MICROBIOLOGICAL ASPECTS OF LIGNIN DEGRADATION[1]

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With the current increased emphasis on the utilization of by-products from large industrial operations, the potential utilization by-product lignin is attracting considerable attention. In 1944, 188,500,000 tons of wood were cut in the United States (11). Of this total, more than 100 million tons were left either as waste in the woods or as waste in the manufacture of wood products. The 25 million tons of lignin in this wood is either burned or is slowly converted into soil organic matter by microbiological and climatological agents. An additional 3.4 million tons of lignin was dumped into streams in the form of an aqueous solution of calcium lignosulfonate, which is a by-product of the sulphite pulp mill. This latter amount of lignin is readily available for further use, and is at present largely disposed of in our waterways, creating an increasingly serious pollution problem.

A perusal of the literature to survey the possibilities of a microbiological degradation of lignin reveals a confusing array of data pertaining to the action of bacteria, fungi, and enzymes on lignin. Much of this confusion exists because of the lack of definite knowledge of the structure and properties of lignin. For this reason, many different preparations labeled "lignin" have been used as substrates for the growth of microorganisms. In addition, questionable analytical techniques have been utilized to analyze for residual lignin remaining after the action of bacteria and fungi on lignin-containing tissues.

In the past fifteen years our knowledge of the chemistry of lignin, although by no means complete, has been significantly advanced. It seems useful at this time to review the work reported to date on the microbiological degradation of lignin in the light of these advances in our understanding of this naturally occurring polymer. Reviews on this subject that have appeared in the past are those by Waksman (89), Norman (65), and Phillips (71).

THE CHEMISTRY OF LIGNIN

A detailed discussion of the complex chemistry of lignin is outside the scope of this review. The brief presentation given here is intended only to orient the reader in the present status of our knowledge of the structure of the lignin molecule. For a comprehensive and current treatment of lignin chemistry, the reader is referred to Brauns (12). A less extensive summary of modern concepts of lignin chemistry has recently been presented by Erdtman (23).

From a physical standpoint lignin is an amorphous polymer having an average molecular weight of 1000–10,000, and is very insoluble in water and most organic solvents. It is slightly soluble in organic hydrophilic solvents, particularly

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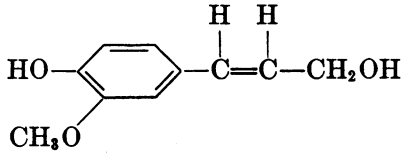
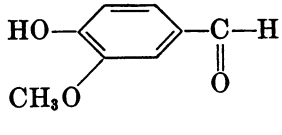
dioxane. It is important to keep in mind that in spite of a period of over 100 years of research, the precise structure of lignin is not yet definitely elucidated. We know that lignin is the polymer of an aromatic compound, or of a group of aromatic compounds, containing one aromatic ring per unit of about 10 carbon atoms. In 1896 Klason (48) put forth the view that lignin is closely related to coniferyl alcohol, a 10-carbon atom compound occurring widely in nature as the glucoside coniferin.



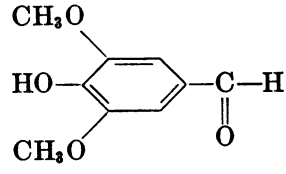
Coniferyl Alcohol

Subsequent developments have confirmed a close relationship between coniferyl alcohol and lignin. Several important researches substantiating this relationship are presented below. These, along with many others, have established the aromatic nature of lignin and its relationship to coniferyl alcohol.

Various workers (49, 85) had observed that small amounts of vanillin were formed when gymnosperm lignin in various forms was treated with alkali. Yields as high as 6 or 7 per cent were reported and this process forms the basis of commercial methods for the manufacture of vanillin from lignin. Freudenberg and co-workers (30) later found that the yield of vanillin could be increased to 25 per cent of the lignin, by oxidizing the wood meal directly with alkaline nitrobenzene mixtures. Hibbert *et al.* (44) were able to isolate mixtures of syringaldehyde and vanillin which totaled 45 per cent of the weight of lignin in deciduous woods (angiosperms). The nature of aldehydes obtained was found to be a distinct differentiating feature between gymnosperms and angiosperms, the former yielding only vanillin and the latter a mixture of vanillin and syringaldehyde.

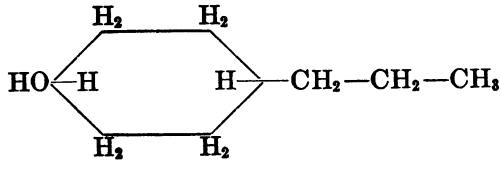


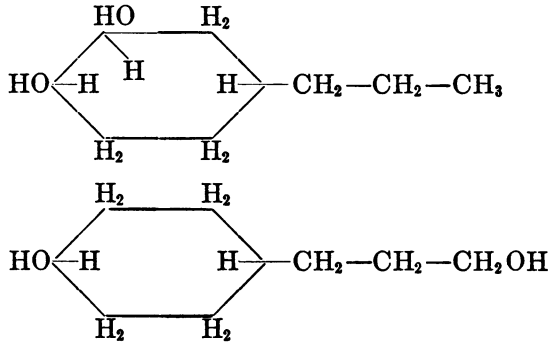
Vanillin



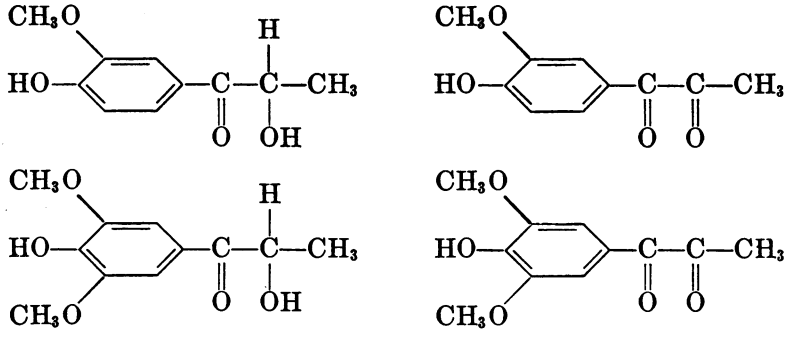
Syringaldehyde

Harris, D'Ianni and Adkins (41) initially hydrogenated lignin at high temperature and pressure and obtained a series of cyclohexyl-propyl compounds in excellent yield. This reaction could be carried out either on isolated lignin or on wood meal, giving amounts of the compounds as high as 80–90 per cent of the weight of the lignin. Some of the isolated compounds were:

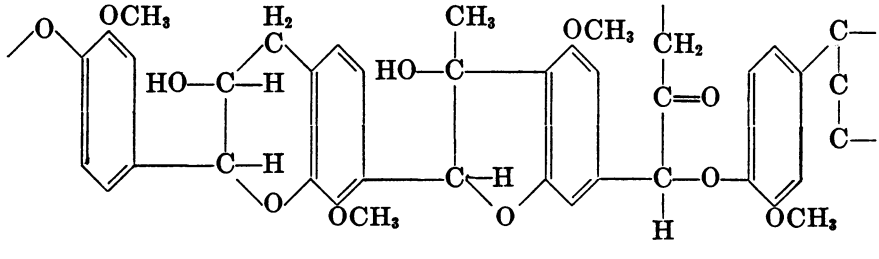




Hibbert and co-workers (44) treated maple wood meal with dry ethanol containing a small amount of hydrochloric acid and reported the isolation, in quantities totaling 10 per cent of the weight of the lignin, of a series of oxygenated propylphenyl derivatives:



The work mentioned above, together with many other investigations, all point to the fact that lignin is probably a condensation product of one or a series of closely related oxygenated phenyl propane compounds. The manner in which these units are linked to form the polymer is not known, but Freudenberg (41) has advanced the following speculative formula which could explain most of the observed facts about lignin:



Alfred Russell (79a) claimed that a synthetic polymer, poly-8-methoxydihydrobenzopyrone, which he obtained by the condensation of vanillin acetate, was identical with gymnosperm lignin. This view was not accepted by most lignin investigators. Recently, C. R. Russell (79b) has synthesized 5-acetyl vanillin, which is supposed to be the monomeric unit in the condensation of

poly-8-methoxydihydrobenzopyrone, but which could not be isolated from intermediate reaction mixtures. Condensation of 5-acetyl vanillin led to products quite different from A. Russell's polymer. This report casts considerable doubt even on the poly-8-methoxydihydrobenzopyrone structure claimed for A. Russell's polymer.

ANALYTICAL ASPECTS

Before proceeding with a review of published work on lignin degradation an attempt will be made to present some of the limitations, from an analytical and chemical point of view, of much of the work that has been done to date.

Numerous claims have been made in the literature purporting to demonstrate the ability of microorganisms to utilize lignin as a sole carbon source, or the existence of enzymes acting specifically on the lignin molecule (5, 9, 36, 55, 60, 91, 92, etc.). Critical examination of this work in the light of modern concepts of lignin chemistry indicates that most of the work reported is based on several dubious assumptions. Some of these assumptions may be listed as follows.

1. Lignin isolated by concentrated acid, alkaline, or sulfite treatment of plant material remains sufficiently unaltered to be used as a substrate for nutritional or enzyme studies.

2. Certain other substances, e.g. tannic acid, are closely related to lignin in structure, and they can therefore be used as alternate substrates in such studies.

3. Various color reactions can be reliably used to ascertain the presence or disappearance of lignin.

Considering the first of these assumptions, it seems apparent that lignin isolated by treatment of plant material with acid, alkali, or sulfite would inevitably contain varying amounts of non-lignin materials. Such materials are likely to be more available to the organisms or enzymes in question than would the lignin. Harris *et al.* (41a) reported that lignin isolated by the treatment of maplewood with cold fuming hydrochloric acid contained carbohydrates; this isolated lignin contained fewer methoxyl groups than could be calculated for "native" lignin as it exists in wood. The same authors found that by using the cuprammonium method (41a) for isolating lignin, which consists of alternately hydrolyzing wood with boiling 1% sulfuric acid and cuprammonium solution (a solution of copper hydroxide in ammonium hydroxide), the isolated lignin contained a significant amount of cellulose. It was shown by Norman and Jenkins (66) that when certain sugars, especially xylose and fructose, are treated with 72 per cent sulfuric acid, as used in the isolation of sulfuric acid lignin (94), insoluble residues calculated as lignin were obtained. Polysaccharides containing pentoses also behave in this way. Such condensation products should therefore be expected when lignin is isolated by treating plant materials with 72 per cent sulfuric acid. Norman and Jenkins (67) further showed that various plant materials, on treatment with 72 per cent sulfuric acid gave isolated lignins containing nitrogen. They report convincing evidence to indicate that this nitrogen arises from a condensation of lignin and protein, resulting in a product resistant to acid hydrolysis. Waksman and Iyer (89) have produced stable lignin-protein complexes by precipitating lignin with acid from alkaline solution in the presence of proteins.

Lignin isolated from pine sawdust by alkaline extraction and subsequent acid precipitation was reported by Pringsheim and Fuchs (75) to have as much as 5 per cent of pentosan as an impurity. Similar objections can be raised to analytical results based on the use of thioglycolic acid (45) as an extractant for lignin. Holmborg (45) and later Bengtsson (6) reported up to 4 per cent of nitrogen in lignin preparations made from fungi by the use of this reagent. The latter author showed the conversion of protein to insoluble products by analyzing meat meal (obviously containing no lignin) for lignin by the thioglycolic acid method. He found 11–17 per cent of "apparent lignin". Where sulfite waste liquor or products derived from it have been used as substrates for microorganisms or enzymes, it is obvious that numerous other materials besides lignin are present, as is evidenced by many analyses (94) made of this liquor.

It must be concluded, therefore, that lignin isolated by acid, alkali, or sulfite reagents often contains degradation and condensation products of carbohydrates and proteins. Therefore, it is questionable whether the utilization of lignin isolated by these procedures can be considered as valid evidence for the ability of microorganisms to utilize lignin. It follows, also, that the use of these various techniques as analytical methods for determining the amount of lignin remaining after microbiological attack of lignin-containing material is subject to the same sources of error discussed above.

The second of the assumptions mentioned above rests on an empirical relationship found between the ability of certain fungi to produce brown halos when grown on a tannic acid medium and their classification as white rots (fungi which can decompose both lignin and cellulose in dead wood). This subject will be treated in detail in a later section of this review, but the obvious limitations of interpretations of results of this type of data should be pointed out here. Bavendamm (3) first reported the possibility of using this reaction to classify white rot fungi, and he surmised that the correlation existed because of a close structural relationship between lignin and tannic acid. Lignin is probably a polymer of guaiacyl and/or syringyl propanol or propanone units, while tannic acid (of the type used in the media mentioned above) is a mixture of galloylated glucoses. The only structural relationship between these two materials is the presence of free phenolic groups, which are more abundant in tannic acid than in lignin. The brown halo produced by some white rot fungi (and other fungi not involved in wood decay) on tannic acid media is most probably due to an oxidation of the phenolic groups of the galloyl moiety by a phenol oxidase excreted by the growing organism into the medium (51).

The final assumption frequently met with in papers dealing with the microbiological degradation of lignin is that color reactions can be reliably used to ascertain the presence or disappearance of lignin. The use of various color reactions has been associated with investigations of the biological degradation of lignin since Czapek (20) reported that a substance which he called "hadromal", extracted from rotted wood, gave an intense red color when treated with phloroglucinol and hydrochloric acid (the Wiesner reagent for lignin). The validity of the phloroglucinol reaction as a specific test for lignin or lignin degradation products is open to serious question. Crocker (18a) showed that many simple aro-

matic aldhydes and phenolic compounds can give this color test, and concluded that the test indicates the presence of small amounts of low molecular weight aldehydes which usually accompany lignin. Similar objections have been raised to other color reactions which have been suggested as more or less specific reagents for lignin (94). It seems reasonable to assume that the various color tests used to detect the presence or absence of lignin indicate only peripheral groupings on the lignin molecule, and are not specific for the molecule as a whole.

A final point which should be made in connection with the chemical aspects of this problem is the recent availability of a method for isolating a part of the lignin in wood by a procedure which minimizes structural changes and condensations with other materials during the isolation process. Brauns (10) in 1939 proposed a method for isolating so-called "native lignin" from wood by a process involving an initial alcohol extraction followed by dioxane-ether and dioxane-water precipitations, in which no drastic agents (heat, acid, or alkali) are used. Although the lignin isolated by this process represents only a small part of the total lignin in wood, Brauns presents convincing evidence (12) to indicate the identity, or at least the close similarity of this material with lignin as it exists in wood. This method of isolating lignin from wood has been criticized by Erdtman (23) as giving only a small yield, and therefore not being really typical of the rest of the lignin in wood, and also for being potentially contaminated with other compounds closely resembling lignin. Despite these criticisms native lignin prepared according to Brauns appears to be the only product currently available for obtaining a lignin fraction with minimal modification.

The chemical and analytical aspects of the problem briefly discussed above are presented at the beginning of the paper so that the various researches subsequently reviewed can be evaluated against a background of the limitations posed by the chemical-analytical techniques in use throughout the period in which most of this work was done.

DEGRADATION OF IN SITU LIGNIN IN PLANT TISSUES

The decomposition of lignin in plant residues, extensively studied biologically, presents a confusing picture. Nevertheless many investigators have established the fact that lignin in nature is subject to degradation. Conflicting experimental evidence is available indicating utilization of lignin from a variety of plant tissues; claims for disappearance of lignin range from negligible amounts to appreciable quantities. The source of contradiction in some of these studies appears to be the inherent difficulties in the methods for the estimation of lignin before and after microbiological attack. This has been discussed in the previous section entitled Analytical Methods. However, it is reasonable to assume that lignin does not accumulate quantitatively in or on the soil but is subject to some slow transformation processes. Otherwise there would be a tremendous amount of organic matter in soil composed of pure lignin. The status of this field of investigation was evaluated by Norman (65) who concluded that lignin *in situ* represents the most resistant major constituent of plants; during brief periods of microbiological attack there is little if any decomposition, while after prolonged attack slow decomposition occurs.

1. Decomposition of lignin in wood

Some of the very early investigations for evidence of lignin degradation in wood (95, 96) employed the qualitative test described by Czapek (20) *i.e.* the extraction of a substance from rotted wood designated "hadromal" which gave an intense red color with phloroglucinol and hydrochloric acid. The value of this test was discredited by the studies of Nutman (68), Baxter (4) and others (40) who obtained positive tests with extracts obtained from sound wood. However, the degradation of lignin in wood by some of the higher fungi (Basidiomycetes) has been established as an unequivocal fact. The wood destroying fungi have been divided into two groups, brown rots and white rots, the names illustrating the color of rot formed in the wood. Falck and Haag (26, 27) correlated this difference in appearance with chemical differences showing that the brown rot fungi attacked the cellulose but not lignin whereas the white rot fungi primarily attacked lignin, later cellulose.

A systematic examination of the chemistry of the white rots of wood has been reported by Campbell (15, 16, 17). Various types of wood were subjected to decay by white rot fungi for a period of several months after which time the wood was reduced to flour and subjected to analysis by solubility of the decomposed wood in dilute alkali together with other analytical criteria. A significant amount of the lignin was degraded, *e.g.*, *Polyporus versicolor* decomposed 35 per cent of the lignin in beechwood after 10 weeks incubation, and 27–28 per cent of the lignin in oak sapwood was decomposed by *Stereum hirsutum* after 13 months. It was found that the white rot fungi as a class are not consistent in the manner of their attack on wood; there was no general uniformity with regard to the order or proportion of the major components decomposed, though all the fungi degraded lignin to some extent. From the evidence obtained the white rots were classified into the following three groups:

Group 1. Lignin and pentosans attacked in early stages; the incidence of attack on cellulose proper is delayed. Representative fungus: *Polyporus versicolor.*

Group 2. Cellulose and its associated pentosans are attacked in early stages; the incidence of attack on lignin, and pentosans not associated with cellulose, is delayed. Representative fungus: *Armillaria mellea.*

Group 3. Lignin and cellulose are both attacked in early stages but in varying proportions. Representative fungi: *Ganoderma applanatum*, *Polyporus adustus*, *Pleurotus ostreatus* and *Polystictus abietinus.*

Wiertelak (93) examined the effect of decay caused by *Trametes pini* and *Polystictus hirsutus* on sawdust and found that these white rots effected a marked decrease in lignin along with a slow consumption of cellulose. Concomitant degradation of lignin and cellulose was also found to be the case with *Ustilina vulgaris* (Campbell and Wiertelak, 17). Scheffer (80) in an extensive study of *Polyporus versicolor* on red gum sapwood found lignin to be utilized but the relative proportions of the principal components were not materially altered. More recently Heuser, *et al.* (43) found that *Polyporus paragamenus* reduced the lignin content of aspenwood (white portion) from an original lignin content of

17.5 per cent down to 3.4 per cent after 20 months incubation though its attack was not confined to lignin. Fahraeus, Nilsson and Nilsson (25) reported that *Polyporus abietinus*, *Stereum rugosum* and *Marasmius scorodonius* decomposed approximately 80 per cent of the lignin (thioglycolic acid method) in birch sawdust in 6 months, along with considerable amounts of cellulose.

While it was long assumed that the white rot fungi were principally concerned with the degradation of lignin, it is apparent from the preceding information, that although there is no doubt that they attack the lignin, other wood components are simultaneously degraded and sometimes to an even greater extent than the lignin. There is no clear cut evidence of a situation with the white rot fungi where the sole wood component degraded, *in situ*, is lignin. None of the studies reported give any information relative to intermediate products formed during the metabolism of the lignin.

The white rot fungi vary in their host specificity. Some species such as *Polyporus versicolor* grow on almost any variety of timber while others exhibit specificity for a particular type (18).

2. Decomposition of lignin in litter

Extensive investigations on the capability of soil-inhabiting Hymenomycetes to degrade lignin contained in residues of dead plants have been carried out by Lindeberg (54). In these studies sterilized litter (pine needles, beech leaves, etc.) was inoculated with pure cultures of fungi and incubated for periods of 6–7 months after which an analysis was made for lignin, cellulose, and loss in dry matter. Lignin determinations were performed by the thioglycolic acid procedure. From a total of 46 species investigated, 44 decomposed both lignin and cellulose to a considerable degree (50 per cent) and in some instances the lignin decomposition was far in excess of the cellulose degraded, *e.g. Clavaria gracilis* decomposed approximately 40 per cent of the lignin and no cellulose and *Collybia butyraceae* decomposed 77 per cent of the lignin and only 16 per cent of the cellulose. The case of *Clavaria gracilis* presents a rather unusual situation since it represents one of the few instances where the lignin is utilized exclusively. Harris (42) in an analysis of beech litter subject to natural infection by *Marasmius peronatus* also concluded that this organism utilizes lignin as its sole source of energy. The cellulose and other carbonaceous substances in the leaves were not materially altered. The degradation of lignin appeared to proceed at a greater rate than could be accounted for by assimilation and oxidation to CO_2 and water (lignin loss 24 per cent, total weight loss 15 per cent).

From the foregoing it would appear that the soil-inhabiting Hymenomycetes are capable of degrading lignin in litter as are the Basidiomycetes in wood, and some of the Hymenomycetes exhibit preference for the lignin, even to the exclusion of other components.

3. Bacterial utilization of in situ lignin

Decomposition of lignified materials (corn stalks, oat hulls, corn cobs, and wheat straw) by soil microorganisms (presumably bacteria) was reported by Phillips, Weihe and Smith (72). A decrease in lignin as high as 48 per cent (de-

termined by the fuming hydrochloric acid method of Willstatter and Zechmeister) was found.

Anaerobic fermentation of lignin in corn stalks during a 600 day period was reported by Boruff and Boswell (8) and a significant reduction in lignin (52 per cent) was observed. The 600 day experiment of Boruff and Boswell indicated lignin utilization by gas forming bacteria since a reduction of 52 per cent in lignin probably falls outside the errors involved in the determination of lignin in the presence of proteinaceous material. It is possible, however, that the reduction was less than 52 per cent and this clearly demonstrates the slowness of biological reactions involving lignin as they occur in nature. However, their results could not be confirmed by Levine *et al.* (53). This discrepancy might possibly be reconciled on the basis of differences in the microbiological flora since the inoculum in either case was an unknown mixed culture, or possibly to discrepancies in analytical technics employed.

What appears to be unequivocal evidence for thermophilic degradation of lignin by bacteria was reported by Virtanen and co-workers (86, 87, 88). Their approach was to reduce wood to a dust by grinding with an emery-paper machine. This finely divided wood was incorporated in an inorganic salts medium and inoculated with garden soil. Analysis for lignin content and methoxyl content revealed a significant disappearance of lignin (11.4 per cent) as well as a decrease in the methoxyl content of the isolated lignin. The successful demonstration of lignin utilization in this instance can probably be attributed to the fine particle size of the substrate (wood) employed, allowing for an intimate contact of lignin with microorganisms. Cellulose fermentation was also followed in these experiments and the highest figures obtained for decomposition (in birch dust) were 67.6 per cent of the cellulose and 85 per cent of the pentosans. These data indicate that the lignin exhibits little if any inhibitory effect on the microorganisms attacking cellulose as is suggested by some (69) and also have a bearing on the debatable topic of a chemical bond existing between lignin and cellulose *in situ*. Virtanen and Hukki's (87) data might be interpreted as evidence against such a bond and in support of a physical hypothesis (33) where the lignin and cellulose form an interpenetrating system, the lignin preventing a portion of cellulose from coming in contact with microorganisms and as a consequence, this portion of cellulose does not undergo degradation. In this connection, Heuser *et al.* (43) found that pure cellulose was only slightly attacked by a white rot fungus (*Polyporus paragamenus*) and concluded that both pentosans and lignin would seem to favor its attack on cellulose in wood.

The identity of the bacteria involved in these claims of lignin degradation *in situ* has not been established and the possibility exists that such degradations are the result of synergistic or symbiotic activity.

DEGRADATION OF ISOLATED LIGNINS BY MICROORGANISMS AND ENZYMES

Lignin, prepared in various ways, has been exposed to bacteria, fungi and enzyme preparations from these organisms. The results have been inconclusive but mainly negative and are summarized below.

Garren (34, 35) prepared lignin by the sulfuric acid method from pine wood

and incorporated it in an agar medium containing mineral salts. The white rot fungus *Polyporus abietinus* was able to make sparse growth on this preparation in 26 days. This observation, together with the fact that *P. abietinus* was found to produce a brown halo in stock agar containing 0.5 per cent tannic acid, led Garren to conclude that this organism could decompose lignin in its isolated state.

Smith and Brown (84) also studied the utilization of isolated sulfuric acid lignin in sterile soil, sand culture and liquid culture. No evidence of decomposition was observed with *Trichoderma lignorum*, *Aspergillus terreus* or *Penicillium vinaceum*. A lignin preparation which had been slightly oxidized with hydrogen peroxide supported growth of *Stereum purpurum* to a small extent.

Using mixed bacterial populations from seven American lakes, Zobell and Stadler (97) suggested that sulfuric acid lignin could be utilized to the extent of 4 to 15 per cent in 30 days by these organisms. Criteria for utilization were oxygen consumed, bacterial count, and decrease in lignin content. The same observations were reported on alkali lignin.

A decrease of methoxyl content of sulfuric acid lignin was reported by Fernandez and Reguiero (28) by the action of enzyme extracts of *Polyporus hispidus* and *Auricularia mesentericus*. Similar results were observed in a lignin preparation made by the degradation of cellulose by snail cellulase.

This work on sulfuric acid lignin is difficult to evaluate. Although different organisms were used in Garren's research and in Smith's and Brown's work, it would not be safe to conclude from the evidence that *Polyporus abietinus* can utilize sulfuric acid lignin while the organisms used by Smith and Brown cannot. The growth reported by Garren was very slow and sparse and could easily have been due to very small amounts of carbohydrate and other impurities that are known to be present in sulfuric acid lignin. As regards the hydrogen peroxide oxidized lignin that could be utilized to a small extent by *Stereum purpurum* it is not clear what products of this oxidation were being utilized. A better case for lignin utilization of acid lignin is made by Zobell and Stadler. Using the best available techniques, they detected a maximum of 15 per cent disappearance of this type of lignin in 30 days. It should be kept in mind that the analysis for lignin in the presence of high-nitrogen residues like bacterial cells is open to serious error. They do not mention how much apparent lignin is obtained by analyzing microbial cells which have been grown in a lignin-free medium. The use of oxygen absorption and bacterial numbers is a criterion of growth, and does not unequivocally indicate lignin utilization, in view of the small percentages of acid lignin that were being utilized.

A few studies have been made on the utilization of other types of isolated lignin by microorganisms. Berl and Koerber (7) could not obtain fermentation of hydrochloric acid lignin with either an aerobic species or an anaerobic (*Amylobacter navicula*) cellulose fermenter. Alkali lignin prepared from sawdust was exposed by Pringsheim and Fuchs (75) to an enrichment technique-derived mixed culture of bacteria from garden soil. They reported that part of the alkali lignin was rendered alcohol soluble and that both the alcohol soluble and the alcohol insoluble portions had a lower methoxyl content than the original material. Their original preparations contained 5 per cent pentosans and these completely dis-

appeared during the 8-day incubation period. Investigating the anaerobic fermentation of hydrochloric acid lignin and of sulfuric acid lignin, Boruff and Boswell (8) found only a slow and incomplete fermentation with mixed gas-forming bacteria. Levine *et al.* (53) were unsuccessful in developing a specific lignin-decomposing flora using alkali lignin. An unidentified species of bacteria was reported by Waksman and Hutchings (90) to be able to make slow growth on phenol lignin (lignin isolated from wood by the action of phenol and hydrochloric acid). The conversion of lignin to mycelium and CO_2 was described as practically quantitative. Other kinds of isolated lignin could not act as sole sources of carbon by various organisms, using enrichment techniques.

The work described above is essentially negative; in no case is there unequivocal evidence for utilization of lignin. The bacteria in the experiment of Pringsheim and Fuchs probably made their growth at the expense of the 5 per cent of pentosans in the preparation. In the case of Waksman's work, the possibility of the utilization of the phenol moiety cannot be excluded since it is known that phenol actually reacts with the lignin molecule in the preparation of phenol lignin.

A few experiments involving isolated lignosulfonic acid and lignosulfonic acid in sulfite waste liquor have been reported. Although *Fomes pini*, *Fomes annosus* and *Stereum hirsutum* were reported by Kazanskii and Mikhailova (46) to be unable to grow on neutralized sulfite waste liquor, these workers reported that infusorial earth mixed cultures could make slow growth (in 50–60 days) if the liquor were evaporated to dryness and then redissolved in water to make a 13 per cent solution (0.25 per cent KH_2PO_4 and 0.17 per cent $(NH_4)_2HPO_4$ were added). The observation is reported that part of the alcohol-insoluble sulfonic acids were transformed into alcohol-soluble ones. Such a conversion of lignosulfonic acid to alcohol soluble material might indicate a partial desulfonation. Ledingham and Adams (1, 2, 52) report a series of researches in which a large number of wood-rotting organisms were tested for their ability to grow in sulfite liquor and on isolated sodium lignosulfonate (2). The sulfite liquor was freed of sulfur dioxide, mineral salts were added and the media adjusted to pH 5.0. A *beta*-naphthylamine precipitation method was used to determine the residual lignosulfonate left in the culture solution. With this set of conditions, these investigators observed that *Endoconidiophora adiposa* could "decompose" up to 10 per cent of the lignosulfonate in 20 days. They observed that when the available sugar (present to the extent of 1–2 per cent in waste sulfite liquor) was depleted, disappearance of lignosulfonate stopped. This study was extended to several other organisms (52), and with certain species of *Fusarium* and *Alternaria* 12 to 18 per cent disappearance of lignosulfonate was noted. In a later paper, chemically defined media containing 3.75 per cent isolated sodium lignosulfonate and 2 per cent dextrose together with a mineral salt mixture, were used. An ultraviolet absorption method was developed to ascertain extent of disappearance of lignosulfonate from the media. In this case, much lower percentages of decomposition were reported, the highest being 6.9 per cent decomposition by *Fusarium culmonum* in 20 days.

It can be seen from the ultraviolet studies of Ledingham and Adams that the

beta-naphthylamine method for determining lignosulfonate is subject to considerable error. Data are presented indicating that in analyses on identical samples of culture filtrate, the *beta*-naphthylamine method indicated 23 per cent decomposition of the lignosulfonate, while the ultraviolet measurements indicated 3 per cent. A phenomenon which needs to be considered in studies on lignosulfonate is the possibility of a reaction between the proteinaceous mycelium and the lignosulfonate. This would be somewhat like the tanning of leather, for which process lignosulfonate derivatives are already in use (11). Day, Gottlieb and Pelczar (21a) have found that *Polyporus versicolor*, grown on a chemically defined liquid medium containing 2 per cent glucose and 0.5 per cent sodium lignosulfonate produces an apparent reduction of the lignosulfonate approaching 80 per cent. Examination of the mycelium revealed that the lignosulfonate had reacted with the mycelium to give a complex which accounted for all the lignosulfonate which had disappeared from solution under the experimental conditions described.

The papers discussed above have dealt with lignin obtained by the standard methods, namely treatment with sulfuric and hydrochloric acids, sodium hydroxide and alkaline sulfite. As mentioned earlier in this paper, a method for extracting so called "'native-lignin" has been proposed by Brauns (10) which involves only the use of inert solvents in the cold. Day, Pelczar and Gottlieb (21) reported the results of an investigation on the degradation of native lignin prepared according to Brauns. An initial survey of many white rot fungi indicated the absence of a consistent and appreciable ability to use native lignin as a sole source of carbon. A technique of adaptation involving culture of the organisms on media containing both glucose and native lignin was used in an attempt to increase the utilization of lignin. The glucose was gradually withdrawn in several serial subcultures until two of the organisms, *Poria subacida* and *Polyporus abietinus*, were able to utilize native lignin as the sole energy source. It was later found (70) that several strains of *Polyporus versicolor* made rapid growth on media containing native lignin as the sole carbon source without the necessity of previous adaptation. In a more detailed study of this phenomenon (38) it was reported that adaptation to native lignin caused *Poria subacida* and *Polyporus abietinus* to become simultaneously adapted to nearly all of 16 lignin materials isolated by various laboratory and commercial techniques.

Studies in progress (50) in the authors' laboratories indicated that pure cultures of bacteria (not yet identified) make significant growth in an inorganic salts medium containing either native lignin, pure sodium lignosulfonate, or conidendrin (13) as the sole carbon source. In the case of conidendrin, quantitative analysis from shake cultures has revealed a disappearance of more than 90 per cent of the conidendrin in 10 days.

CORRELATION OF OXIDASE REACTION WITH LIGNIN UTILIZATION

Some of the higher fungi are capable of oxidizing phenolic substances to dark colored products. Bavendamm (3) recognized the difference between the brown rots and the white rots in this respect. By cultivating these organisms in an agar

medium containing either tannic or gallic acid, the white rot fungi produced a large darkened zone around the mycelial mat. No zone of darkening was associated with the growth of the brown rots. This darkening phenomenon on tannic acid was also interpreted as an indication that the fungus could utilize lignin, and that this correlation existed because of a structural similarity between lignin and tannic acid. However, it is now recognized that the only structural similarity between these compounds is the presence of free phenolic groups.

Bavendamm (3) advocated this method (cultivation of fungi on gallic or tannic acid media) for determination of their ability to utilize lignin. Subsequent observations employing this technique have shown that his generalization is, for the most part, correct when applied to the higher fungi.

The most extensive examination of wood rotting fungi with respect to their reaction on gallic or tannic acid media was performed by Davidson, Campbell and Blaisdell (22). These studies were performed with a view toward supplementing methods for the identification of wood-decaying fungi, attempting to correlate the type of decay with the oxidase reaction. Some 210 fungi were studied. Of the fungi known to be associated with white rots, 96 per cent (156) gave a positive reaction while 80 per cent (39) of those associated with brown rots gave a negative reaction. Those giving a positive reaction showed considerable variation in intensity of reaction and time of development. Advantage was taken of variations in growth behavior on the tannic and gallic acid media for the establishment of 10 different groups among the 210 fungi examined. The variable nature of this phenomenon with some fungi was observed by Robak (76). Of the fungi examined by him, three species, namely strains of *Lenzites sepiaria*, *Stereum sanguinolentum*, and *Trametes serialis* deviated from the typical reaction by showing no reaction or gradations to positive reactions on the gallic acid medium or a negative reaction on the tannic acid medium. It is concluded that the oxidases secreted by these wood-destroyers are not identical and that the time of secretion and quantity of enzyme produced may vary from strain to strain and within one and the same strain. Ledingham and Adams (52) in their study of lignosulfonate degradation by wood destroying and soil fungi attempted to correlate this characteristic with the oxidase reaction. Only a slight positive correlation was found between the tannic acid reaction for identifying lignin decomposing fungi and their ability to break down lignosulfonate after 60 days growth. Variations were also found in lignosulfonate decomposition by species giving the same tannic acid reaction. There is some question as to whether the quantitative evaluation of lignosulfonate degradation does actually measure metabolized lignin. This situation, which has been encountered in our work with *Polyporus versicolor* on sodium lignosulfonate is discussed in the preceding section of this paper and the possibility exists that a considerable amount of the lignosulfonate may be removed by adsorption on the mycelium.

The nature of the enzyme responsible for the positive reaction and referred to as a polyphenol oxidase, was studied by Lindeberg (57) from litter-destroying Hymenomycetes. He had previously shown that 44 of 46 species were capable of degrading lignin and cellulose. The behavior of these organisms was studied on

catechol and hydroquinone agar. The results indicated that all species produced an *ortho*-diphenol oxidase and certain species (only a few) produced small amounts of *para*-diphenol oxidase. Fahraeus (24) concluded, from the range of phenolic substrates oxidized by the white rot fungi that the enzyme involved was "laccase". However, no purified enzyme was obtained for definitive studies.

Attempts to improve or modify the gallic acid or tannic acid media have also been reported. Preston and McLennan (74) evaluated 18 dyes belonging to the following groups: nitro, azo, anthraquinone, quinone-imine, phenyl methane and xanthene. The dyes were incorporated in an agar medium (.007 per cent dye concentration) followed by cultivation of the fungi. The white rot fungi were able to decolorize all dyes investigated while the brown rots effected no decolorization. Decolorization of the dyes was not due to a pH change and was not an effect of a reversible oxidation-reduction system. Extensive studies were performed with gentian violet and neutral red. The correlation of white rot activity with decolorization of the dye was good. An attempt to identify the agent responsible for the decolorization of the dyes was carried out by Law (51) who concluded that destruction of the dyes was associated with a specific phenol oxidase possessed by all white rot fungi examined.

Fahraeus (24) surveyed several simple phenolic compounds, high molecular weight phenolic compounds and some dyes when incorporated into agar for the purpose of facilitating isolation of lignin decomposing fungi. Of all compounds tested, tannic acid proved the best, having a low toxicity for fungi while being inhibitory to bacteria. *Alpha*-naphthol was found to be inhibitory for brown rots but not white rots. No fungi tested (with the exception of white rots) were able to develop at a concentration more than 0.0005 M. The non-toxicity of this compound for white rots was attibuted to that fact that they possess an oxidase capable of altering the molecule. Addition of *alpha*-naphthol to an agar medium facilitated the isolation of lignin decomposing fungi.

From the foregoing it may be concluded that there is reasonably good agreement between the lignin decomposing ability of wood rotting organisms and their ability to produce a positive test on tannic acid media. However, it should be borne in mind that a positive tannic acid reaction is not confined to wood rotting fungi.

MISCELLANEOUS STUDIES

This section will deal with miscellaneous studies which, although not directly concerned with the microbiological degradation of lignin, are nevertheless pertinent to the relationships between microorganisms and lignin.

A small amount of work has been done on enzymes from fungi which act on lignin and lignin-like material. Gottlieb and Geller (39) reported an enzyme preparation from mushroom spawn which could catalyze a reaction between native lignin and oxygen, and which seemed to be different from the phenol oxidases heretofore reported. It was later found (37) that the enzyme actually acts on a low molecular weight water soluble compound closely related to and closely associated with native lignin. The enzyme shows considerable specificity for lignin-related model compounds, like guaiacol and eugenol. The same enzyme was detected in filtrates from *Polyporus versicolor* containing native lignin as a

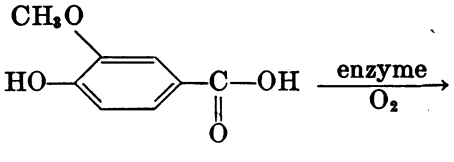
sole carbon source in a chemically defined liquid medium. Parallel experiments in which glucose was the sole carbon source showed no enzyme activity of this kind. This observation indicates a relationship between the enzyme and the utilization of lignin which is more than fortuitous, although the specific role which the enzyme plays is obscure at present.

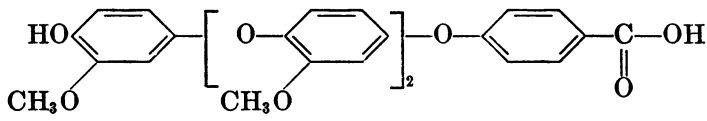
Lindeberg (56) observed an extracellular catecholase produced by various litter-decomposing Hymenomycetes. He reported different pH optima for the catecholase produced by different species. The optima generally coincided with the pH of the natural substrates for the various fungi. In a later paper (58), Lindeberg reported that the mycorrhizal forming fungi *Boletus Grevilei* and *Boletus variegatus* do not form extracellular catecholases. These species are more sensitive to the toxic action of enzymatically oxidized (by the catecholase) gallic acid than are the litter fungus *Marasmius foetidus* and the white rot *Polyporus zonalis*. Both of the latter fungi produce extracellular catecholase. Based on these observations, Lindeberg discusses the possibility of the formation of antibiotic quinones by the action of extracellular catecholases on certain phenols, which products could inhibit the growth of mycorrhizal fungi.

Lindeberg and Korjus (59) reported an increase in the weight of mycelium of *Marasmius foetida* caused by the presence of gallic acid in a glucose medium. The data indicate that the phenomenon is not a buffering effect, nor a vitamin type effect, nor a utilization of gallic acid for a carbon source. *Marasmius foetida* is one of the organisms producing an extracellular catecholase which oxidizes gallic acid to a yellow-brown material.

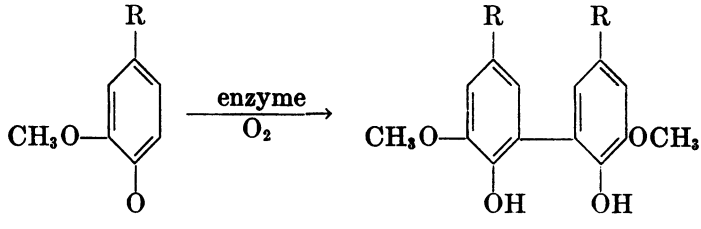
The presence of phenol oxidases in some wood rotting fungi has been studied by Law (51). Preston and McLennon (74) had reported the decolorization of certain dyes by white rots and the inability of brown rots to accomplish this decolorization under similar circumstances. Law found that the decolorization is due to a specific phenol oxidase. The properties of the phenol oxidase indicate that it is similar to laccase (47), though having somewhat different substrate specificity. Brown rots contained an oxidase, which according to its reactions, resembles the tyrosinase of mushroom and tomato.

In an interesting series of papers Freudenberg and Richtzenhain (31, 32), and later Richtzenhain (77, 78, 79) reported researches on the enzyme-catalyzed polymerization of lignin-related compounds. The enzyme was obtained by extracting the sporophores of *Agaricus campestris* with water and subsequent fractionation of the water extract with alcohol to give a dry powder. When incubated in slightly alkaline or neutral media, in the presence of an abundance of oxygen, on phenolic substrates like vanillic and syringic acid and ferulic acid, the enzyme would catalyze the absorption of oxygen. A condensation resulted involving decarboxylation and the formation of ether linkages. The available data led to the following speculated condensation (*e.g.*, for vanillic acid):





It was later reported that other types of dehydrogenation could be catalyzed by this enzyme, such as the dehydrogenation of pyrogallol dimethyl ether to the related alcohol, aldehyde and acid and also dimeric products. Dehydrogenations in the guaiacol series led to the following products:



R = CH_3, C_2H_5, or C_3H_7

Recently Freudenberg (29) reported that the reaction between coniferyl alcohol and this enzyme resulted in a product that was indistinguishable from native lignin as prepared by the method of Brauns. What the possible role of such a dehydrogenating enzyme could be in the physiology of fungi is difficult to ascertain at present, but the reactions reported above might give some insight into what happens in the cambial tissue of the plant, where lignin is elaborated. It should be borne in mind, however, that most of the reactions were carried out under laboratory conditions involving 60 hours exposure to oxygen, bubbled through a solution at an alkaline pH (near 8.0). These can hardly be called physiological conditions.

In connection with oxidase activity and the genesis of lignin in plants, Manskaya (61) found that peroxidase and polyphenol oxidase activity, using coniferin as a substrate, is high in the precambial and cambial tissues. A partial polymerization of coniferyl alcohol by extracted Willstätter peroxidase is reported. *In vivo* analyses indicated that in the spring the coniferin level was high and the oxidase level was low. In summer the situation was reversed.

A possible relationship between lignin and fungi is the existence in fungus mycelium of a material which resembles plant lignin in its resistance to solubilization by mineral acids. Kucher (48a) reported 25 per cent of the weight of mycelium of *Polyporus fomentarius* as lignin. Thom and Phillips (84a) analyzed a series of wood rotting fungi for their apparent lignin content by the fuming hydrochloric acid method and got values of from 3.40 per cent for *Polyporus sulfureus* to 54.08 per cent for *Trametes pini*. Phillips (71) studied the lignin from *Trametes pini* and found it to contain practically no methoxyl, and that it could be methylated to a maximum of 27 per cent methoxyl. Pinck and Allison (73) reported a similar range of apparent lignin contents in a series of soil fungi grown in a chemically defined medium with sugar as the sole carbon source. Schubert and Nord (83) treated the mycelium of *Trametes pini* (analyzing 23.7 per cent

sulfuric acid lignin) with alcohol by the method of Brauns (10) to prepare native lignin and obtained 1 per cent of the weight of the mycelium as a substance resembling native lignin. The material had 12.76 per cent methoxyl, resembled native lignin from soft woods in its solubility, but did not give the characteristic color reaction with phloroglucinol.

Schubert and Nord (81) reported that the action of brown rots like *Lentinus lepideus*, *Lenzites sepiaria* and *Poria vaillantii* on white Scot pinewood results in a two-fold increase in the amount of native lignin which can be isolated by the method of Brauns. Various analytical data, including ultraviolet and infrared analyses (82) are presented to indicate the identity of the native lignin from the decayed wood with that isolated from sound wood. By increasing the time of decay of wood, amounts of native lignin up to 22.7 per cent of the total lignin could be extracted (63). Since a maximum of 3 per cent of the weight of total lignin in sound wood can be extracted as native lignin, a serious objection has been raised as to whether it is representative of the other 97 per cent of the lignin. This work would seem to indicate that the other 97 per cent is essentially the same, but is prevented from being extracted with alcohol by its close association with cellulose. When the cellulose is decomposed by the brown rot organism, the native lignin can then be extracted. Schubert and Nord reason that the identity of the native lignin isolated from decayed wood with that from sound wood proves the absence of a chemical bond between lignin and cellulose. The reasoning is that if such a bond were present, one would expect to find differences in the two lignins, presumably caused by the presence of a fragment from the cellulose at the point of linkage. It would seem however, that if a chemical bond did exist between cellulose and lignin, an enzymatic hydrolysis of such a bond by the fungus could give rise to native lignin identical with that from sound wood. Therefore, the identity of the two lignin preparations may not have a direct bearing on the question of the presence or absence of a chemical bond between lignin and cellulose.

Nord and Vitucci (62) report that methyl *p*-methoxy cinnamate is formed by the action of *Lentinus lepideus* on either wood, glucose or xylose. Acetaldehyde (64) seems to be the key intermediate in this reaction. This fact is used by the authors to substantiate a direct role for cellulose in the formation of lignin in plants, on the basis of the similarity of the cinnamate ester to the aromatic moieties known to be present in lignin. Accepting the fact that acetaldehyde is an intermediate in the formation of aromatic structures, it is difficult to see why the metabolic degradation of cellulose would have to be the only source of acetaldehyde available for the synthesis of aromatic structures like lignin. It can be just as readily assumed that the acetaldehyde available from other sugars (before their condensation to cellulose), or available from an even earlier product of photosynthesis, is used to synthesize lignin.

CONCLUDING REMARKS

From the foregoing account of many and variegated studies on lignin degradation, one hesitates to make unequivocal summarizations. It is obvious that few

dogmatic generalizations can be made. Nevertheless, a few remarks are necessary to leave the reader with an impression of something less than total confusion.

Many investigations have been cited to prove that the higher fungi do degrade lignin in nature, albeit slowly. Nothing is known about the intermediary metabolism of this degradation. Even though the analytical techniques employed are subject to serious errors in interpretation, nevertheless many of the lignin degradation data reported fall far outside the limits of these errors. It seems well established, too, that the tannic acid reaction and similar cultural reactions can be used as a general indication of white rot activity for wood rotting fungi. It should be recognized, however, that there are notable exceptions and that the reaction is not infallible. Thus far, no specific or identified bacterial species has been reliably associated with the natural degradation of lignin. Similarly, no enzyme preparation has been reported which catalyzes the cleavage of a carbon-carbon or a carbon-oxygen bond in either isolated or *in situ* lignin to bring about a significant decrease in its molecular size.

It is to be hoped that with the availability of a method for isolating lignin in an unchanged state, together with the current emphasis and improvements in microbiological and enzymatic techniques, the problem of the elucidation of the microbiological degradation of lignin will approach a solution in the future.

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