenoxy)1,3-propanediol (IV) were isolated as degradation products, suggesting that cleavage of the alkyl-phenyl bond had occurred. Cleavage of the alkylphenyl bond also occurred during the metabolism of α-deoxyguaiacylglycol-β-guaiacyl ether (V) to 2-(o-methoxyphenoxy) ethanol (VI). The latter compound was also observed as a product of the metabolism of the α-hydroxy analog, guaiacylglycol-β-guaiacyl ether (VII). Similar results were obtained using α-deoxyguaiacylglycerol-β -guaiacyl ether (VIII). The nature of the γ-carbon of the side chain does not appear to be critical since (IV), which lacks a γ-carbon appears to be metabolized by a mechanism similar to (V). These results suggest that cleavage of the alkyl-phenyl bond proceeds via an α-hydroxylated intermediate.

Interestingly, the α-deoxy model compounds (V, VIII) were only metabolized in nitrogen-limited stationary cultures whereas the α-hydroxylated model compounds (VII, I), which were the products of the first reaction in the metabolism of V and VIII, respectively, were metabolized in nitrogen sufficient, agitated cultures. Similarly, VI, IX and XI were metabolized in nitrogen sufficient, agitated cultures. This strongly suggests that regulation of the metabolism of certain lignin model compounds and lignin by nitrogen is at the step of hydroxylation of the α-carbon of the side chains.

4-Ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether (XIII), which contains a phenolic ether, was metabolized by P. chrysosporium only in nitrogen limited cultures (1.2 mM N) and not in nitrogen sufficient media (12mM N) (27, 28). The β-ether linkage of XIII was cleaved to produce 4-ethoxy-3-methoxyphenyl glycerol (XVI) and guaiacol (XVII). The α-deoxy and γ-deoxy analogs (XIV and XV, respectively) were

metabolized at rates comparable to XIII and resulted in the formation of analogous products. The product of the metabolism of the α-deoxy analog, 4-ethoxy-3-methoxyphenyl-2,3-dihydroxypropane (XVIII), was metabolized through two pathways. In one pathway the α-carbon of XVIII is hydroxylated to form 4-ethoxy-3-methoxyphenyl glycerol (XVI) which is then cleaved between the α and β carbons to form 4-ethoxy-3-methoxybenzyl alcohol (XIX). Alternatively, β,γ cleavage of the side chain of XVIII occurred to form 4-ethoxy-3-methoxyphenyl-2-hydroxy ethane (XX). In analogous reactions, guaiacol and 4-ethoxy-3-methoxyphenyl-1,2-dihydropropane (XXI) were the products of the metabolism of the γ-deoxy analog (XV). The side chain of XXI was then cleaved between the α and β carbons to form (XIX). The triol was metabolized by both cleavage of the α,β bond and the β,γ bond.

These results indicate that the catalyst which initiates the metabolism of β-guaiacyl ether linked model compounds containing phenolic ethers is quite nonspecific and requires neither the α nor γ hydroxyl group of the side chain. Previous studies have shown that the cleavage of the β-ether bond in other model compounds is catalyzed by phenol oxidases and by cell-free culture filtrates. The reactions were shown to proceed by oxidation (radicalization) of the free phenolic hydroxyl group (75). The mechanism by which XIII, XIV and XV were metabolized was clearly different since cleavage of the phenolic ether to generate a free phenolic hydroxyl groups was not a prerequisite for β-ether cleavage. The agents which catalyze the cleavage of the side chains of XVIII and XXI were also shown to exhibit low specificity as both the α,β dihydroxy and the β,γ dihydroxy homologs were metabolized.

These studies on the metabolism of β-guaiacyl ether linked dimeric lignin model compounds suggest that model compounds and analygous

substructures in lignin which have free phenolic hydroxyl groups are metabolized via cleavage of the α,β bond of the side chain. In contrast when the structure has a phenolic ether present, the β-ether linkage of the side chain is cleaved.

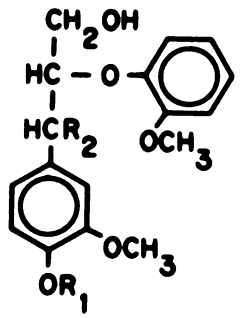
Phenylcoumaran linked dimeric lignin model compounds.

The phenylcoumaran substructure accounts for 6% and 9-12% of the intermonomer linkages in birch and spruce wood lignins, respectively (1). The initial reactions in the metabolism of methyldihydroconiferyl alcohol (XXII), a model compound with a phenylcoumaran linkage, by P. chrysosporium were elucidated by Nakatsubo et al (96). The first step in the conversion of XXII was the formation of XXIII via hydroxylation of the cinnamyl alcohol moiety. Both erythro and threo forms were produced suggesting that the oxidation of the double bond was not stereoselective or that subsequent reactions cause recemization. To rationalize these results Nakatsubo et al (96) suggested that the insertion of oxygen at the α and β carbons is probably not enzymatically controlled. The reaction probably proceeds by nonstereoselective expoxidation of C_α - C_β followed by nonenzymatic hydrolysis of the epoxide. The side chain is then cleaved between the α and β carbons in a reaction analogous to that observed for β ether compounds by Weinstein et al (113) and Enoki et al (27, 28) and the α carbon is oxidized to a carboxylic acid (XXIV).

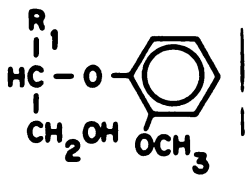
The nonstereoselective oxidation of the cinnamyl alcohol moiety is extremely interesting. It is consistent with the widely held belief that the ligninolytic system of white-rot fungi must be nonspecific and

supports the suggestion (2, 55, 95) that oxygen radicals are involved in lignin degradation.

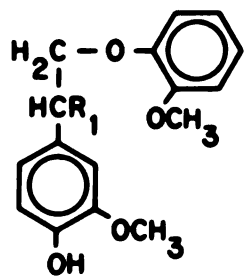
Figure 5. Structures of dimeric lignin model compounds and products of their metabolism (compounds I - XXIV).



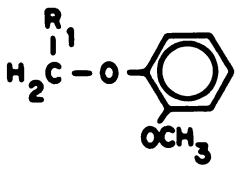
I	$R_1 = R_2 = -OH$		Guaiacylglycerol-β-guaiacyl ether
II	$R_1 = -CH_3$	$R_2 = -OH$	Veratryl glycerol-β-guaiacyl ether
VIII	$R_1 = -OH$	$R_2 = -H$	α-deoxyguaiacylglycerol-β-guaiacyl ether



III	$R_1 = -COOH$	2-(o-methoxyphenoxy) propionic acid
IV	$R_1 = -CH_2OH$	2-(o-methoxyphenoxy)-1,3-propanediol

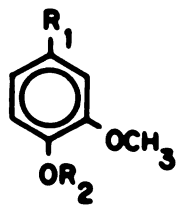


V	$R_1 = -H$	α-deoxyguaiacylglycol-β-guaiacyl ether
VII	$R_1 = -OH$	guaiacylglycol-β-guaiacyl ether



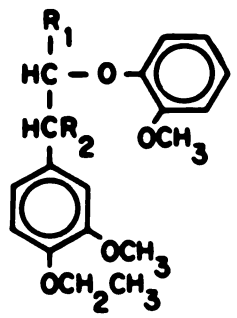
VI R_1 = $-CH_2OH$ 2-(o-methoxyphenoxy) ethanol

IX R_1 = $-COOH$ 2-(o-methoxyphenoxy) acetic acid



XI R_1 = $-CH_2OH$ R_2 = $-CH_3$ veratryl alcohol

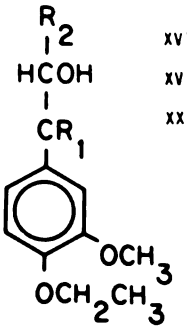
XVII R_1 = $-H$ R_2 = $-OH$ guaiacol



XIII R_1 = $-CH_2OH$ R_2 = $-OH$ 4-ethoxy-3-(methoxyphenyl)glycerol-:-guaiacyl ether

XIV R_1 = $-CH_2OH$ R_2 = $-H$ 1-(4'-ethoxy-3'-methoxyphenyl)-2-(2"-methoxyphenoxy)-3-hydroxypropane

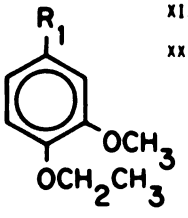
XV R_1 = $-CH_2$ R_2 = $-OH$ (4'-ethoxy-3'-methoxyphenyl)-2-(2"-methoxyphenoxy)-1-hydroxypropane



XVI R_1 = -OH R_2 = $-CH_2OH$ 4-ethoxy-3-methoxyphenyl glycol

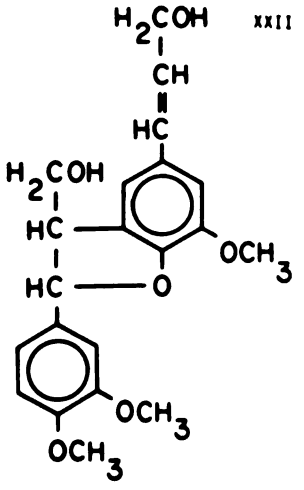
XVIII R_1 = -H R_2 = $-CH_2OH$ 4-ethoxy-3-methoxyphenyl-2,3-dihydroxypropane

XXI R_1 = -OH R_2 = $-CH_3$ 4-ethoxy-3-methoxyphenyl-1,2-dihydroxypropane

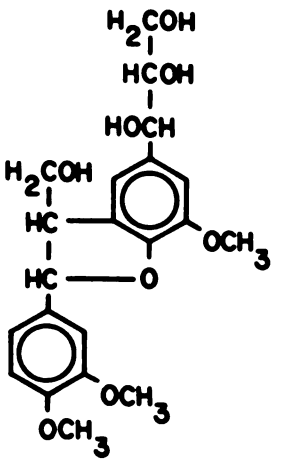


XIX R_1 = $-CH_2OH$ 4-ethoxy-3-methoxybenzyl alcohol

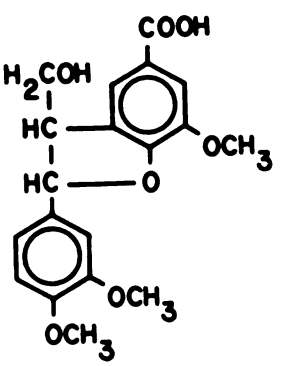
XX R_1 = $-CH_2CH_2OH$ 4-ethoxy-3-methoxyphenyl-2-hydroxyethane



XXII methyldehydroconiferyl alcohol


H2COH
HCOH
HOCH
H2COH
HC
HC
O
OCH3
OCH3
OCH3

XXIII


COOH
H2COH
HC
HC
O
OCH3
OCH3
OCH3

XXIV

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CHAPTER I

Biological Studies on Lignin Degradation: A Simple Procedure for the Synthesis from Ferulic Acid of Coniferyl Alcohol, a Precursor of Synthetic Lignin

A relatively simple procedure for the conversion of ferulic acid to coniferyl alcohol, a precursor of synthetic lignin, is described. In this procedure, unlabeled or (2'-^{14}C) ferulic acid was reacted with N,O-bis-(trimethyl-silyl) acetamide in dioxane-pyridine (10:1) to form the trimethylsilyl ester of ferulic acid which was then reduced to coniferyl alcohol with $LiAlH_4$. Excess $LiAlH_4$ was destroyed with NaOH, the organic solvent layer was evaporated and the precipitated aluminum salts were removed by filtration. The coniferyl alcohol in the filtrate was extracted into ethyl ether, which was washed with water and dried with Na_2SO_4. The yield of coniferyl alcohol was approximately 47%. The purity and identity of the coniferyl alcohol was confirmed by gas chromatography, high pressure liquid chromatography and mass spectrometry. The unlabeled or (2'-^{14}C) coniferyl alcohol synthesized was polymerized to produce unlabeled or (2'-^{14}C) synthetic lignin, respectively. The synthetic lignin was similar to synthetic lignins described in the literature in elemental composition, gross structural characteristics and in its susceptibility to degradation by Phanerochaete chrysosporium.

INTRODUCTION

In recent years, investigators have used synthetic lignin (a dehydrogenated polymer of coniferyl alcohol), which contains inter-monomer linkages similar to those in naturally occurring lignins (4,10), as a model substrate for studies on the biological degradation of lignin (2,6,8,13,14,16). Although unlabeled or ^{14}C-coniferyl alcohol, the common precursors for synthetic lignin, are available commercially, they are relatively expensive. Hence, there is a need for developing a relatively simple and inexpensive procedure for the synthesis of unlabeled or ^{14}C-coniferyl alcohol.

Existing methods for the synthesis of coniferyl alcohol are relatively complex (1,3,5,7,10,13). In contrast, the reduction of ferulic acid (4-hydroxy-3-methoxycinnamic acid) to coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol) has several advantages: (1) Only a few steps are required for the conversion of ferulic acid to coniferyl alcohol; (2) Ferulic acid is relatively inexpensive and is readily available commercially; and (3) An established simple procedure (15) exists for synthesizing (2'-^{14}C) ferulic acid which could be used as a precursor for synthesizing (2'-^{14}C) synthetic lignin.

In this paper we describe a simple method for the synthesis of coniferyl alcohol from ferulic acid which was then utilized in the preparation of synthetic lignins for use in studies of lignin biodegradation.

MATERIALS AND METHODS

Synthesis of (2'-^{14}C) ferulic Acid. (2'-^{14}C) ferulic acid was synthesized using the procedure of Pearl and Beyer (15). In this procedure vanillin and (2'-^{14}C) malonic acid were added to pyridine which contained a small amount of piperidine and stirred in the dark. After 3 weeks, the reaction was acidified with HCl and the precipitated ferulic acid was filtered from the solution, washed with distilled water and dried under vacuum in an oven. The yield of ferulic acid was 78% (based on the initial amount of vanillin added).

Synthesis of coniferyl alcohol. The trimethylsilyl derivative of ferulic acid was synthesized by adding 2.50 g ferulic acid (Aldrich Chemical Co., Milwaukee, WI), 50 ml dioxane-pyridine (10:1) and 40 ml N,O-bis-(trimethylsilyl) acetamide (BSA; Sigma Chemical Co., St. Louis, MO) to a 500 ml round bottom flask which was closed with a drying tube containing silica gel. The reaction mixture was stirred for 30 min at room temperature, cooled in a dry ice-ethanol bath, and 1.5 g $LiAlH_4$ (Aldrich Chemical Co.) was gradually added to reduce the trimethylsilyl ester to coniferyl alcohol. After evolution of H_2 had subsided, the reaction mixture was refrigerated for 12 h. The residual $LiAlH_4$ was destroyed by slowly adding 30 ml of 1 M NaOH to the reaction mixture held in an ice bath. After evaporation of the organic layer under a stream of air, the aluminum salts were removed by filtration on a Whatman GF/C filter (Whatman Inc., Clifton, NJ), washed with 70 ml of 1 M NaOH and the washings were added back to the alkaline reaction mixture. The pH of the reaction mixture was adjusted to 7.0 with 1 M HCl and extracted four times with 500 ml portions of ethyl ether.

The ethyl ether extracts were then washed with 125 ml distilled water, pooled and dried with anhydrous Na_2SO_4. The volume of the ethyl ether extracts was reduced from 2 1 to 500 ml by evaporation under a stream of air.

^{14}C-coniferyl alcohol was synthesized exactly as described above except that 2-^{14}C ferulic acid, instead of unlabeled ferulic acid, served as the substrate.

Analysis of coniferyl alcohol. The coniferyl alcohol was quantified by ultraviolet spectroscopy, gas chromatography (GLC) and high performance liquid chromatography (HPLC). GLC and HPLC were also used to determine the purity of the coniferyl alcohol.

For quantification by UV spectroscopy, 10-20 µl of the coniferyl alcohol sample was added to 3.0 ml of absolute ethanol and the absorbance at 292 nm was measured with a Varian 634 S spectrophotometer (Varian Instruments, Palo Alto, CA). The yield of coniferyl alcohol was calculated using an extinction coefficient of 5.85×10^2 l $mole^{-1}cm^{-1}$ (10).

GLC analyses were performed on a Varian 2440 gas chromatograph (Varian Instruments, Palo Alto, CA) which was interfaced with a Varian CDS-111 computing integrator. A 2 m x 0.32 mm I.D. stainless steel column packed with 5% SE-30 on 100/120 mesh G-AW-DMCS was used. The chromatograph oven temperature was held at 215°C or was programmed to hold at 180° C for 2 min and then to increase to 280°C at 4°C min^{-1}. Samples of coniferyl alcohol solution in ethyl ether (10-80 µl) were added to a small vial, the solvent was evaporated and 100 µl of dioxane-pyridine (10:1) and 50 µl of BSA were added. The vial was then sealed

and allowed to incubate at room temperature for 30 min prior to analysis. Coniferyl alcohol standards were prepared in an identical manner.

HPLC analyses were performed using a Varian 5000 liquid chromatograph (Varian Associates, Palo Alto, CA) fitted with a Rheodyne model 7125 injector, 254 nm ultraviolet detector and interfaced with a Spectra-Physics model 4100 computing integrator. A 30 cm x 4 mm MCH-10 Micropac C_{18} reverse phase column (Varian Associates) was used with 50:50 methanol-water (flow rate 2.0 ml min^{-1}) as the eluting solvent. For preparing samples for HPLC analysis an appropriate amount of coniferyl alcohol sample (dissolved in ethyl ether) was added to a vial, the solvent was evaporated and 500 µl of methanol was added. Coniferyl alcohol standards were prepared in an identical manner.

Samples for gas chromatography-mass spectrometry analysis were prepared as described above for GLC analysis. The analysis was performed using a Hewlett-Packard 5840A gas chromatograph with a Hewlett-Packard 5985 GC-MS (Hewlett-Packard Co., Palo Alto, CA). The column packing was the same as that described above. A 6 ft x 0.25 in glass column was used, and He at 29 ml min^{-1} was the carrier gas. The chromatograph oven was temperature programmed to hold at 185°C for 2 min and to increase at 6°C min^{-1} to 230°C. The injector temperature was 240°C.

Synthesis of dehydrogenative polymers of coniferyl alcohol. The volume of the coniferyl alcohol solution in ethyl ether was reduced from 500 ml to 10-20 ml under a stream of air. A known amount of previously degassed (boiled and bubbled with N_2 while cooling) 0.01 M sodium phosphate buffer (pH 6.5) was added. The remaining ethyl ether was evaporated

under a gentle stream of air. The procedure of Kirk et al. (10) was used for the polymerization of the coniferyl alcohol except that the quantities of the reagents used were proportionally altered depending on the amount of coniferyl alcohol used. The synthetic lignins were stored at -10°C as suspensions in distilled water and were lyophilized as needed before use.

Side chain (2'-^{14}C) labeled lignin was prepared from ^{14}C-coniferyl alcohol in a manner identical to that described above. The specific radioactivity of the ^{14}C-synthetic lignin was determined by adding a known amount (approximately 50 μg) of this lignin to dioxane-based liquid scintillation cocktail (DB-LSC, 6) to which a small amount of water had been added (0.3 ml water to 1.5 ml cocktail). 50 μl of this solution was then transferred to aqueous DB-LSC (0.5 ml water per 10 ml DB-LSC) and the radioactivity was determined to within ± 1% by counting three replicate vials using a Searle Model 300 liquid scintillation counter (Searle, Arlington Heights, IL).

Characterization of synthetic lignin. Elemental analysis (C,H,N) of the synthetic lignin was determined using a Carlo-Erba model 1104 elemental analyzer or by Galbraith Laboratories (Knoxville, TN). The oxygen content was determined by difference [100-(%C + %H + %N)]. The values for C, H, and O were then normalized to 100%.

Infra-red (IR) spectra of the synthetic lignin and milled wood lignin (spruce-MWL, provided by Dr. T. K. Kirk) in KBr pellets (approximately 2 mg of dry lignin per 200 mg KBr) were determined using a Perkin-Elmer model 700 IR spectrophotometer (Perkin-Elmer, Norwalk, CT).

Degradation of (2'-^{14}C) synthetic lignin by Phanerochaete chrysosporium. A culture of Phanerochaete chrysosporium Burds. (ME-446) was provided by Dr. T. K. Kirk, Forest Products Laboratory, U.S. Department of Agriculture, Madison, WI and was maintained by periodic transfer on malt extract agar. The inoculum consisted of a conidia suspension which was prepared as described by Kirk et al. (10) except 7-10 day old cultures of P. chrysosporium cultures were used and the final optical density at 600 nm of the condial suspension was 0.5 (1.8 cm light path). the conidial suspensions were stored at -10°C until used and thawed at room temperature prior to adding 0.5 ml per 10 ml of culture medium.

Degradation of ^{14}C-synthetic lignin by P. chrysosporium was conducted in a medium which contained the following per liter of distilled water: KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCl_2$, 0.01 g; 2,2-dimethyl-succinate, 1.46 g; D-glucose, 10.0 g; NH_4NO_3, 0.048 g; L-asparagine, 0.09 g; mineral solution (11), 1.0 ml; and vitamin solution (11), 0.5 ml. The final concentration of nitrogen was 2.4 mM and the final pH was adjusted to 4.5 with NaOH. The medium was dispensed (10 ml per flask) in 125 ml Erlenmeyer flasks and sterilized by autoclaving at 121°C for 15 min.

The (2'-^{14}C) synthetic lignin (specific activity of 2.8×10^5 dpm mg^{-1}) and identically prepared unlabeled synthetic lignin were mixed to obtain a lignin preparation of desired specific activity. This synthetic lignin and (U-ring-^{14}C) synthetic lignin (0.78×10^5 dpm mg^{-1}, supplied to us by Dr. T. K. Kirk) were added separately to N,N-dimethylformamide (DMF) to give 5% solutions. These DMF solutions were slowly added to sterile distilled water (7 µl DMF/0.5 ml water) and

one-half ml (0.35 mg) of these aqueous suspensions were added to each flask. Using this procedure, no contamination of the cultures from the addition of lignin was observed.

Culture flasks were closed with rubber stoppers which permitted periodic flushing of the culture headspace as previously described (11) except that the effluent tubing was connected to a 1 cc syringe barrel with a 1 in 25 gauge needle. Every third day of the experiment the flasks were flushed with CO_2-free air at a rate of 60 ml min^{-1} for 45 min. The respired CO_2 was trapped in 10 ml of a liquid scintillation coctail which contained ACS (Amersham-Searle, Arlington Heights, IL)-methanol-ethanol amine (Aldrich Chemical Co., Milwaukee, WI) (50:40:10). The efficiency of $^{14}CO_2$ trapping using this procedure was 97% (L. J. Forney and C. A. Reddy, 1980, unpublished data). The degradation data are expressed as % of added ^{14}C recovered as $^{14}CO_2$ after correcting for the efficiency of $^{14}CO_2$ trapping, and counting efficiency (typically 85-89%) as determined by sample channels ratio method. Values from four replicate flasks were used to calculate the mean for each data point.

RESULTS

Synthesis of coniferyl alcohol. Ferulic acid reacts with N,O-bis-(trimethylsisyl) acetamide (BSA) to form the trimethylsilyl (TMS) ester of ferulic acid (Fig. 1). The phenolic hydroxyl group of ferulic acid also reacts with BSA to form a TMS ether. The ester was then reduced by adding $LiAlH_4$ with the concomittant formation of an alcohol group. After allowing the reduction reaction to proceed for 12 h, 1 M NaOH was added to destroy the excess reducing agent and to regenerate the phenolic hydroxyl group by hydrolyzing the phenolic ether bond. The coniferyl alcohol was recovered as described in Materials and Methods.

The purity of the coniferyl alcohol, obtained was determined by GLC and HPLC. Gas chromatography revealed that the coniferyl alcohol sample was pure as evidenced by a single peak which had a retention time identical to that of an authentic coniferyl alcohol. Temperature programming of the chromatograph oven from 180°C (hold for 2 min) to 280°C at 4°C min^{-1} failed to show the presence of any other compound (data not shown). Using HPLC elution of the sample with 50% aqueous methanol showed the presence of one major peak which had the same elution volume as the coniferyl alcohol standard (Fig. 2). The identity of the minor compound which eluted at 3.6 min in chromatograms of both sample and standards is unknown although it is not ferulic acid (which elutes at 1.4 min). Elution of the sample with 100% methanol failed to reveal additional compounds in the ether extract (data not shown). These results indicate the absence of any extraneous compounds capable of absorbing at 254 nm (i.e., aromatic compounds).

The mass spectra of the coniferyl alcohol sample and coniferyl alcohol standard showed nearly identical fragmentation patterns with parent ions of mass 324 which corresponds to the mass of the TMS derivative of coniferyl alcohol (Fig. 3). Presumably, one TMS group is bonded to the alcoholic oxygen and a second to the phenolic oxygen of coniferyl alcohol. The base peak, m/e = 73 is due to TMS ions. These results indicate that the synthesis product is coniferyl alcohol.

The yield of coniferyl alcohol was routinely determined using UV spectroscopy and gas chromatography. The values obtained using these two independent methods were in close agreement and averaged 47.1 ± 3.0% (five trials).

Synthesis and characterization of synthetic lignin. The elemental composition of the synthetic lignin was 62.2% C, 6.2% H and 31.4% O which agreed well with published values for milled spruce wood lignin (9) and for synthetic lignin of Kirk et al. (10, Table 1).

The infra-red spectra of the synthetic lignin and milled spruce wood lignin were very similar (Fig. 4). However, as previously reported for synthetic lignin the shoulder at 1725 cm^{-1} in the spectrum of milled spruce wood lignin was absent in the spectrum of synthetic lignin described here.

Degradation of ^{14}C-synthetic lignin by Phanerochaete chrysosporium. The usefulness of the (2'-^{14}C) synthetic lignin obtained in this study as a model substrate for studies on biological degradation of lignin was evaluated by determining the rate at which it was degraded by the typical white-rot fungus Phanerochaete chrysosporium. The rate of degradation of (U-^{14}C-ring) synthetic lignin, prepared by the procedure of Kirk et al. (10), was also determined to serve as a

comparison. The (2'-^{14}C) synthetic lignin was degraded at a rate comparable to that of the (U-^{14}C-ring) synthetic lignin (Fig. 5). With both the lignin preparations, ligninolytic activity appeared after 6 d of incubation and increased over the next several days. The rate of lignin degradation was very similar for the two lignin preparations.

DISCUSSION

In studies on the biochemistry of lignin degradation, substantial quantities of coniferyl alcohol are needed to prepare synthetic lignin. To simplify the chemical synthesis of coniferyl alcohol, we examined the feasibility of reducing the terminal carboxyl group of ferulic acid to form coniferyl alcohol. Previous attempts to directly reduce the terminal carboxyl group of cinnamic acid derivatives has resulted in a low yield of product (3) due to the susceptibility of the carbon-carbon double bond in the propyl side chain to reduction when an acidic -OH is adjacent to it. However, if the acid group is first converted to an acid chloride or an ester, moderate yields of α,β-unsaturated aromatic alcohols are possible (1,3,17). Since formation of trimethylsilyl esters is simple and familiar to many scientists, it appeared that reduction of the trimethylsilyl ester of ferulic acid to form coniferyl alcohol might be the basis of a relatively simple procedure. The results show this to be the case.

The yield of coniferyl alcohol (47%) is typical for the reduction of acid chlorides and esters of cinnamic acid derivatives (1,3,12,17). It may be possible to increase the yield of coniferyl alcohol through the use of other reducing agents and by employing more stringent measures to exclude water from the silylation reactions and silylated intermediates.

The data showed that the synthetic lignin obtained by polymerization of the coniferyl alcohol produced in this study, was similar in elemental composition and gross structural characteristics to spruce milled wood lignin and other synthetic lignins described in the literature (9,10). Previous investigators have shown (U-^{14}C-ring) and

(2'-^{14}C-side chain) labeled lignins to be degraded at comparable rates by P. chrysosporium. In agreement with these findings (11) we found that P. chrysosporium degraded the (2'-^{14}C) synthetic lignin (prepared using methods described here) at a rate comparable to that observed for (U-^{14}C-ring labeled) synthetic lignin of Kirk (10).

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Table 1. Comparison of the elemental composition of the synthetic lignin with that of other lignins

Type of lignin	%C	%H	%O
Synthetic lignin[a]	62.2	6.3	31.4
Synthetic lignin[b]	63.2	5.8	31.0
Spruce milled wood lignin[c]	62.9	6.1	31.1

[a]Synthetic lignin prepared by the procedures described in this study.

[b]Kirk et al. (10).

[c]Kirk and Chang (9).

Figure 1. Reaction sequence for the synthesis of coniferyl alcohol from ferulic acid.

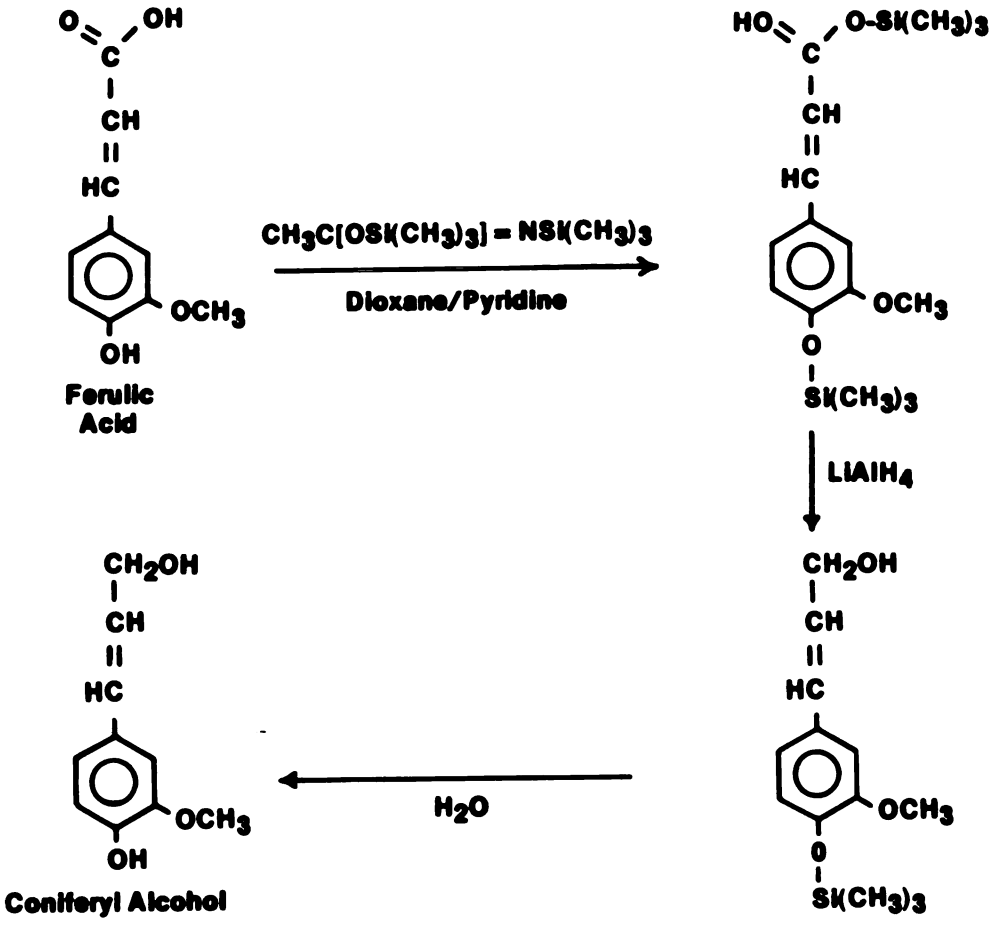

Ferulic Acid
$CH_3C[OSi(CH_3)_3] = NSi(CH_3)_3$
Dioxane/Pyridine
$LiAlH_4$
H_2O
Coniferyl Alcohol

Figure 2. Analysis by high performance liquid chromatography of coniferyl alcohol (a) standard and (b) that synthesized by the methods described here. The detector was set at 0.04 AUFS which allowed for the detection of trace levels of impurities although the signal due to the presence of coniferyl alcohol is off scale in both chromatograms.

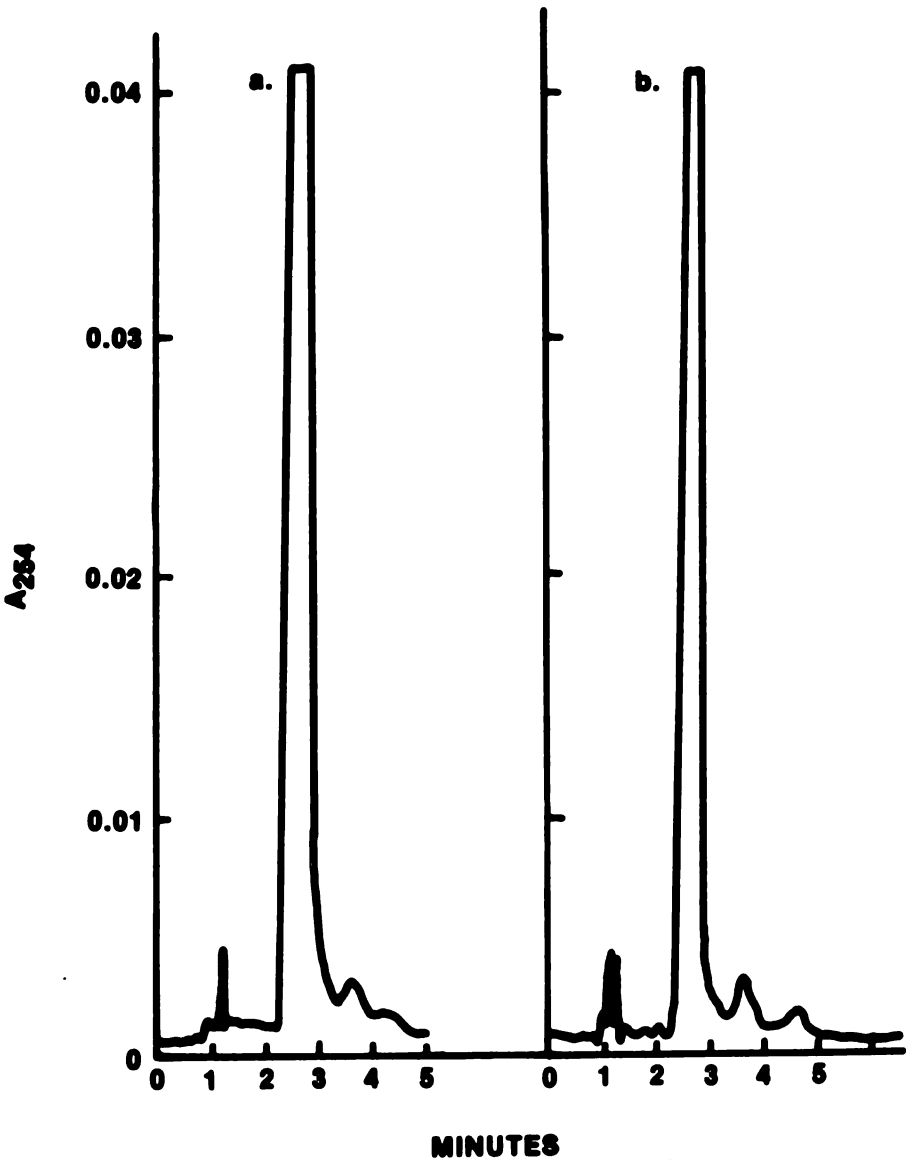

a.
b.
0.04
0.03
0.02
0.01
0
A_{254}
0 1 2 3 4 5
0 1 2 3 4 5
MINUTES

Figure 3. Mass spectra of (a) coniferyl alcohol synthesized by the methods described here and (b) that of an authentic coniferyl alcohol standard.

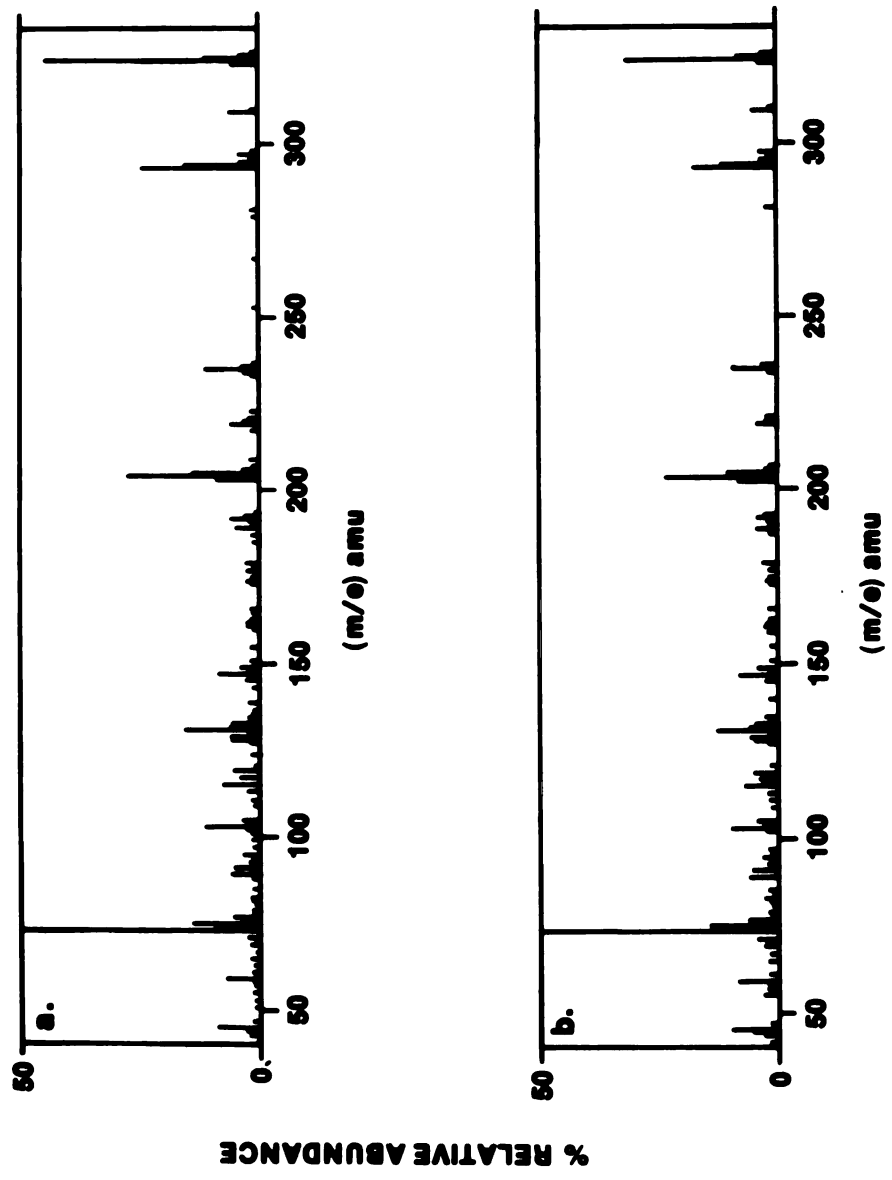

a.
b.
50
0
50
0
50
100
150
200
250
300
(m/e) amu
(m/e) amu
% RELATIVE ABUNDANCE

Figure 4. Infra-red spectra of (a) synthetic lignin prepared by the methods described here (—) and (b) milled spruce wood lignin (····).

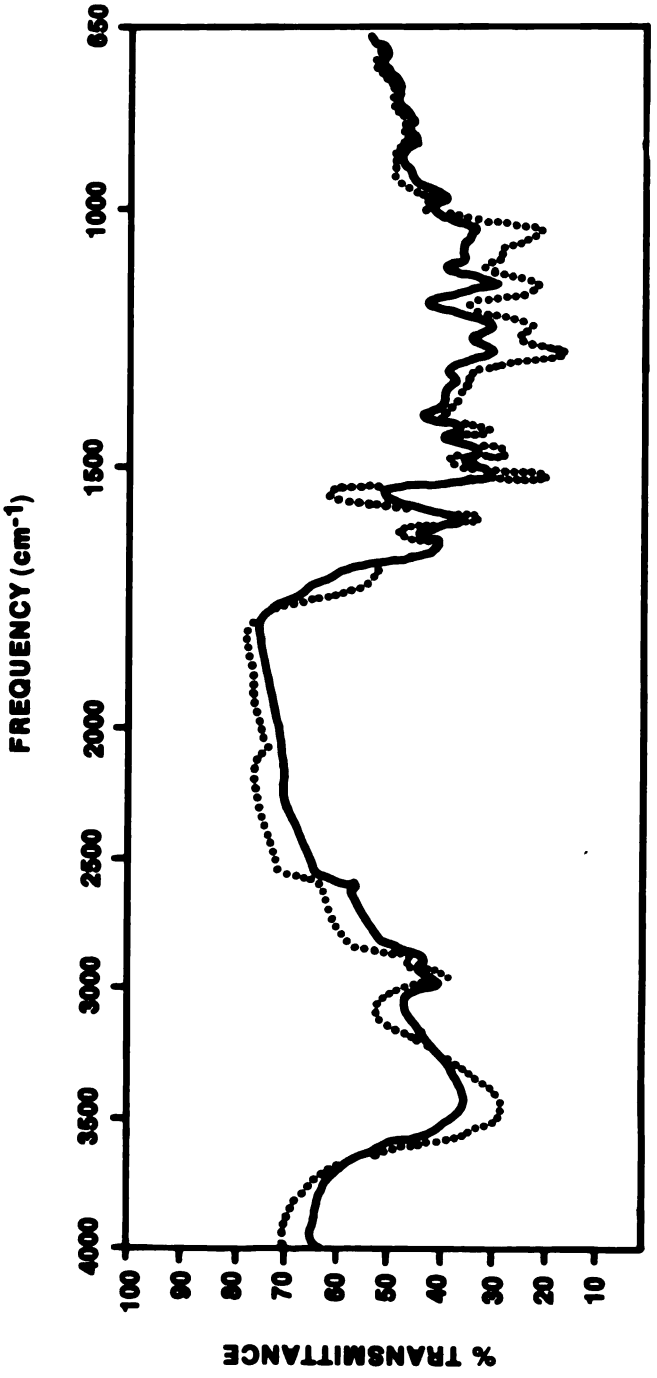
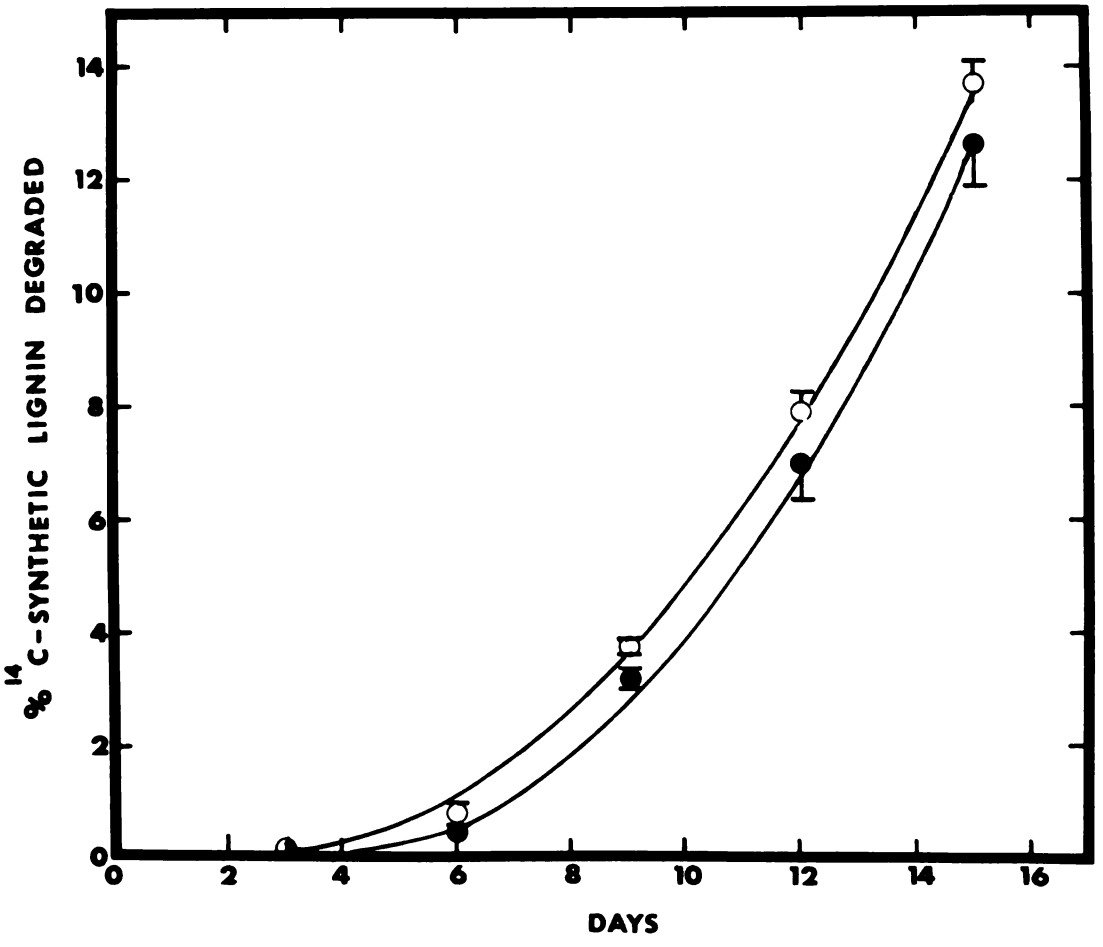

FREQUENCY (cm-1)
4000
3500
3000
2500
2000
1500
1000
650
% TRANSMITTANCE
100
90
80
70
60
50
40
30
20
10

Figure 5. Degradation of (2'-^{14}C) synthetic lignin (O), prepared in this study, and (U-ring-^{14}C) synthetic lignin of Kirk (●) by Phanerochaete chrysosporium.


% ^{14}C-SYNTHETIC LIGNIN DEGRADED
0
2
4
6
8
10
12
14
16
DAYS

CHAPTER II

The Involvement of Hydroxyl Radicals Derived from Hydrogen Peroxide in Lignin Degradation by the White-Rot Fungus Phanerochaete Chrysosporium

SUMMARY

The possibility was investigated that hydroxyl radical (·OH) derived from hydrogen peroxide (H_2O_2) is involved in lignin degradation (^{14}C-lignin$\longrightarrow$ $^{14}CO_2$) by Phanerochaete chrysosporium. When P. chrysosporium was grown in low nitrogen medium (2.4 mM N), an increase in H_2O_2 production in cell extracts was observed to coincide with the appearance of ligninolytic activity and both activities appeared after the culture entered stationary phase. The production of ·OH in ligninolytic cultures of P. chrysosporium was demonstrated by α-keto-γ-methiolbutyric acid dependent formation of ethylene. Hydrogen peroxide-dependent ·OH formation was also shown in cell extracts of ligninolytic cultures. The radical species was demonstrated to be ·OH by the ·OH-dependent hydroxylation of p-hydroxybenzoic acid to form protocatechuic acid and by using 5,5-demethyl-1-pyrroline-N-oxide (DMPO) and EPR spectometry to detect the production of the nitroxide radical of DMPO. These reactions were inhibited by ·OH scavenging agents and were stimulated when azide was added to inhibit endogenous catalase. Lignin degradation by P. chrysosporium was markedly supressed in the presence of the ·OH scavenging agents mannitol, benzoate and the nonspecific radical scavenging agent butylated-hydroxytoluene. The above results indicate that ·OH derived from H_2O_2 is involved in lignin biodegradation by P. chrysosporium.

Lignin is an integral part of all vascular plants and is among the most abundant and widely distributed organic polymers in nature (1, 47). Due to the importance of lignin in the biospheric carbon cycle and the interest in the use of lignocellulosic materials for the production of various fuels and chemicals (6), research on the biodegradation of lignin has greatly accelerated in recent years. A large number of basidiomycetous fungi, collectively known as white-rot fungi, have been shown to degrade lignin extensively (3, 36). Considerable progress has been made toward understanding the physiological and nutritional parameters which affect lignin degradation by white-rot fungi (5, 17, 31, 33, 35); however, little is known about the basic biochemical mechanisms involved in the process.

In view of the molecular size and structure of lignin it is unlikely that lignin is directly taken up by microbial cells. Hence, it is reasonable to postulate that extracellular agents are involved in the initial breakdown of the lignin polymer. Yet, extracellular ligninolytic enzymes capable of extensively transforming lignin have not been isolated from fungal cultures of wood decomposing fungi (2, 3, 28, 36). Analyses of lignin residues following fungal attack and solubilized products of lignin degradation have shown that the extracellular degradation of lignin is oxidative in nature (30, 34). These results further showed that the extracellular agents attacking lignin agents are nonspecific and nonstereosolective as evidenced by the following observations: a) lignin is extensively degraded despite the variety of intermonomer linkages (1) and racemic asymmetric carbons in the polymer (480; and b) a

variety of structurally different lignins and lignin model compounds are metabolized efficiently (15, 42). In consideration of the above features, it appears unlikely that the reactions involved in the initial degradation of the lignin polymer are mediated by specific enzymes. Hence, it has been postulated that the actual extracellular agents attacking the lignin polymer might be activated oxygen species (2, 28, 46) such as superoxide ($O_2^{\cdot-}$) or singlet oxygen ($^1O_2$), however, conclusive evidence is lacking. Koenigs (37, 38) observed hydrogen peroxide (H_2O_2) production by cultures of a number of wood-decomposing fungi and suggested the possible involvement of H_2O_2 in lignin degradation by white-rot fungi. In an effort to elucidate the role of extracellularly produced activated oxygen species in lignin degradation, we tested the hypothesis that hydroxyl radical (·OH) derived from H_2O_2 is involved in lignin degradation by white-rot fungi. The above hypothesis can be rationalized by the fact that the ·OH is highly reactive and is known to react rapidly with a wide variety of organic compounds in oxidative reactions (29, 52). This would be in keeping with the well established oxidative degradation of lignin (30, 34). Furthermore, the ·OH radical is nonspecific, nonstereoselective (29, 52), and is known to participate in a number of biological reactions (8, 11, 44, 51).

The ·OH can be produced by one electron reduction of H_2O_2 (52) and in biological systems is thought to be generated in two types of reactions (8, 16, 25, 52). In the Fenton reaction (reaction 1), H_2O_2 is reduced by Fe (II), or other reduced transition metals, to produce ·OH (52). In the iron-catalyzed Haber-Weiss reaction (25;

reaction 2), superoxide ($O_2^{-}\cdot$) reduces Fe(III) to Fe(II) which in turn reduces H_2O_2 to form ·OH in a manner similar to that in reaction 1. This reaction is greatly stimulated by chelators such as EDTA (25).

1. Fenton Reaction

$$H_2O_2 + Fe(II) \longrightarrow \cdot OH + OH^{-} + Fe(III)$$

2. Iron-catalyzed Haber-Weiss reaction

a. $Fe(III)\text{-chelate} + O_2^{-}\cdot \longrightarrow Fe(II)\text{-chelate} + O_2$

b. $Fe(II)\text{-chelate} + H_2O_2 \longrightarrow \cdot OH + OH^{-} + Fe(III)\text{-chelate}$

$$H_2O_2 + O_2^{-}\cdot \longrightarrow \cdot OH + OH^{-} + O_2$$

Phanerochaete chrysosporium causes a typical white-rot decay of wood and has been used in numerous studies on the biochemistry of lignin degradation (5, 17, 31, 33, 35). We used this organism in this study to answer the following questions: (1) Is there a correlation between the appearance of H_2O_2 production and ligninolytic activity cells of P. chrysosporium? (2) Is ·OH produced by ligninolytic cultures of P. chrysosporium? (3) Does the addition of ·OH scavenging agents to ligninolytic cultures affect the rate of lignin degradation?

Our results indicate that ·OH, derived from H_2O_2, is produced in cultures of P. chrysosporium and that ·OH is involved in lignin degradation by the fungus.

MATERIALS AND METHODS

Organism and culture conditions. Phanerochaete chrysosporium Burds. ME446 (ATCC 34541) was provided by Dr. T. K. Kirk and was maintained through periodic transfer on malt extract agar as previously described (35). Unless indicated otherwise, the fungus was grown in shallow liquid cultures (25 ml por 250 ml Erlenmeyer flask) in low N medium (35) which contained 0.6 mM NH_4NO_3 and 0.6 mM asparagine 2.4 mM N final concn.) The flasks were foam stoppered, autoclaved for 15 min at 121°C and inoculated with a conidial suspension in water (5% inoculum) which had been prepared from 7-10 d old malt extract agar cultures as previously described (21). The cultures were incubated in air (21% O_2) at 39°C, without agitation for various periods of time as indicated in the text.

Ligninolytic Activity. Lignin degradation was determined by measuring the $^{14}CO_2$ produced during the metabolism of [2'-^{14}C]-synthetic lignin. The [2'-^{14}C]-synthetic lignin was prepared as previously described (21) and 0.35 mg (39,300 dpm) of this lignin, suspended in 0.5 ml of sterile water, was added to 10 ml of sterile low N medium. The medium was inoculated with 0.5 ml of the conidial suspension and the flasks were stoppered prior to incubation with manifolds which permitted periodic flushing of the culture headspace. At 3 d intervals, the headspace of the cultures was flushed with CO_2-free air and the respired $^{14}CO_2$ was trapped in a scintillation cocktail containing 2-aminoethanol as previously described (21).

H_2O_2 production. The specific activity for H_2O_2 production in cell extracts of P. chrysosporium was determined using a modification of the catalase-aminotriazole assay of Cohen and Somerson (10). Cells of P. chrysosporium were harvested after various periods of incubation by centrifugation at 12,000 X g for 10 min, washed twice with sodium phosphate buffer (50mM, pH 5.5), resuspended in 10 ml of the same buffer, and stored at -10°C until assayed. Freezing the cells had little effect on H_2O_2 production by the cell extracts. The cells were thawed as needed, homogenized with a glass tissue homogenizer and ruptured by a French pressure cell at 20,000 psi. Unbroken cells and cell debris were removed by centrifugation at 12,000 X g for 10 min and the supernatant cell extract was used for the H_2O_2 assays. The reaction mixture contained: 125 µg catalase (bovine heart, 6600 U mg^{-1}; Sigma Chemical Co., St. Louis, MO), 11 µmol glucose, 50 µmol 3-amino-1,2, 4-triazole (Sigma Chemical Co.) and 0.9 ml sodium phosphate buffer. The reactions were initiated by adding 0.1 ml of cell extract and were incubated at 39°C for 5 to 120 min. At various times, 200 µl samples were removed and placed in 1.0 ml of 0.45 M ethanol to inhibit the further formation of non-catalatic H_2O_2-catalase-aminotriazole complexes. After 10 min at room temperature, 0.5 ml of this solution was added to 4.5 ml of 6 mM H_2O_2 in sodium phosphate buffer and incubated for 180-295 s at 0°C (on ice) followed by termination of the reaction by the addition of 1.0 ml 6 N H_2SO_4. The residual H_2O_2 was determined by titration with 7.5 mM $KMnO_4$. The assay was standardized by substituting glucose oxidase (β-D-glucose: O2 1-oxidoreductase, E.C.1.1.3.4) from Aspergillusniger (Sigma Chemical Co.) for the cell extract, in the

reaction mixture. Glucose oxidase solution was desalted prior to use by passing through a Sephadex G-25 column. Boiled cell extracts served as negative controls.

Enumeration of conidia and fungal cell mass determinations. The number of conidia (aleuriospores) formed in cultures of P. chrysosporium was determined in triplicate cultures on 11 consecutive days beginning on d 3. The cultures were diluted with an equal volume of water and homogenized in a Waring blender for 30 sec. A portion of the homogenized culture was placed in a haemocytometer and the conidia were counted using phase contrast microscopy.

The growth of P. chrysosporium in low N medium was determined by gravimetric determination of fungal cell mass. On 14 consecutive days, starting on day 1, three replicate 10 ml cultures in low N medium were filtered through tared 0.22 μm micropore filters (Millipore Corp., Bedford, MA). The cells on the filter were washed with 10 ml of 10 mM 2,2-dimethylsuccinate buffer (pH 4.5) and were dried to a constant weight under vacuum at 60°C. The cell mass was calculated by difference.

Determination of ·OH formation in cultures. The ·OH is known to react with α-keto-γ-methiolbutyric acid (KTBA) to produce ethylene gas (12). To detect ·OH formation in cultures of P. chrysosforium the organism was grown in 5 ml of low N medium contained in 50 ml foam stoppered serum vials. After 14 d of incubation the foam stoppers were replaced with rubber septa and the vials were sealed with aluminum caps. To different flasks, 0.5 ml of water or the ·OH scavenging agents benzoate (5 mM final concn.) or mamnitol (50 mM final concn.) were added and the reactions were initiated by

injecting 0.5 ml KTBA (3.3 mM final concn.). Identical but autoclaved fungal cultures also served as negative controls. Reactions were terminated at various times after reincubation by injecting 1.0 ml 6 N H_2SO_4 per vial. The amount of ethylene gas present in 0.2 cc samples of the headspace of each vial was determined using a Varian 2440 gas chromatograph (Varian Associates, Palo Alto, CA) equipped with a 2 m X 3.2 mm O.D. stainless steel column packed with Porapak N (Waters Associates, Milford, MA) and a flame ionization detector. The carrier gas was N_2 at 30 ml min^{-1}. The detector gas flow rates were 300 ml air min^{-1} and 30 ml H_2 min^{-2}. The chromatograph oven was maintained at 200°C, with the injector and detector both set at 140°C.

CX-distilled water. Distilled water, used to prepare reagents for hydroxylation (Table 3 and 4) and EPR experiments (Fig. 3) was freed of trace minerals and other contaminants by passage through a Milli-Q water purification system (Millipore Corp., Bedford, MA) and then through a column packed with Chelex-100 resin (Sigma Chemical Co., St. Louis, MO). This purified distilled water is hereafter referred to as CX-distilled water. Solutions of Fe(II) were prepared in CX-distilled water and sparged with either N_2 or Ar to minimize the dissolved O_2 concentration. All organic reagents solutions were adjusted to pH 4.5 prior to use.

Preparation of cell extracts. Cell extracts (used in experiments described in Table 2 and Fig. 3) were prepared from 14 d old cultures of P. chrysosporium. The cells were harvested by centrifugation (12,000 x g for 10 min), washed once with 20 ml of 10 mM 2,2-dimethylsuccinate (pH 4.5), once with 20 ml of CX-distilled water, and finally resuspended in 20 ml of the latter. Glass beads

(0.5 mm; Scientific Products, Romulus, MI) were then added to the cell suspension (ten times the weight of the cell pellet) and the cells were ruptured by blending in a precooled Waring blender for 3-30 sec periods separated by 30 sec cooling periods. The unbroken cells and glass beads were removed by centrifugation for 10 min at 48,200 xg. The cell extracts were held on ice and used within 3 h. Protein determinations were made using the procedure of Lowry et al. (41).

Assay of ·OH production by measuring hydroxyltion of p-hydroxybenzoic acid. The ·OH is known to react with various aromatic compounds resulting in the hydroxylation of the aromatic ring (52). Thus, the production of ·OH was determined by measuring hydroxylation of p-hydroxybenzoic acid (pHB) to form 3,4-dihydroxybenozic acid (protocatechuic acid; PCA). The basic reaction mixture contained 10 µmol p-hydroxybenzoic acid (Aldrich Chemical Co., Milwaukee, WI), 10 µmol glucose, 0.1 µmol $FeCl_2$ or $FeCl_3$, 1 µmol EDTA, 200 µl glucose oxidase (0.06 u ml^{-1}) or 200 µl cell extract and CX-distilled water to 1.0 ml. Where indicated mannitol (20 or 200 µmol), benzoate (2 or 20 µmol), α-keto-γ-methiolbutyric acid (2 or 10 µmol), or 0.1 µmol NaN_3 were also added to the reactions. The reactions were preincubated at 39°C for at least 10 min, initiated by adding cell extract or glucose oxidase, incubated for 120 min and were terminated by adding 100 µl 6 N H_2SO_4. PCA was identified as the reaction product by comparison with authentic PCA (Aldrich Chemical Co., Milwaukee, WI) using high pressure liquid chromatography (HPLC) as described below and gas chromatography using previously described methods (21).

The amount of PCA in the reactions was determined by HPLC using a Hibar II RP-18 (250 mm X 4.6 mm) column (Anspec, Inc., Ann Arbor, MI) with water/methanol/acetic acid (200:20:10) at 2.0 ml min^{-1} as the eluting solvent. The chromatograph was comprised of a Rheodyne Model 7125 injector (Rheodyne, Inc., Berkeley, CA), a Milton-Roy pump (Milton-Roy Corp., Riveria Beach, FL) and a Laboratory Data Control UV detector with a 254 hm filter (Model LDC III, Laboratory Data Control, Riveria Beach FL).

Detection of ·OH by EPR spectometry. The ·OH rapidly reacts with the nitrone spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Aldrich Chemical Co., Milwaukee, WI) and the resultant DMPO-OH adduct can be detected by EPR spectometry (18). The DMPO was purified prior to use by vacuum distillation and stored at -10°C. The reaction mixture in a final volume of 1 ml contained: 0.1 ml cell extract, 10 µmol glucose, 0.1 µmol $FeCl_2$, 1.0 µmol EDTA, 0.1 µmol NaN_3, 60 µmol DMPO and 30 mM NaCl. Where indicated, 100 µmol ethanol, 50 µmol mannitol, 100 µmol benzoate or 10 µmol p-hydroxybonzoic acid were also added. The reactions were initiated by the addition of Fe(II) and the spectra were recorded immediately using a Varian Century E122 EPR spectrometer (Varian Associates, Palo Alto, CA) set at the following conditions: 3370 G magnetic field, 9.41208 GHz, 30 mW microwave power, 100 kHz modulation frequency, 2.5 modulation amplitude, 0.25 s time constant and 8 min scan time.

In a second experiment, the ·OH was generated chemically by the use of Fenton's reagent and the DMPO-OH adduct formed was detected as described above. The reaction mixture contained in a final volume of 1 ml; 60 µmol DMPO, 0.1 µmol $FeCl_2$, 0.1 µmol H_2O_2

and 30 mM NaCl. The spectrum was recorded using 15 mW microwave power and a 2 min scan time.

Effect of ·OH scavenging agents on ligninolytic activity. The effect of ·OH scavenging agents on the metabolism of lignin by P. chrysosporium was determined by measuring the amount of $^{14}CO_2$ produced following the additions of [2'-^{14}C] synthetic lignin to ligninolytic cultures of the fungus, in the presence or absence of ·OH scavenging agents. After 13 d of incubation, 0.5 ml of a synthetic lignin suspension (described above) and either 0.5 ml of 10 mM dimethylsuccinate (pH 4.5), 50 mM mannitol, 500 mM mannitol, 10 mM benzoate, 10 mM butylated hydroxytoluene (BHT), or 1 mM BHT, were added to the cultures. The flasks were then stoppered with sterile gassing manifolds and reincubated. After 24 and 48 h of incubation, the respired $^{14}CO_2$ was flushed from each flask and quantified as described above. Four replicate flasks were used for each treatment and the results are expressed as the mean ± S.E.

To rule out the possibility that the inhibition of [^{14}C]-synthetic lignin degradation was due to nonspecific inhibition of fungal metabolism by mannitol, benzoate or BHT, the effect of these compounds on the metabolism of D-[U-^{14}C]-glucose was determined in a manner identical to that described above except that D-[U-^{14}C] glucose (22 µl, 1.02 X 10^5 dpm, Schwarz/Mann, Orangeburg, NY) was substituted for [^{14}C]-synthetic lignin.

RESULTS

Relationship between culture growth phase, ligninolytic activity and H_2O_2 production. In low N media, ligninolytic activity of *P. chrysosporium* has been shown to be derepressed after primary growth ceases due to nitrogen starvation and apparently is a secondary metabolic event (17,33). If ·OH derived from H_2O_2 was involved in the degradation of lignin, one would anticipate little H_2O_2 production in the primary growth phase and an increase in specific activity of H_2O_2 production to occur in concert with the appearance of ligninolytic activity, after the culture entered the stationary phase. The results showed that this was in fact the case. After 2 d of incubation, the nitrogen in the culture was exhausted (data not shown), fungal growth slowed beginning on d 3 and the culture appeared to enter stationary phase after 6 d (Fig. 1). The formation of conidia in the cultures followed the end of primary growth and reached a maximum of 1×10^6 ml^{-1} on d 8, further indicating that the culture was in stationary phase. Lignin degradation began on d 5 or 6 and increased rapidly thereafter. There was little H_2O_2 production activity (3-8 nmol min^{-1} mg $protein^{-1}$) in extracts of cells from 6-7 d old cultures, i.e., before the appearance of ligninolytic activity. Once the culture entered the stationary phase, the specific activity for H_2O_2 production increased and reached a maximum level of approximately 70 nmol in 20 d old cultures (Fig. 1). Boiled cell extracts did not produce H_2O_2.

These results indicate that a temporal relationship existed between the appearance of H_2O_2 production activity and ligninolytic

activity. Since 13-14 d old cultures were actively ligninolytic and contained high levels of H_2O_2 production activity (or approximately 50 nmol min^{-1} mg $protein^{-1}$) they were used in the experiments described below.

Demonstration of ·OH in cultures of P. chrysosporium. If ·OH derived from H_2O_2 was involved in lignin degradation, the radical should be demonstrable in ligninolytic cultures of P. chrysosporium. Appreciable levels of ethylene were produced in 14 d old cultures (Fig. 2) after adding KTBA, thus demonstrating that ·OH is produced in the extracellular environment of actively ligninolytic cultures of P. chrysosporium. The fact that the observed ethylene production from KTBA was a ·OH dependent process was confirmed by showing that it was inhibited in the presence of ·OH scavenging agents benzoate or mannitol. When 10 mM benzoate or 50 mM mannitol was present in the culture, the production of ethylene from KTBA was inhibited by 94 and 15%, respectively (Table 1). When cultures were autoclaved prior to adding the KTBA or when KTBA was not added to the culture, no ethylene was produced.

When P. chrysosporium was grown in a high N medium (identical to low N medium except that the N content was increased to 24 mM), less than 1% of the added [^{14}C]-synthetic lignin was recovered as $^{14}CO_2$ in the first 14 d of incubation whereas identical cultures grown in low N medium degraded or approximately 14% of the added lignin. If there is a correlation between ·OH production and ligninolytic activity by P. chrysosporium, less ethylene would be expected to be produced when KTBA was added to cultures grown in high N medium. The results showed that cultures grown in high N

medium produced approximately 100-fold less ethylene from KTBA than cultures grown in low N medium (data not shown).

Hydroxylation of p-hydroxybenzoic acid to protocatechuic acid by ·OH. Since the above results suggested that the ·OH radical may play a role in ligninolytic activity we sought to obtain several independent lines of evidence to demonstrate conclusively that the radical species in question was ·OH. In one experiment the ·OH-mediated hydroxylation of p-hydroxybenzoic acid (pHB) to form protocatechuic acid (PCA) was investigated using cell extracts of P. chrysosporium (9, Table 2). The ·OH may be produced through the reduction of H_2O_2 by Fe(II). Alternatively, the cell extracts may produce $O_2^{-}\cdot$ or other reductants capable of reducing Fe(III) to Fe(II) which in turn reduce H_2O_2 and yield the ·OH as in the iron-catalyzed Haber-Weiss reaction (25). Therefore, the ability of Fe(II) and Fe(III) to participate in ·OH formation by cell extracts was examined.

As shown in table 2, the amount of PCA formed in complete reaction mixtures containing Fe(II) (44.7 pmol PCA ml^{-1}) was much greater than that in complete reaction mixtures containing Fe(III) (14.0 pmol PCA ml^{-1}). The lower amount of PCA formed in reactions containing Fe(III) was probably due to the need to reduce Fe(III) to Fe(II) required for the reductive cleavage of H_2O_2 to form the ·OH. When the cell extract was omitted from reaction mixtures containing Fe(II) 14.5 pmol PCA ml^{-1} was formed. No PCA was formed on the deletion of cell extract from reaction mixtures containing Fe(III). The formation of PCA in the former reaction mixtures was probably due to the autooxidation of Fe(II).

The ·OH scavenging agents benzoate, mannitol and KTBA were all shown to inhibit the formation of PCA in reaction mixtures containing either Fe(II) or Fe(III) (Table 2) indicating that the formation of PCA from pHB in these reaction mixtures was a ·OH dependent process.

Endogenous catalase activity in cell extracts was found to be completely inhibited by 0.1 mM NaN_3. Inhibition of catalase by the inclusion of NaN_3 in the reaction mixture would allow for the production of a higher concn. of H_2O_2 and in turn, higher levels of ·OH production. In the presence of 0.1 mM NaN_3, the formation of PCA in reactions containing either Fe(II) or Fe(III) increased two-fold and three-fold, respectively.

The formation of PCA in reactions containing Fe(II) or Fe(III) decreased on deletion of glucose or EDTA. When pHB, cell extract or iron was deleted, or when boiled cell extract was used in these reaction mixtures, little or no PCA, other than that resulting from Fe(II) autooxidation, was formed.

The above results suggest that the hydroxylation of pHB to form PCA was a ·OH dependent process and that the ·OH was derived from H_2O_2. To independently confirm these conclusions, an experiment identical to that described above was performed except that glucose oxidase and glucose were substituted for cell extract as the source of H_2O_2. The results showed that comparable amounts of PCA were formed when the specific activities for H_2O_2 production were similar in reaction mixtures containing cell extract or glucose oxidase (Tables 2 and 3). The formation of PCA in reaction mixtures

containing glucose and glucose oxidase was also inhibited by benzoate, mannitol or KTBA and was greatly stimulated by the addition of glucose and EDTA. PCA formation was negligible when Fe(II) was deleted. In contrast to results obtained using cell extract, the inclusion of 0.1 mM NaN_3 resulted in no increase in the amount of PCA formed in reactions containing glucose oxidase, since there was no catalase present. The results of the previous experiment suggested that PCA formation in reaction mixtures containing Fe(III) may be dependent on the reduction of Fe(III) to Fe(II) by cell extract. If so, no PCA formation would be expected when Fe(III) was substituted for Fe(II) in the present experiment. Results showed that this was the case (data not shown) indicating that there was no mechanism for reducing Fe(III) to Fe(II) in these reactions.

Demonstration of ·OH production using DMPO. The ·OH reacts with DMPO to produce a relatively stable nitroxide radical (18). The DMPO-OH adduct can be detected by EPR spectrometry and identified by its characteristic g-value and hyperfine splitting constants of the resultant spectrum. The EPR spectrum of reactions containing H_2O_2, Fe(II) (Fenton's reagent; reaction 1) and DMPO showed a quartet with a symmetrical 1:2:2:1 signal intensity, a g-value of 2.006 and hyperfine splitting constants of $A_N=A_H^B=14.9$ G indicating the production of the DMPO-OH adduct (Fig. 3a). The EPR spectrum of complete reaction mixtures containing cell extract (Fig. 3b) was very similar to that observed in Fig. 3a indicating that ·OH was produced in these reactions.

The ·OH reacts with ethanol to form the α-hydroxyethyl radical which in turn can react with DMPO to produce a stable DMPO-(α-hydroxyethyl) adduct, the spectrum of which is distinct from that of the

DMPO-OH adduct (18). When 100 μmol ml^{-1} of ethanol was added to the complete reaction mixture, the DMPO-(α-hydroxyethyl) adduct which had hyperfine splitting constants of A_N = 15.8 G and A_H = 22.8 G was observed (Fig. 3c). These results confirm the identity of the radical species being produced as ·OH.

Production of ·OH in the complete reaction mixture was dependent on the presence of Fe(II) (Fig. 3d) and cell extract (data not shown) and did not occur when boiled cell extract was added to the reaction mixture (Fig. 3e). As shown above, the inhibition of catalase in cell extract by NaN_3 allows for higher levels of ·OH to be produced. Thus, the intensity of the signal was markedly diminished when NaN_3 was omitted from the reaction mixture (Fig. 3f). The production of ·OH in the absence of EDTA was approximately one-third that seen in complete reaction mixtures (Fig. 3g) and was essentially eliminated in the absence of glucose (Fig. 3h). The formation of the DMPO-OH adduct was inhibited when mannitol, benzoate or pHB were added to the reactions (Figs. 3i, 3j, and 3k, respectively). The spectrum observed with mannitol was presumably that of the DMPO-mannitol radical adduct (J. Bucher, M. Tien, T. Moorehouse, and S. Aust, manuscript in preparation). The results indicate the H_2O_2-dependent formation of ·OH in cell extracts from ligninolytic cultures of P. chrysosporium.

Inhibition of lignin degradation by ·OH scavenging agents. The results presented above showed a relationship between ligninolytic activity, H_2O_2 production activity, H_2O_2-dependent ·OH production by cell extracts and ·OH production in cultures of P. chrysosporium. If ·OH is involved in lignin degradation, then one would

expect the degradation of lignin to be inhibited by ·OH scavenging agents. The results (Table 4) showed that the ·OH scavenging agents, mannitol and benzoate and the nonspecific radical scavenging agent BHT, markedly inhibited lignin degradation during the 24 h following their addition to ligninolytic cultures. There was little difference in the inhibition observed after 24 h vs. 48 h of incubation. BHT and benzoate were the most effective scavenging agents examined. Lignin degradation was inhibited 59-62% by 0.1 mM BHT and 91% by 1.0 mM BHT. Benzoate was almost as effective as BHT. Lignin degradation was inhibited by 34-40% and 48-53%, respectively, when mannitol was added at final concn. of 5 mM and 50 mM. Thus, much higher concentrations of mannitol were necessary for significant inhibition of lignin degradation as compared to those of benzoate and BHT.

An alternate, albeit less likely, explanation for the above results is that the inhibition of lignin degradation by mannitol, benzoate and BHT was due to a nonspecific inhibition of cellular metabolism and that these agents have little or no specific effec on lignin degradation. To investigate this possibility, the effect of these agents on the metabolism of D-[U-^{14}C]-glucose by identical cultures of P. chrysosporium was determined. As shown in Table 5, they had little or no effect on the metabolism of D-[U-^{14}C]-glucose. Benzoate (1mM final concn.) slightly inhibited glucose metabolism; however, the inhibitory effect on $^{14}CO_2$ release from [^{14}C]-synthetic lignin was significantly greater. In addition, these compounds were shown to have no effect on the production of H_2O_2 by cell extracts (data not shown).

DISCUSSION

In this study, extracts of cells from stationary phase cultures of P. chrysosporium, grown in low N medium, were shown to produce significant amounts of H_2O_2. This finding extends the list of wood-decomposing fungi which are known to produce H_2O_2 (37, 38). Hydrogen peroxide is known to be a product of numerous enzymatic reactions (37). It has also been shown to be produced nonenzymatically by dismutation of hydroperoxy radicals (the conjugate acid of $O_2^{-}\cdot$) as shown in reaction 3 (7).

(3) $HOO\cdot + HOO\cdot \longrightarrow O_2 + H_2O_2 \quad k = 8.6 \times 10^5\ M^{-1}s^{-1}$

Certain metals and metal complexes (25, 39) as well as superoxide dismutase (EC 1.15.1.1; 8, 22) have been shown to catalyze reaction 3. For example, Cu^{+2}_{aq} catalyzes this reaction at a rate of $2 \times 10^9\ M^{-1}s^{-1}$. Significant levels of metal ions are known to be present in habitats of white-rot fungi, thus the formation of H_2O_2 from $O_2^{-}\cdot$ in nature may occur through reaction 3. However, whether or not $O_2^{-}\cdot$ is a precursor of H_2O_2 in cultures of P. chrysosporium is not known.

A temporal relationship between the appearance of H_2O_2 production activity and ligninolytic activity was observed in cultures grown in low N medium. In agreement with previously published results (33), ligninolytic activity appeared in response to nitrogen starvation and after the culture had entered the stationary phase. Similarly, H_2O_2 production increased rapidly following the cessation of primary growth and after the formation of conidia in the culture

was initiated. R. L. Kelley and C. A. Reddy (manuscript in preparation) obtained similar results using an independent assay for H_2O_2 production (H_2O_2-dependent peroxidatic oxidation of o-dianisidine). Furthermore, both ligninolytic activity and H_2O_2 production were seen in cultures grown in low N medium regardless of whether or not lignin was present in the medium. Collectively, these data strongly suggest a close correlation between H_2O_2 production and lignin degradation by the fungus.

In the presence of reduced transition metals H_2O_2 is readily reduced to form ·OH (26, 51, 52). The production of ·OH in ligninolytic cultures of P. chrysosporium was indicated by the production of ethylene from KTBA. Inhibition of the reaction by mannitol and benzoate, known ·OH scavenging agents (25, 26, 50, 51), confirmed that the production of ethylene observed in the ligninolytic cultures is a ·OH-dependent reaction. Furthermore, ·OH was barely detectable in cultures grown under conditions in which lignin was not degraded, indicating a link between ·OH production and ligninolytic activity.

The production of ·OH in cell extracts from ligninolytic cultures was demonstrated by the ·OH dependent hydroxylation of pHB. The hydroxylation of pHB to PCA in reactions containing cell extract required the presence of either Fe(II) or Fe(III). The production of ·OH in reactions containing Fe(II) and cell extract probably proceeds through a Fenton-type reaction. In reactions containing Fe(III), the cell extract apparently reduces Fe(III) to Fe(II) followed by the production of ·OH through a Fenton-type reaction. The reduction of Fe(III) by cell extract may be mediated by $O_2^{\overline{}}\cdot$ (13, 50) or by other reactions of endogenous metabolism.

The validity of the production of ·OH from H_2O_2 was supported by the fact that a 2-3 fold stimulation in ·OH production was observed when the endogenous catalase activity in the cell extract was inhibited by 0.1 mM NaN_3 (Table 3). Furthermore, PCA was formed in identical reactions when glucose oxidase and glucose were employed as a source of H_2O_2 instead of cell extract. In reactions in which glucose oxidase and glucose were used to generate H_2O_2 (Table 4), PCA was formed only in the presence of Fe(II), and not when Fe(III) was substituted for Fe(II). This observation is consistent with the conclusion that the cell extract can reduce Fe(III) to Fe(II). Previous investigators have found that the reductive cleavage of H_2O_2 to form ·OH can be enhanced in the presence of metal chelates (25). Similarly, in these experiments, the formation of PCA was stimulated in the presence of EDTA.

Verification of ·OH production in cell extracts was obtained by using EPR spectrometry. The production of the DMPO-OH adduct via the addition of ·OH to DMPO was dependent on the addition of cell extract, DMPO, glucose, Fe(II) and NaN_3 to the reaction mixtures. The observed requirements for NaN_3, which would allow for higher concentrations of H_2O_2 in the reactions, and Fe(II) is additional evidence that the production of ·OH proceeds through the reductive cleavage of hydrogen peroxide with Fe(II) serving as the electron donor. Based on the above results, it appears that in ligninolytic cultures of *P. chrysosporium* the production of ·OH occurs through the reduction of H_2O_2 produced by the fungus.

The ·OH scavenging agents mannitol and benzoate and the non-specific radical scavenging agent BHT significantly inhibited the

degradation of [2'-^{14}C] synthetic lignin but had little or no effect on the metabolism of D-[^{U}-^{14}C]-glucose or the production of H_2O_2 by the fungus. The results strongly suggest that the inhibition of lignin degradation by these compounds is due to their ability to rapidly react with ·OH and indicate that ·OH is involved in the degradation of lignin by P. chrysosporium.

The involvement of ·OH in lignin degradation seems reasonable in that a relatively nonreactive species, H_2O_2 (24, 29) is produced and excreted by the fungus. Recent studies (R. L. Kelley and C. A. Reddy, 1981, unpublished data) have shown that various culture parameters which affect lignin degradation by P. chrysosporium similarly affect the production of H_2O_2 and ·OH in a manner which is consistent with the involvement of ·OH in lignin degradation. Furthermore, the involvement of ·OH in lignin degradation would explain the lack of success by previous investigators in isolating enzymes capable of extensively transforming lignin and the inability of cell-free culture filtrates to degrade lignin (27, 28, 36). For example, hydroxylation of the aromatic rings within lignin has been shown to occur during the extracellular transformations of the polymer by white-rot and brown-rot fungi (34, 36), yet monooxygenase or dioxygenase activity has not been demonstrated in the cell-free culture filtrates of these fungi (3, 36). As demonstrated previously (52) and in this study, ·OH readily adds to aromatic rings. Indeed, ·OH reacts nonspecifically and rapidly with many organic compounds in highly exothermic reactions (29, 52). This would account for the apparent low specificity and oxidative nature of the ligninolytic system of white-rot fungi previously reported (15, 30, 34, 36).

Recently, Amer and Drew (2) reported that $O_2^{-}\cdot$ produced by the white-rot fungus Coriolus versicolor, was "exported" to the extracellular environment and speculated that $O_2^{-}\cdot$ may be involved in lignin degradation by this fungus. The mechanism by which $O_2^{-}\cdot$ was produced in cultures of C. versicolor was not established. However, it is known that $O_2^{-}\cdot$ is produced in several enzymatic reactions (8, 22) and can also be formed through the oxidation of H_2O_2 by ·OH (52) as shown in reaction 4.

(4) $\cdot OH + H_2O_2 \longrightarrow H_2O + HO_2\cdot \quad k = (1.2\text{-}4.5) \times 10^7\ M^{-1}s^{-1}$

Superoxide radicals have been implicated in many "oxygen radical"-mediated processes including lipid peroxidation (32, 50), depolymerization of polysaccharides (43) and phagocytosis by polymorphonuclear leucocytes (4). However, it has long been known that $O_2^{-}\cdot$ is relatively nonreactive in aqueous systems (13, 16) and the ability of $O_2^{-}\cdot$ per se to initiate the above processes has recently been questioned (16, 22). It has been proposed that essentially all of the in vitro manifestations of $O_2^{-}\cdot$ can be explained by the involvement of $O_2^{-}\cdot$ in the iron-catalyzed Haber-Weiss reaction (Reaction 2; 16, 22) to produce ·OH. Thus, if $O_2^{-}\cdot$ is involved in lignin degradation, it is possible that it serves as a reductant for the production of ·OH, via the iron-catalyzed Haber-Weiss reaction (Reaction 2).

The involvement of singlet oxygen ($^1O_2$) in the photochemical oxidation of lignin and humic acids is well established (23, 49). Involvement of $^1O_2$ in lignin degradation by P. chrysosporium was

first suggested by Nakatsubo et al (46). However, no evidence for the production of $^1O_2$ by P. chrysosporium was provided and it is not clear at the present time how and if this organism produces $^1O_2$. The enzymatic production of $^1O_2$ is unlikely since 22.5 kcal mol^{-1} is necessary to excite O_2 from its triplet ground state ($^3\Sigma$) to the lowest energy singlet state ($^1\Delta_g$) (29). It has been proposed that $^1O_2$ is produced abiologically through the spontaneous dismutation of $O_2^{-}\cdot$ (40; reaction 5) and the uncatalyzed Haber-Weiss reaction (40; reaction 6).

(5) $O_2^{-}\cdot + O_2^{-}\cdot \longrightarrow H_2O_2 + O_2$

(6) $O_2^{-}\cdot + H_2O_2 \longrightarrow \cdot OH + HO^{-} + O_2$

Recent studies by Foote et al (20) have shown that less than 0.07% of the O_2 formed in reaction 5 would be in the $^1\Delta$ state and the possibility that any $^1O_2$ is formed has been questioned by Millson and Kearns (45). Furthermore, the uncatalyzed Haber-Weiss reaction (reaction 6) has been shown to proceed very slowly with a rate constant of $< 1\ M^{-1}S^{-1}$ and is probably of little importance in biological systems (7, 14). Therefore, it would appear unlikely that significant levels of $^1O_2$ are produced in ligninolytic cultures of P. chrysosporium through reactions 5 or 6.

Singlet oxygen has been shown to be produced in some systems during the propagation and termination of radical chain reactions (19). For example, it is generally accepted that $^1O_2$ is produced during the breakdown of α-hydroxy hydroperoxy acid or peroxy radicals which can be produced after an initial reaction of a compound

with some activated oxygen species (19, 50; reaction 7). These reactions have been shown to occur in the peroxidation of lipids, for example.

(7) $ROO\cdot + ROO\cdot \longrightarrow ROOR + {}^1O_2$

Thus, the inhibition of lignin degradation by P. chrysosporium by the $^1O_2$ trap anthracene-9,10-bis-enthanesulfonic acid (AES) observed by Nakatsubo et al (46) may be due to the quenching of $^1O_2$ produced during radical propagation or termination reactions which probably occur in lignin degradation. It should also be pointed out that AES is likely to scavenge ·OH due to the extended π electron system of the molecule and this may explain, at least in part, the observed inhibition of lignin degradation.

The results presented here indicate that the ·OH derived from H_2O_2 plays an integral role in the decomposition of lignin by P. chrysosporium and perhaps other white-rot fungi. The specific reactions in which ·OH participates are not known at this time. However, we suggest that the primary role of ·OH is in the initial oxidation and depolymerization of the lignin polymer which occur in the extracellular environment to produce low molecular weight moieties. Some of the low molecular weight moieties which result could then be taken up by the fungal cells and metabolized further.

Table 1. Effect of ·OH scavenging agents on the production of ethylene gas from KTBA by cultures of *P. chrysosporium*.[a]

Treatment	nmoles ethylene ml^{-1}[c]	% of complete
Complete[b]	18.36 ± 0.71	100
+ 50 mM Mannitol	15.69 ± 0.24	65.5
+ 5 mM Benzoate	1.16 ± 0.04	6.3
- KTBA	0.00	0.0
Boiled cultures	0.00	0.0

[a]Cultures were grown for 14 d in 5 ml volumes in low N medium contained in 50 ml bottles (see Materials and Methods),

[b]The complete reaction mixture consisted of a 14 d old culture to which KTBA (3.3 mM final concn.) had been added.

[c]The amount of ethylene produced per ml of headspace during 10 h of incubation; numbers were expressed as mean ± S.E.

Table 2. The effect of ·OH scavenging agents and azide on the hydroxylation of p-hydroxybenzoic acid to form protocatechuic acid by cell extracts of P. chrysosporium.

Addition	Final concn. (mM)	Protocatechuic Acid Formed[b] (10^{-12} moles ml^{-1} reaction mixture)	
		0.1 mM Fe(II)	0.1 mM Fe(III)
Complete[a]	-	44.7	14.0
Benzoate	2	41.2	10.5
Benzoate	20	21.0	4.1
Mannitol	20	34.5	10.5
Mannitol	200	7.9	4.5
KTBA	2	41.9	12.1
KTBA	10	17.8	7.1
NaN_3	-	85.9	44.2
- Fe	-	6.1	3.9
- Glucose	-	38.3	3.4
- p-hydroxybenzoate	-	0.0	0.0
- EDTA	-	18.5	4.5
+ Boiled Cell Extract	-	19.2	2.2
- Cell Extract	-	16.3	1.1

[a]The complete reaction mixture contained: 10 µmol p-hydroxybenzoic acid, 10 µmol glucose, 0.1 µmol $FeCl_2$ OR $FeCl_3$, 1 µmol EDTA, 200 l cell extract (74 nmol H_2O_2 min^{-1} ml cell $extract^{-1}$) and CX-distilled water to 1.0 ml.

[b]After 120 min incubation at 39°C.

Table 3. Effect of ·OH scavenging agents and azide on the hydroxylation of p-hydroxybenzoic acid to form protocatechuic acid in reactions containing glucose oxidase.

Addition	Final Concn. (mM)	Protocatechuic Acid Formed[b] (10^{-12} moles ml^{-1} reaction mixture)
Complete[a]	-	33.0
Benzoate	2	24.2
Benzoate	20	19.5
Mannitol	20	26.3
Mannitol	200	17.8
KTBA	2	18.8
NaN_3	0.1	28.8
- Fe(II)	-	1.1
- Glucose	-	18.5
- p-hydroxybenzoate	-	0.0
- EDTA	-	12.1
- Boiled enzyme	-	14.9
- Enzyme	-	16.3

[a]The composition of the complete reaction mixture was the same as in Table 2 except 200 µl glucose oxidase (60 nmol H_2O_2 $min^{-1}ml$ glucose oxidase solution[1]) was substituted for cell extract.

[b]After 120 min incubation at 39°C.

Table 4. Effect of ·OH scavenging agents on ligninolytic activity and Phanerochaete chrysosporium.[a]

Addition	Final Concn. (mM)	% of control[b]			
		^{14}C-lignin		^{14}C-glucose	
		24 h[c]	48 h	24 h	48 h
Complete	-	100	100	100	100
+ Mannitol	5	60	66	114	121
+ Mannitol	50	47	52	88	99
+ Benzoate	1	13	16	72	86
+ BHT	0.1	38	41	91	98
+ BHT	1.0	9	9	92	94

[a]Complete reaction mixtures consisted of cultures of P. chrysosporium were grown for 13 d in low N medium to which the ·OH scavenging agents and either [^{14}C] lignin or [^{14}C] glucose had been added. The metabolism of these substrates was assayed after 24 and 48 h of incubation by quantification of the respired $^{14}CO_2$,

[b]% of control = $\frac{\text{dpm (no additions)-dpm (with addition)}}{\text{dpm (no additions)}} \times 100$

[c]Time interval after following addition of ·OH scavenging agent.

Fig. 1 Relationship between the specific activity for H_2O_2 production in cell extracts (▲), the metabolism of [2'-^{14}C] synthetic lignin to [$^{14}CO_2$] (●) and mycelial dry weight (□). P. chrysosporium was grown in low N medium as described in Materials and Methods.

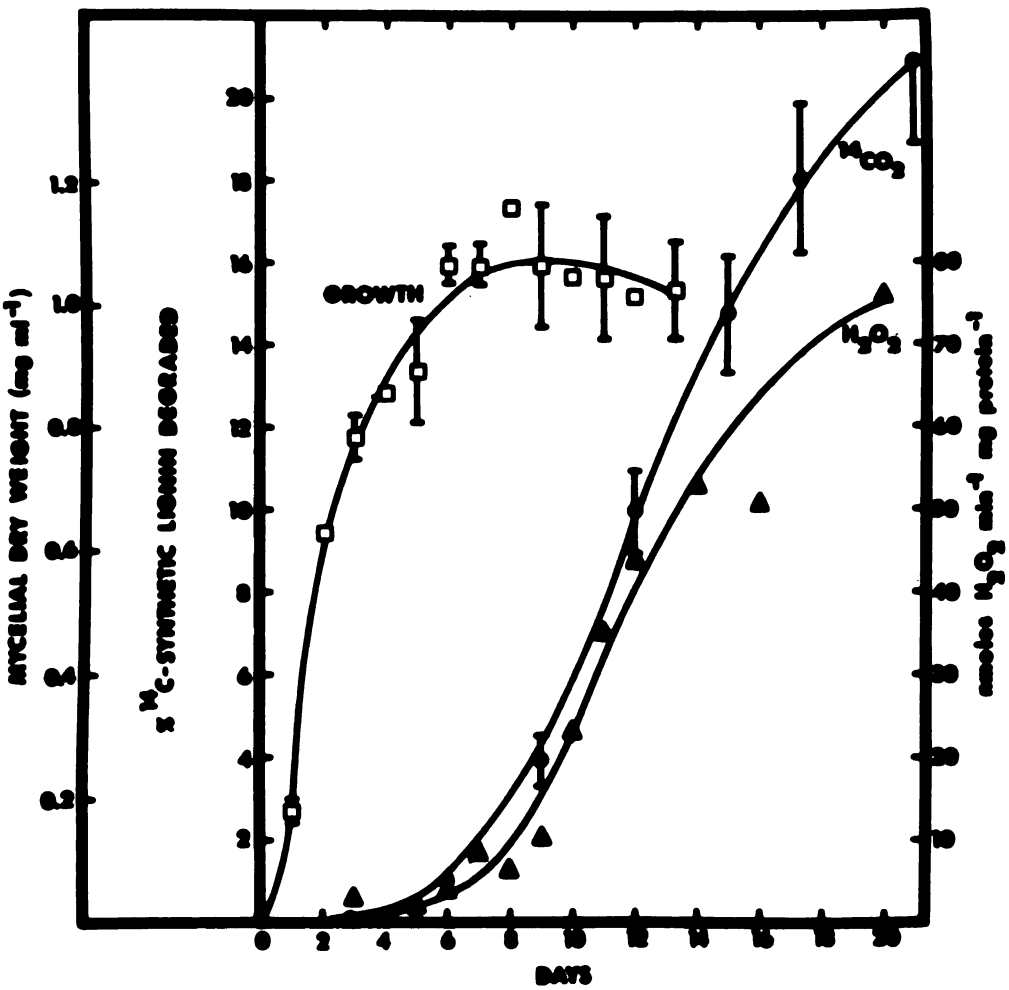

MYCELIAL DRY WEIGHT (mg ml⁻¹)
GROWTH
DAYS

Fig. 2 The production of ·OH in ligninolytic cultures of P. chrysosporium. The ·OH was detected by the ·OH-dependent formation of ethylene following the addition of α-keto-γ-methiolbutyric acid (KTBA) (3.3 mM final concentration) to cultures grown for 14 d in low N medium. The ethylene produced was detected using gas chromatography and is expressed as nmoles ethylene per ml of headspace.

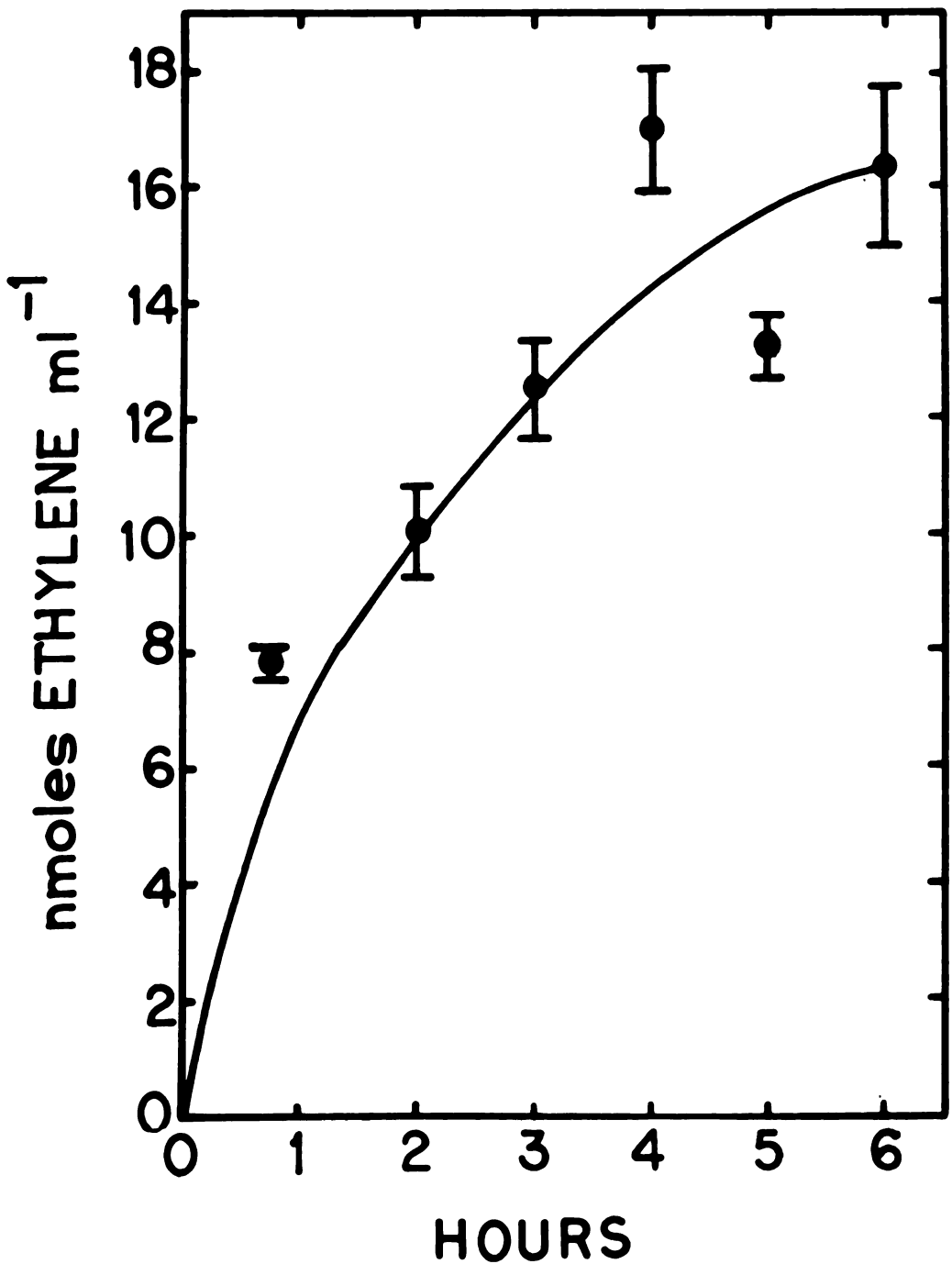

nmoles ETHYLENE ml^{-1}
0
2
4
6
8
10
12
14
16
18
0
1
2
3
4
5
6
HOURS

Fig 3. Electron paramagnetic resonance spectra of DMPO-OH adduct formed in reactions containing H_2O_2 and Fe(II) (Fenton's reagent or cell extract (B-K) as described in Materials and Methods. Fenton's reagent, A; complete, B; plus 100 mM ethanol, C; minus Fe(II), D; plus boiled cell extract, E; minus azide, F; minus EDTA, G; minus glucose, H; plus 50 mM mannitol, I; plus 100 mM benzoate, J; and plus 10 mM p-hydroxybenzoate, K. the following gain settings were used (a) = 6.3×10^2, (b) = 1.6×10^4, (C)-(K) = 4.0×10^4.

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CHAPTER III

Ultrastructural Localization of Hydrogen Peroxide Production in Ligninolytic Cells of *Phanerochaete chrysosporium*

ABSTRACT

Previous studies have shown that the hydroxyl radical derived from hydrogen peroxide (H_2O_2) is involved in lignin degradation by Phanerochaete chrysosporium. In the present study, the ultrastructural sites of H_2O_2 production in cells from ligninolytic cultures was demonstrated by cytochemically staining cells with 3,3'-diaminobenzidine (DAB). Hydrogen peroxide production, as evidenced by the presence of deposits of oxidized DAB, appeared to be localized in the periplasmic space of cells from ligninolytic cultures grown for 14 d in nitrogen-limited medium. When identical cells were treated with DAB in the presence of aminotriazole, periplasmic deposits of oxidized DAB were not observed, suggesting that the deposits of oxidized DAB resulted from the H_2O_2-dependent peroxidatic oxidation of DAB by catalase. Cells from cultures grown for 3 or 6 d in nitrogen-limited medium or for 14 d in nitrogen-sufficient medium had little ligninolytic activity, low specific activity for H_2O_2 production and did not contain periplasmic oxidized DAB. The results suggest a positive correlation between the occurence of oxidized DAB deposits in the periplasmic space, the intensity of the staining reaction, the specific activity for H_2O_2 production in cell extracts and ligninolytic activity.

INTRODUCTION

Previous studies have shown that the ligninolytic system of Phanerochaete chrysosporium is synthesized after the cessation of primary growth and in response to nitrogen starvation, apparently as a part of secondary metabolism (an idiophasic event; 10). Furthermore, a temporal relationship between the appearance of ligninolytic activity and hydrogen peroxide (H_2O_2) production activity in nitrogen-limited cultures of P. chrysosporium has been demonstrated (7). Hydrogen peroxide-derived hydroxyl radical (·OH) was shown to play an integral role in lignin degradation as evidenced by the fact that benzoate and mannitol, which scavenge ·OH, inhibited lignin degradation (7).

Hydroxyl radical has been shown to be highly reactive and to cause damage to cellular constituents such as proteins, lipids and DNA (1,5,13). In many cells these effects are minimized by compartmentalizing the H_2O_2 production activity and catalase within subcellular organelles such as peroxisomes and glyoxysomes (17). In light of the observation that high levels of H_2O_2 are produced by cell extracts of ligninolytic cultures of P. chrysosporium (contrasted with minimal levels of H_2O_2 produced by non-ligninolytic cultures) it was of interest to determine the subcellular location of the H_2O_2 production activity in P. chrysosporium. Such information would be valuable in understanding how H_2O_2 production is localized in P. chrysosporium in a way to minimize its cytotoxic effects, yet allows for the involvement of H_2O_2 in the production of ·OH in the extracellular environment.

In this study, the ultrastructural sites of H_2O_2 production activity in cells of P. chrysosporium were demonstrated by cyto-

chemically staining the cells with 3,3'-diaminobenzidine (DAB). The results of this study suggest that H_2O_2-production activity, as evidenced by the presence of deposits of oxidized diaminobenzidine (DAB) is localized in the periplasmic space of cells from ligninolytic cultures but not in cells from nonligninolytic cultures.

MATERIALS AND METHODS

Organism and culture conditions. Phanerochaete chrysosporium Burds. ME 446 (ATCC 34541) was obtained from T. K. Kirk (Forest Products Laboratory, U. S. Department of Agriculture, Madison, WI) and was maintained through periodic transfers on malt extract agar slopes as previously described (11).

The composition of the nitrogen-limited basal medium used for these experiments has been described previously (11) and contained 0.6 mM asparagine plus 0.6 mM NH_4NO_3 (2.4 mM N). High N medium was identical to the basal medium except that it contained 6 mM asparagine plus 6 mM NH_4NO_3 (24 mM N). The medium (50 ml) was dispensed into 500 ml Erlenmeyer flasks, which were then foam-stoppered and autoclaved for 15 min at 121°C. The medium was inoculated with a conidial suspension of P. chrysosporium in water as previously described (6). The cultures were incubated in air at 39°C, without agitation, for various periods of time as indicated in the text.

Cytochemical staining techniques. Hydrogen peroxide production activity in cells of P. chrysosporium was demonstrated by cytochemical staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Aldrich Chemical Co., Milwaukee, WI) using a procedure modified from Van Dijken and Veenhuis (19). The cells were harvested by centrifugation at 12,000 X g for 10 min, and washed once with 50 mM sodium phosphate (pH 5.5). The pellet was resuspended in the same buffer, homogenized with a glass tissue homogenizer and centrifuged as above. The cells were fixed in 3% glutaraldehyde in 0.1 mM cacodylate buffer (pH 7.0) and were treated with aerated DAB solutions [2 mg DAB and 100 μmoles glucose per ml of sodium phosphate buffer (70 mM; pH 6.6) at 39°C for 1 h]. In control

experiments, glutaraldehyde-fixed cells were preincubated for 30 min in phosphate buffer (described above) containing 50 µmoles 3-amino-1,2,4-triazole (aminotriazole, Sigma Chemical Co., St. Louis, MO) per ml. These cells were treated with DAB solution identical to that described above except that it contained an additional 50 µmoles aminotriazole ml^{-1}. After 30 min, the cells from both treatments were transferred to fresh DAB solutions. These DAB-treated cells were post-fixed with 2% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.0) at room temperature for 45 min, washed with the same buffer and held in 1% aqueous uranyl acetate overnight. The uranyl acetate was decanted and the cells were embedded in 1% Noble agar. A thin layer of solidified agar was cut into 2 mm squares, dehydrated in a graded alcohol series and propylene oxide followed by embedding in Poly-bed (Polysciences, Warrington, PA). After sectioning, the specimens were post-stained for 10 min with 2% uranyl acetate and 3 min with lead citrate and examined using a Philips EM 300 electron microscope.

H_2O_2 production. The specific activity for H_2O_2 production in cell extracts of P. chrysosporium was determined using a modified catalase-aminotriazole assay as previously described (3,7).

Degradation of ^{14}C-synthetic lignin. The degradation of (2'-^{14}C)-synthetic lignin (1.0×10^5 dpm mg^{-1}) to $^{14}CO_2$ was determined as described previously (6).

RESULTS

Cytochemical staining of H_2O_2 production activity. DAB is peroxidatically oxidized by catalase or peroxidase in a H_2O_2-dependent reaction to form an osmiophilic polymer of oxidized DAB (14,16) which becomes electron dense after treating with osmium. Hence, any subcellular locations which are the sites of catalase or peroxidase and H_2O_2 production activity will appear as dark regions in electron micrographs (14). This is the basis of the cytochemical staining employed in this study to visualize sites of H_2O_2 production activity in cells of P. chrysosporium.

Experiments were conducted to determine the relationship between levels of ligninolytic activity, H_2O_2 production and the presence of deposits of oxidized DAB within the cells. Less than 1% of the ^{14}C-synthetic lignin was degraded during the first 6 d of incubation whereas 5.1% and 11.8% of the added ^{14}C-lignin was degraded after 10 and 14 d incubation, respectively, by cells grown in nitrogen-limited medium (Table 1). Furthermore, extracts prepared from cells grown for 3, 6, 10, and 14 d in nitrogen-limited medium had specific activities (nmol min^{-1} mg $protein^{-1}$) for H_2O_2 production of 2.9, 3.1, 22.1, and 52.3, respectively (Table 1). Thus only cells from 10 and 14 d-old cultures of P. chrysosporium, which were actively ligninolytic, had significant levels of H_2O_2 production activity whereas 6 d cultures did not have appreciable levels of ligninolytic activity or H_2O_2 production activity. When cells from 10-14 d-old nitrogen-limited cultures were stained with DAB, electron-dense deposits of oxidized DAB were observed between the cell wall and cytoplasmic membrane of the fungal cells (Fig. 1A and C). In contrast, in cells from 3 and 6 d-old

cultures these periplasmic deposits were not seen (Fig. 1D and E). The deposits of oxidized DAB were found to be distributed rather evenly along the length and circumference of the cells (Fig. 1A and C). The H_2O_2 production activity of cells grown in nitrogen-limited cultures for 10 d was less than half that found in cells after 14 d incubation (Table 1). As one might expect, the staining intensity and number of deposits of oxidized DAB in the periplasmic space of 10 d-old cells was qualitatively less than that observed with 14 d-old cells (Fig. 1C). These data suggest a positive correlation between the numbers of periplasmic deposits of oxidized DAB which appear to be the sites of H_2O_2 production, and ligninolytic activity of P. chrysosporium.

Aminotriazole is a known inhibitor of catalase (15). Therefore, when cells from 10 d and 14 d-old nitrogen-limited cultures were incubated with DAB in the presence of aminotriazole, deposits of oxidized DAB would not be expected to be formed if only catalase (and no peroxidase) was present in the periplasmic space. The results (Fig. 1B) indicated that the electron-dense regions observed in the electron micrographs (Fig. 1A and 1C) were due to the peroxidatic oxidation of DAB by catalase.

Little lignin degradation occurred in cultures of P. chrysosporium when grown in high nitrogen medium (Table 1). Also the specific activity for H_2O_2 production decreased in these cultures from 12.5 on d 3 to 6.1 on d 14. Furthermore, in these 14 d-old cultures the specific activity for H_2O_2 production was approximately 8-fold lower than that observed in similar cells grown in nitrogen-limited medium. If there was a positive correlation between the number of deposits of oxidized DAB periplasmic and ligninolytic activity, as indicated by the above results, then one would expect few or no periplasmic

deposits in cells from 14 d-old nonligninolytic cultures of P. chrysosporium, grown in high N medium. The results showed that this was the case (Fig. 1E).

DISCUSSION

Lignin was not degraded during the first six days of incubation in cultures of P. chrysosporium grown in nitrogen-limited medium (2.4 mM N) and the H_2O_2 production activity of these cells was also low. Similarly, cells from 14 d-old cutlures of P. chrysosporium grown in high nitrogen medium (24 mM N) degraded less than 1% of the added ^{14}C-synthetic lignin and exhibited low levels of H_2O_2 production activity. Deposits of oxidized DAB were not observed in the periplasmic space of cells from either of the above nonligninolytic cultures. The development of ligninolytic activity in nitrogen-limited cultures was concomitant with an increase in the specific activity for H_2O_2 production, which was in turn correlated with the appearance of periplasmic deposits of oxidized DAB. There was no apparent difference in the number of cytoplasmic oxidized DAB-positive microbodies between cells from ligninolytic cultures and nonligninolytic cultures. These observations suggest that the H_2O_2 production activity associated with lignin degradation is localized in the periplasmic space of cells from ligninolytic cultures (L.J. Forney and C.A. Reddy, unpublished data). Most, but not all of the hyphae examined from 14 d-old culture contained deposits of oxidized DAB in the periplasmic space. In cells from 10 d-old cultures, the deposits of oxidized DAB were observed in a lower proportion of the cells examined and, when present, were less numerous and smaller (Fig. 1C) than in 14 d-old cells.

In the presence of H_2O_2, the oxidation of DAB can be accomplished by several enzymes, including peroxidase and catalase (14,16). When cells containing high specific activities for H_2O_2 production were treated with DAB in the presence of aminotriazole, deposits of oxidized DAB were not observed in the periplasmic space of the cells. Since aminotriazole specifically inhibits catalase (15), these data indicate that peroxidatic oxidation of DAB was most likely effected by catalase and was not an artifact of the staining procedure. Furthermore, if significant levels of peroxidase had been present in the periplasmic space, deposits of oxidized DAB would have been observed in the presence of aminotriazole. Since this was not the case, little or no peroxidase was present in the periplasmic space.

Catalase and H_2O_2 production activities are known to be located in subcellular organelles such as peroxisomes and glyoxisomes (8,17). The definition of deposits of exodized DAB in the periplasmic space of cells used in this study suggests that the H_2O_2 production activity is present in membrane-limited organelles. However, due to the intensive oxidation of DAB it was not possible to determine with certainty whether or not these activities are contained within a unit membrane. Further studies are needed to clearly demonstrate this point.

The mechanism by which P. chrysosporium produces H_2O_2 has not been established. However, wood decomposing fungi have been shown to produce numerous enzymes, including various carbohydrate oxidases (12) cellobiose-quinone oxidoreductase (18) and aromatic alcohol oxidases (4,9), which oxidize various substrates with the concomitant production of H_2O_2. Hydrogen peroxide could also be produced through the

enzymatic or chemical dismutation of superoxide radicals (1,2). Further investigations are needed to determine the specific enzymatic reaction(s) which play a role in H_2O_2 production by P. chrysosporium.

To our knowledge, these results represent the first time that H_2O_2 production activity and catalase have been demonstrated to be localized in the periplasmic subcellular structures of a fungus. This unusual arrangement would seem to be advantageous to P. chrysosporium. With the H_2O_2 production activity located outside the cytoplasmic membrane the fungus is able to avoid high levels of H_2O_2 in the cytoplasm where it could participate in cytotoxic processes, yet the enzymes responsible for the production of H_2O_2 are retained by the cell. Furthermore, the H_2O_2 could readily diffuse from the cells whereupon it could undergo reductive cleavage to produce ·OH which is known to be involved in lignin degradation.

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Table 1. Effect of culture age and nitrogen concentration on DAB reaction, hydrogen peroxide production and ^{14}C-synthetic lignin degradation by P. chrysosporium.[a]

	Culture Age							
	3 d		6 d		10 d		14 d	
	2.4 mM	24 mM	2.4 mM	24 mM	2.4 mM	24 mM	2.4 mM	24 mM
DAB reaction[b]	-	-	-	-	+	-	+++	-
H_2O_2 production[c]	2.9	12.5	3.1	n.d.[e]	22.1	n.d.	52.3	6.1
% ^{14}C-lignin degraded[d]	0.1	0.2	0.7	0.4	5.1	0.6	11.8	0.9

[a]Cultures were incubated in air at 39°C without agitation either in nitrogen-limited (2.4 mM N) or high nitrogen (24 mM N) medium.

[b](-) = No oxidized DAB deposits; (+) = moderate oxidation of DAB; (+++) = extensive oxidation of DAB.

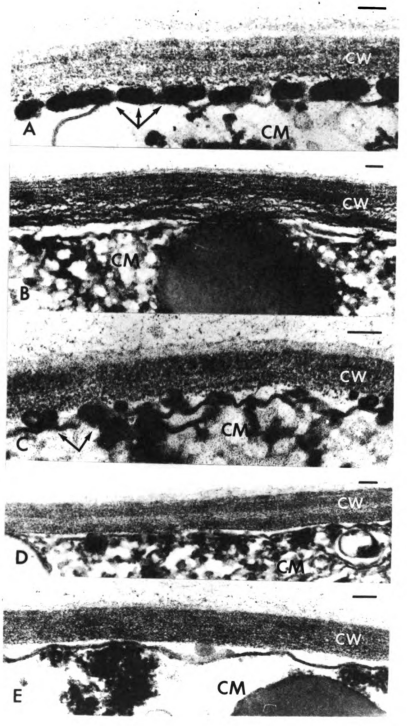
[c]Expressed as nmoles H_2O_2 min^{-1} mg $protein^{-1}$.

[d]^{14}C-lignin recovered as $^{14}CO_2$.

[e]n.d. = not determined.

FIGURE LEGEND

Figure 1. Transmission electron micrographs of P. chrysosporium showing details of cell wall (CW), periplasmic area and cytoplasmic membrane (CM). Deposits of oxidized DAB (see text for details) are indicated by arrows. Bar = 0.1 μm. Cells grown in nitrogen-limited medium for 14 d (A; 90,100X), 10 d (C; 113,400X), and 6 d (D; 56,700X); (B) is identical to (A) except for the addition of aminotriazole to the staining solution (60,000X); cells grown for 14 d in medium not limited in nitrogen (E; 80,000X).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APPENDICES

APPENDIX 1

APPENDIX 1

Speculation on the involvement of oxygen radicals in lignin degradation by wood decomposing fungi

How microorganisms degrade a polymer as structurally complex as lignin is a very intriguing area of research. It has become clear, based on the results of earlier studies, that lignin is metabolized in a manner different from that of other biopolymers (14, 23). In this paper, some of the relevant structural features of lignin and the apparent requirements they may impose on the ligninolytic systems of microorganisms are discussed.

Within plant tissue, lignin exists in a matrix with hemicelluloses (1, 35). Fungal hyphae are probably unable to penetrate the intact lignin-polysaccharide matrix, and so much of the polymer probably remains physically inaccessible to the fungus. Furthermore, lignin is insoluble in water (25), has a high molecular weight (1, 3, 25) and is three dimensional (1), thus making transport into the cell unlikely. These features of lignins in plant tissues would appear to dictate that the agents which attack lignin be extracellular and freely diffusible.

In consideration of the structural complexity of the lignin polymer, the agents responsible for lignin degradation probably have rather broad substrate specificity. More than 24 different intermonomer linkage types have been shown to occur in lignin (1), many of which are not readily hydrolyzed (1, 25). Due to the nonenzymatic free radical

mechanism by which the polymer is formed, the linkages are nonrepeating and are randomly distributed throughout the polymer (1). Furthermore, the polymer is racemic despite the presence of asymmetric carbons in the aliphatic three carbon side chains (33). Indeed, each lignin molecule can be considered to be structurally unique. Yet despite the structural complexity of lignin, white-rot fungi are able to extensively oxidize and degrade (greater than 90%) the polymer (1, 8, 20, 21). These findings suggest that most, if not all, of the intermonomer linkages in lignin can be attacked by white-rot fungi.

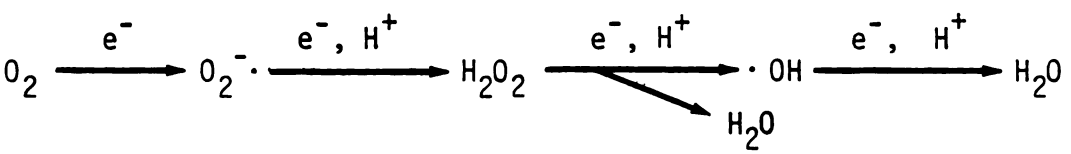
Certain general observations suggest that at least certain components of the ligninolytic system have relatively low specificity. Lignin decomposing fungi are capable of degrading structurally different lignins including lignins in various genera of plants (e.g. gymnosperm, angiosperm and grass lignins), within different tissues of the same plant (e.g. early, late and compression wood) (20) and industrially modified lignins (e.g. kraft lignins and lignosulfonates) (26). Thus, the ligninolytic system is able to recognize structurally different polymers as substrates. Furthermore, Keyser et al (19) suggested that the relatively low rates of lignin degradation by fungi, and the apparent inability of lignin to serve as a growth substrate for fungi, may be indicative of the low activity of the ligninolytic system and, indirectly, that the system possesses low specificity.

As discussed above (see Literature Review), cell-free culture filtrates of wood decomposing fungi are low in protein (3, 15) and are unable to extensively transform lignin (14, 15). In fact, conclusive evidence for the involvement of any enzyme in lignin degradation is lacking.

Thus it would appear that the agent(s) which effect the initial transformations of lignin should be extracellular, freely diffusible, and be relatively nonspecific and nonstereoselective in their mode of attack. These apparent requirements have led to the suggestion that the extracellular transformations of lignin which occur during its degradation by white-rot fungi may be mediated by "activated oxygen species" such as oxygen radicals (2, 11, 14, 28).

Oxygen chemistry

The ground state of dioxygen is a triplet state with one electron pair shared and with two unpaired electrons occupying different antibonding orbitals but having the same spin quantum number (16). Concerted reactions between dioxygen in its ground state and organic compounds, which normally are in a singlet state, are spin-forbidden and therefore kinetically unfavorable unless dioxygen is first activated (16). This can be accomplished in at least three ways, by: (1) Transferring energy to ground state oxygen to excite it to a singlet state (either $^1\Delta g^+$ or $^1\Sigma g$); (2) The reaction of oxygen with the unpaired d electrons of a transition metal or stable organic free radical (as in monooxygenases and dioxygenases; 16) or (3) Partial reduction of oxygen to form superoxide radical ($O_2^-\cdot$), hydrogen peroxide or hydroxyl radical ($\cdot OH$) (Reaction 1; 12).



Oxygen radicals have been implicated in numerous important biological processes (12) including phagocytosis by polymorphonuclear leucocytes

(4, 32), lipid peroxidation (18, 34), inflammatory responses (e.g. certain types of arthritis) (27) and xenobiotic and drug metabolism (5, 7). It has also been suggested that oxygen radicals may have a role in aging (30), spontaneous mutations in DNA (6, 36) and carcinogenesis (30).

The involvement of hydroxyl radical in lignin degradation

The mechanism by which oxygen radicals might be produced in cultures of white-rot fungi were discussed earlier (see Chapter II). Evidence presented here (see Chapter II) indicated that ·OH plays an integral role in lignin degradation by P. chrysosporium and perhaps other wood-decomposing fungi. The ·OH is a strong oxidant and reacts nonselectively with many organic compounds at rates limited by diffusion (37). The following reactions which have been shown to occur during lignin degradation by white-rot fungi could be effected by ·OH (9, 14, 29, 37): (a) Hydroxylation of aromatic rings; (b) Aromatic ring cleavage; (c) Oxidation of carbons α to aromatic rings to carbonyls; (d) Cleavage of C-C single bonds; (e) Oxidation of carbons to carboxylic acids; and (f) Demethylation of methoxyl groups. Furthermore, the involvement of ·OH in lignin degradation may explain why the process is apparently obligately aerobic (13) (see Literature Review) and why investigators have been unable to isolate enzymes capable of extensively degrading lignin (14).

In consideration of the apparent involvement of ·OH in lignin degradation, it now appears possible to offer reasonable interpretations of some of the earlier results on lignin degradation.

Inhibition of lignin degradation by o-phthalate

Fenn and Kirk (10) have shown that twice as much lignin is degraded by P. chrysosporium when 10 mM 2,2-dimethylsuccinate was used to buffer the medium as compared to that observed with 10 mM o-phthalate as the buffer. The degradation of methoxyl, side-chain and ring labelled lignins to $^{14}CO_2$ were inhibited to a similar degree in the presence of o-phthalate but the oxidation of glucose, acetovanillone and apocynal were unaffected (10). In other experiments, P. chrysosporium was grown in medium buffered with 2,2-dimethylsuccinate for 6 d at which time o-phthalate (9 mM final concentration) and ^{14}C-synthetic lignin were added. The degree of inhibition of lignin degradation observed was approximately the same as when the cultures were grown from d 0 in medium containing o-phthalate. Similar results were obtained when cycloheximide (an inhibitor of eucaryotic protein synthesis) was added to the cultures 1 h prior to the addition of o-phthalate and ^{14}C-synthetic lignin. These results indicate that o-phthalate inhibits the existent ligninolytic system.

Hydroxyl radical rapidly react with aromatic compounds ($k\text{-}10^9\text{-}10^{10}\ M^{-1}s^{-1}$; 37). o-Phthalate is an aromatic compound and as such would be expected to efficiently scavenge ·OH. Since .OH have been shown to play a role in lignin degradation, the inhibition of lignin degradation by o-phthalate may be due to its ability to scavenge .OH.

Cosubstrate requirement for lignin degradation

Data available to date indicate that lignin does not serve as a carbon source for growth of white-rot fungi (22). A cosubstrate appears to be required not only for growth but also to support lignin

degradation (17, 31). However, studies using specifically labelled ^{14}C-synthetic lignin have shown that lignin carbon does enter into central metabolic pathways in white-rot fungi since it was incorporated, with rearrangement of the labelled carbons, into the secondary metabolite veratryl alcohol (17) and was respired as $^{14}CO_2$ (17, 19, 31). It is possible that the function of the cosubstrate, in addition to providing carbon and energy for cell growth, is to serve as a source of reducing equivalents for the partial reduction of O_2 to $O_2^{-}\cdot$, or H_2O_2 which then lead to the production of ·OH. The latter in turn mediate the degradation of lignin.

Effect of oxygen partial pressure

Dioxygen has been shown to aggravate the damage caused in biological systems by oxygen radicals. For example, Kong and Davison (24) examined the effect of various partial pressures of oxygen on the resultant increase in permeability in the plasma membrane of erythrocytes to glyceraldehyde-3-phosphate following the production of radicals by γ-irradiation. When erythrocyte suspensions were incubated in an atmosphere of 0.2 and 1.0 atm of oxygen, the membrane permeability increased 2.8 and 60-fold respectively, as compared to anaerobic conditions. The observed increase in damage caused by oxygen was thought to be due to increased branching of radical chain reactions. Similar effects may in part explain the higher rate of lignin degradation by P. chrysosporium when incubated under higher partial pressures of oxygen (0.4-1.0 atm) as compared to air or 0.05 atm of oxygen. Following initiation by ·OH, the increased branching of radical chain reactions in lignin which would be likely to occur in the presence of high partial pressures of oxygen,

may allow for more extensive oxidation and depolymerization of the polymer. The depolymerization of lignin is presumably the rate-limiting step in lignin degradation. As a result, the more extensive depolymerization of lignin under high partial pressures of oxygen would in turn results in more rapid metabolism of lignin carbon to CO_2.

Thus, the data obtained in previous investigations appear consistent with the involvement of .OH in the degradation of lignin by wood-decomposing fungi. Further studies are needed to determine the specific reactions effected in lignin by .OH, the mechanism of lignin depolymerization, identification of the depolymerization products which are subsequently metabolized by the fungi, and what, if any, other microbially produced agents are involved in the extracellular degradation of the polymer.

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APPENDIX 2

APPENDIX 2

Assay of Ligninolytic Activity (^{14}C-lignin → $^{14}CO_2$) by Phanerochaete chrysosporium: Evaluation of methods for the recovery and quantification of $^{14}CO_2$

Introduction

The biodegradation of lignin is frequently assayed by determining the amount of ^{14}C-synthetic lignin or ^{14}C-(lignin labelled) lignocellulose respired as $^{14}CO_2$ (2). Due to the low rates of degradation observed, it is sometimes necessary to measure very low levels (50-100 dpm) of radioactivity and thus it is critical that the methods used to recover and quantitate the $^{14}CO_2$ be carefully optimized and employed. These studies were undertaken to evaluate the effect of several parameters on $^{14}CO_2$ quantification.

Evaluation of methods to trap $^{14}CO_2$

Two different methods for trapping $^{14}CO_2$ were evaluated. In one method, the $^{14}CO_2$ was trapped directly in KOH and in the second method $^{14}CO_2$ was flushed from the headspace of the flask and trapped in scintillation cocktail which contained one of several organic amines. In the reaction mixtures used in these experiments, the $^{14}CO_2$ was produced by acidification of alkaline solutions to which $NaH^{14}CO_3$ had been added.

1. Use of KOH to trap $^{14}CO_2$. To evaluate the trapping of $^{14}CO_2$ directly in KOH, a known amount of $NaH^{14}CO_3$ in 5 ml of slightly alkaline water was added to a 50 ml Erlenmeyer flask which was then closed with a serum stopper from which a small polypropylene well (Kontes, Co., Vineland, NJ) containing 300 µl 0.5 M KOH was suspended. To acidify the reaction mixture, one ml of 1 M HCl was injected through the serum stopper into the $NaH^{14}CO_3$ solution. The reactions were allowed to equilibrate for at least four hours at room temperature after which the KOH from the center well was transferred to 10 ml of scintillation cocktail which contained ACS (Amersham-Corp., Arlington Heights, IL)-water-0.2 M KOH (200:15:2) (3). The amount of $^{14}CO_2$ recovered was determined by liquid scintillation counting. The counting efficiency of the scintillant was determined to be 89.3% using a ^{14}C-toluene standard. Experimental results showed that 97.1% of the added $NaH^{14}CO_3$ was recovered as $^{14}CO_2$.

Upon addition of acid to the medium, the equilibrium between CO_2 (g), HCO_3^- and $CO_3^=$ shifts in favor of CO_2(g). This would favor the recovery of $^{14}CO_2$, produced through microbial metabolism of ^{14}C-lignin, which may be dissolved in the medium.

In some instances, acidification of the medium or reaction mixture is not desired. The following experiments were conducted to determine how effectively $^{14}CO_2$ could be recovered when $NaH^{14}CO_3$ was added to a buffered medium (pH 6.8), which was not subsequently acidified. Ten ml of 20 mM Na-PO_4 buffer (pH 6.8) and aliquots of $NaH^{14}CO_3$ were placed in 50 ml Erlenmeyer flasks which were immediately sealed with serum stoppers as described above. One ml amounts of 1.0 M HCl

were added to some of the flasks and no additions were made to the remaining flasks. After allowing 48 h for equilibration of the gases, the trapped $^{14}CO_2$ was quantified. There was no difference in the effectiveness of $^{14}CO_2$ recovery between the acidified medium and the buffered medium with 96.0% of the added $NaH^{14}CO_3$ recovered as $^{14}CO_2$ from acidified reactions and 96.9% recovered as $^{14}CO_2$ from reactions which were not acidified.

2. Trapping of $^{14}CO_2$ by flushing of headspace. Previous investigators have shown that the pO_2 in the culture atmosphere markedly affects the rate and extent of lignin degradation by P. chrysosporium (1). In the procedure described above, the flask atmosphere was not replenished with air and remains closed to the atmosphere except for the short periods of time required to change the CO_2 traps (serum stoppers and 0.5 M KOH in the center well). This would probably result in the partial depletion of oxygen in the culture headspace which would have a deleterious effect on lignin degradation. Furthermore, the effectiveness of $^{14}CO_2$ recovery using this method was compromised in some instances due to the poor fit between the serum stopper and the Erlenmeyer flask.

The following experiments were conducted to evaluate the trapping of $^{14}CO_2$ by flushing the flask headspace and trapping the $^{14}CO_2$ in scintillation fluid which contained an organic amine. The experiments were conducted by placing 200 µl of $NaH^{14}CO_3$ in a dry 125 ml Erlenmeyer flask and closing the flasks with rubber stoppers fitted with 2-5 mm glass tubes. One of the tubes extended into the flask to within 1-2 cm of the acid solution and the second tube extended to the bottom of the stopper. On the outside of the flasks, both tubes were connected via latex

tubing to glass wool-plugged filters and the flasks were closed by clamping the rubber tubes. Ten ml of 1 M HCl was then injected into the flasks through the rubber stopper using a syringe with an 18 guage needle. To flush the $^{14}CO_2$ present in the flask headspace, the tube which extended into the flask was connected to a CO_2-free air supply and the second tube was connected to a 1 ml syringe barrel fitted with a 1 in 25 guage needle. The syringe barrel was then placed in a polypropylene scintillation vial containing 10 ml of liquid scintillation cocktail. The CO_2 was scrubbed from the air used to flush the flasks by bubbling it through 0.02 M NaOH which contained 0.002% phenol red and then through distilled water to remove aerosols containing NaOH. The latex tubing delivering the CO_2-free air was then connected to a gassing manifold constructed of brass aquarium gang valves. The flasks were flushed for 60 min at a flow rate of 60 ml min^{-1}. Flushing at 75 or 100 ml min^{-1} did not improve recoveries of $^{14}CO_2$ (data not shown).

The overall efficiencies (counting efficiency and their ability to trap $^{14}CO_2$) of four liquid scintillation counting (LSC) cocktails were determined. The following LSC cocktails were examined: (a) ACS (Amersham-Searle, Arlington Heights, IL)-methanol-ethanolamine (Aldrich Chemical Co., Milwaukee, WI) (5:4:1); (b) 3a20 (Research Products Internation, Elk Grove Village, IL)-methanol-ethanolamine (5:4:1); (c) Count-sorb (Research Products International); and (d) ACS:methanol: phenethylamine (Eastman Kodak Chemicals, Rochester, NY). The results showed that the overall efficiency of LSC cocktails (a), (b), (c) and (d) were 94.8%, 93.8%, 92.8% and 90.1%, respectively. Thus, all the scintillation cocktails were efficient. Cocktails (c) and (d) were excluded from further consideration since phenethylamine and Count-

sorb are rather expensive and phenethylamine is noxious relative to ethanolamine. Cocktail (a) uses ACS, a xylene based, water compatable LSC cocktail; whereas cocktail (b) uses 3a20, a toluene based, liquid scintillation cocktail which is not compatable with aqueous samples except at very low concentrations of water. Therefore, cocktail (a) was chosen for future use on the basis of its greater versatility in biological experiments.

Effect of flushing time on the recovery of $^{14}CO_2$

To optimize the recovery of $^{14}CO_2$ during the flushing of cultures, the $^{14}CO_2$ was produced through the metabolism of $CH_3{}^{14}COONa$ by Nocardia sp. DSM 1069 (4). The basal medium used contained per l of distilled water: yeast extract, 0.1 g; Na_2HPO_4, 1.5 g; NaH_2PO_4, 1.5 g; NaCl, 0.25 g; $(NH_4)_2SO_4$, 1.25 g; $MgSO_4 \cdot 7\ H_2O$, 0.01 g; $FeSO_4 \cdot 7\ H_2O$, 0.002 g; and sodium acetate, 1.36 g (8.6×10^4 dpm). The final pH of the medium was adjusted to 6.8, dispensed into 125 ml Erlenmeyer flasks (10 ml/flask) and sterilized by autoclaving at 121°C for 15 min.

The flasks were inoculated with 0.5 ml of a 24 h old culture which had been grown in the same medium containing nonradioactive acetate and closed with sterile rubber stoppers as described above. The cultures were then incubated for 16 h at 30°C on a rotary shaker water bath. To quantify the respired $^{14}CO_2$, the flasks were flushed with CO_2-free air for a total time of 60 min. After 15, 30 and 45 min of flushing, the CO_2 traps (scintillation vials with cocktail a) for some of the flasks were replaced with fresh CO_2 traps. Thus, the amount of $^{14}CO_2$ trapped after 15, 30, 45 and 60 min was determined. After flushing the flasks, 200 µl of the culture was removed and placed in a vial

containing 10 ml of LSC cocktail (a). The radioactivity present in the vials was determined by liquid scintillation counting using a Searle Model Delta 300 liquid scintillation counter (Searle, Arlington Heights, IL) with preset discrimination cartridge (^{3}H and ^{14}C cartridge).

During the 16 h incubation period, approximately 61% of the added $CH_3{}^{14}COONa$ was respired as $^{14}CO_2$. After 15, 20, and 45 min of flushing, 87, 93, and 96% respectively of the total $^{14}CO_2$ produced had been flushed from the flasks and trapped in the CO_2 traps (Table 1). Flushing the flasks for 60 min did not significantly increase the recovery of $^{14}CO_2$ compared to that observed with 45 min flushing. Total recovery of the added ^{14}C was greater than 95%. Based on these data, a 45 min flushing time (equivalent to 7 total flushings of the headspace) was determined to be optimal for maximal recovery of $^{14}CO_2$ and to ensure adequate aeration of the culture.

Determination of quench correction curves

A quench correction curve for cocktail (a) (ACS-methanol-ethanolamine, 5:4:1) was constructed using the sample channels ratio method. Ten ml of cocktail (a) was added to five replicate vials. ^{14}C-Toluene (100 ul, 2.125 X 10^4dpm, ICN, Davis, CA) was added to each vial. The radioactivity present (counts per minute, cpm) and the sample channels ratio were determined as described above. In four successive trials, 30 ul of CH_3Cl was added to each vial and the cpm determinations were repeated. All determinations of radioactivity were made to ± 1% and the counting efficiencies were calculated. The regression equation describing the plot of counting efficiency vs sample channels ratio was $y = -51.74\ x + 132.23$ ($r^2 = -0.98$). The plot of the counting efficiency

vs sample channels ratio (SCR) was nonlinear at SCR values greater than 0.920 and therefore, the equation should not be used when the SCR is larger than this value.

Table 1. Effect of flushing time on the recovery of $^{14}CO_2$.

Flushing time (min) [a]		$\%^{14}CO_2$ recovered [b]		$\%^{14}C$ recovered [c]
Trap 1	Trap 2	Trap 1	Trap 2	
15	45	87.2±1.8	12.9±1.8	95.9±0.6
30	30	93.4±0.8	6.7±0.8	102.9±1.2
45	15	96.8±0.9	3.3±0.9	96.1±1.1

[a] Flow rate = 60 ml min^{-1} CO_2-free air

[b] $\%^{14}CO_2$ Trap 1+ $\%^{14}CO_2$ Trap 2 = 100%

[c] $\%^{14}C$ Recovered = $\frac{^{14}C \text{ remaining in medium} + ^{14}CO_2 \text{ Trap 2}}{CH_3{}^{14}COONa \text{ Added}}$ X 100

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