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Investigations on Lignin and Lignification. II. The Characterization of Enzymatically Liberated Lignin*

BY WALTER J. SCHUBERT AND F. F. NORD

The solution to the problem of isolating and identifying lignin in its natural state requires a method of extraction that avoids drastic conditions, and utilizes inert solvents. Brauns' extraction of native lignin from woods of various species[1] with alcohol at room temperature seems to have met these requirements; however, only 3% of the total lignin was obtained. This observation increased the numerous existing speculations regarding the nature of the residual lignin and its linkage with the cellulose in the wood.[2]

In a previous paper,[3] we have reported the isolation of chemically unaltered lignin from softwood samples which were decayed by members of the "brown rot" (*i. e.*, cellulose-degrading) fungi.[4] After a limited period of decay, there was approximately a two-fold increase in the yield of alcohol-extractable lignin from the decayed wood, relative to that obtained from sound wood. Comparison studies on the Native Lignin of white Scots pine and on the lignin extracted from this wood after the fungal decay indicated that the two preparations were identical in composition.[3]

This conclusion, however, could not be made unequivocally, since the alcoholic extract of the decayed wood contained the original native lignin, together with that liberated by the enzymatic action. A clear-cut separation of these two fractions will now be reported, and their identity confirmed.

The ambiguity was clarified by first extracting the wood with alcohol until all the native lignin was removed. The native-lignin-free wood was then decayed by the fungi and, after decay, the residue was re-extracted with alcohol. The extract then contained only that lignin which was liberated from its association with the cellulose by the enzymatic activity, and was free of native lignin. The results of these investigations constitute the subject of the present report.

Experimental

The species of wood investigated in these experiments was white Scots pine, and the "brown rot" organisms employed to effect the decay were the *Basidiomycetes: Poria vaillantii* and *Lenzites sepiaria*.

Isolation of Native Lignin.—The wood samples were cleaned of their bark and ground to 60 mesh in a mill. The sawdust was freed of its "extractives" with water and ether. Then, the native lignin was isolated by extracting with 95% ethyl alcohol at room temperature in a percolator-type extractor which will be described elsewhere.[5]

This extraction was continued until the alcoholic solution failed to respond to the phloroglucinol test for lignin. The wood was then considered to be free of native lignin. The lignin so obtained was purified as described previously.[3]

Sterilization and Inoculation of Wood Samples.—Ten-gram samples of the native-lignin-free sawdust were weighed into each of several 500-ml. Fernbach flasks, and to each was added a 25-ml. portion of the nutrient medium of composition previously described.[3] The flasks were plugged with cotton, and sterilized by Tyndallization. After cooling, each flask was inoculated with a 5-ml. spore-mycelial suspension of one of the organisms indicated above.

The inoculated flasks were incubated in the dark at 27–

* The data presented have been abridged from a part of the dissertation submitted by W. J. S. to the Graduate School of Fordham University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1950. Discussed at the Second Lignin Round Table held at the Institute of Paper Chemistry, Appleton, Wisconsin, August, 1950.

(1) (a) F. E. Brauns, THIS JOURNAL, **61**, 2120 (1939); (b) F. E. Brauns, *J. Org. Chem.*, **10**, 211 (1945); (c) M. A. Buchanan, F. E. Brauns and R. L. Leaf, Jr., THIS JOURNAL, **71**, 1297 (1949).

(2) W. J. Wald, P. F. Ritchie and C. B. Purves, *ibid.*, **69**, 1371 (1947); K. Hess and K. E. Heumann, *Ber.*, **75**, 1802 (1942).

(3) W. J. Schubert and F. F. Nord, THIS JOURNAL., **72**, 977 (1950).

(4) F. F. Nord and J. C. Vitucci, *Adv. in Enzymol.*, **8**, 253 (1948); G. B. Craemer, *Pulp and Paper Magazine* (Canada), **51**, 86 (1950).

(5) F. F. Nord and W. J. Schubert, *Holzforsch.*, **5**, No. 1 (1950).

28°. The progress of the decay was followed by periodic analyses of the wood residues.

Analytical Methods.—The wood residues were analyzed in duplicate for their cellulose and lignin contents, each of which was corrected for moisture. Lignin and moisture were both determined by standard methods,[6] while cellulose was determined as before.[7] As the decay proceeded, it became necessary to remove adhering fungal mycelia, and this was accomplished as hitherto.[3]

Isolation of the Enzymatically Liberated Lignin.—The lignin liberated from its association in the wood by the enzymatic activity of the molds was isolated from the decayed wood in a manner identical with that indicated above under "Isolation of Native Lignin." The acetates and phenylhydrazones of the lignins were prepared as previously,[3] and the absorption spectra of the lignins and their derivatives were determined with a Beckman quartz spectrophotometer and a Baird Double-beam infrared recording spectrophotometer.

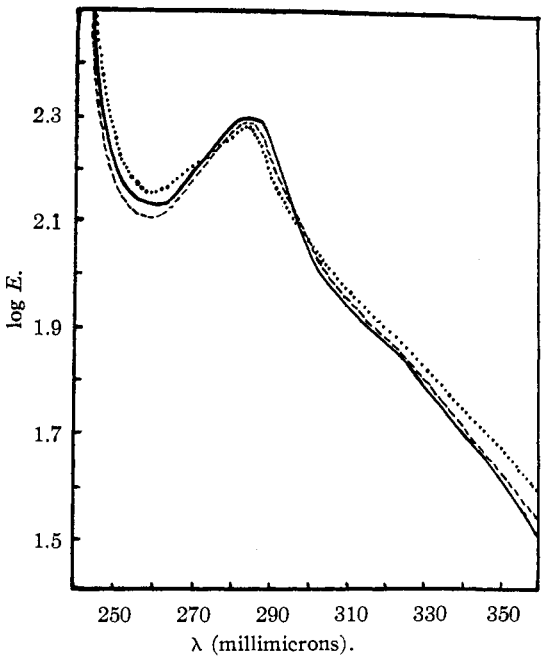

Fig. 1.—Ultraviolet spectra of white scots pine lignins: ——, native lignin; - - - - - - - lignin obtained with *P. vaillantii;* . . . . . lignin obtained with *L. sepiaria.*

Results and Discussion

As reported,[3] 3.2% of the lignin content of sound white Scots pine wood can be isolated by alcoholic extraction. This native lignin was completely removed from a sample of this species of wood, and was characterized in our previous paper.[3] The native-lignin-free wood was then subjected to decay by the cellulolytic mold *Poria vaillantii* for a period of fifteen months, and by *Lenzites sepiaria* for thirteen months. The overall effects of the decay on the chemical composition of the wood are recorded in Table I.

These decayed wood samples were again thoroughly extracted with ethyl alcohol at room temperature. As a result of the "lignin enrichment" of the wood, the yield of lignin isolated from the *Poria vaillantii*-decayed white Scots pine wood now amounted to 22.7% of the residual lignin, while that from the *Lenzites sepiaria*-decayed wood corresponded to 18.3%.

(6) "Methods for the Chemical Analysis of Pulps and Pulpwoods," Forest Products Laboratory, Madison, Wis., 1939.

(7) A. I. Virtanen and O. A. Koistinen, *Svensk Kem. Tid.*, **56**, 391 (1944).

TABLE I

EFFECTS OF *Poria vaillantii* AND *Lenzites sepiaria* ON THE COMPOSITION OF WHITE SCOTS PINE WOOD

Organism	Period of decay, months	Cellulose, %	Lignin, %
Poria vaillantii	0	45.5	33.9
	15	15.2	52.5
Lenzites sepiaria	0	45.5	33.9
	13	18.5	50.1

Thus, after a prolonged period of decay by cellulose-degrading organisms, there is a substantial increase in the yield of alcohol-extractable lignin from the decayed sample when compared with that from sound wood.

It was of importance to determine whether this enzymatically liberated lignin was chemically identical with, or different from, the Native Lignin isolatable from the sound wood.

Therefore, a comparison was made of the native lignin of white Scots pine wood with that liberated from this wood by the organism, *Poria vaillantii.* The results showed that the native lignin of white Scots pine, and the lignin liberated by the mold were both soluble in methyl and ethyl alcohols, in dioxane, pyridine, glacial acetic acid and 4% sodium hydroxide solution, and were both insoluble in water, ether, benzene and petroleum ether. Both lignins reduced Fehling solution, and both gave a red-violet color with the phloroglucinol reagent, and yellow colors with the aniline, *p*-phenylenediamine and diphenylamine reagents.

Moreover, the similarities extended to the analytical data of the lignins and their derivatives, recorded in Table II, and to their ultraviolet spectra, shown in Fig. 1, and their infrared spectra, shown in Fig. 2.

TABLE II

COMPARISON OF THE NATIVE AND ENZYMATICALLY LIBERATED LIGNINS OF WHITE SCOTS PINE WOOD

	Native lignin	Enzymatically liberated lignin
C, %	64.0	64.2
H, %	6.3	6.0
OCH_3, %	14.5	14.4
OCH_3 of acetate, %	10.1	10.2
OCH_3 of phenylhydrazone, %	13.3	13.4

From these data, and the identity of the ultraviolet and infrared curves of the two lignins (Figs. 1 and 2), it must be concluded that the native lignin of white Scots pine wood, and the lignin liberated from this wood exclusively by the enzymatic degradation of the wood-cellulose are identical.

In order to investigate the possibility that the fungally liberated lignin might have been affected in some way by the enzyme system of the organism employed, namely, *Poria vaillantii,* a parallel study was also carried out on the lignin liberated from white Scots pine wood by the mold, *Lenzites sepiaria.* This lignin was found to have a methoxyl content of 14.5%, and an identical absorption spectrum in the ultraviolet region (see Fig. 1).

From these data, and the agreement of the ultraviolet spectra of the lignin samples, it appears that the lignin obtained from the *Lenzites sepiaria*-decayed wood is identical with the *Poria vail-*

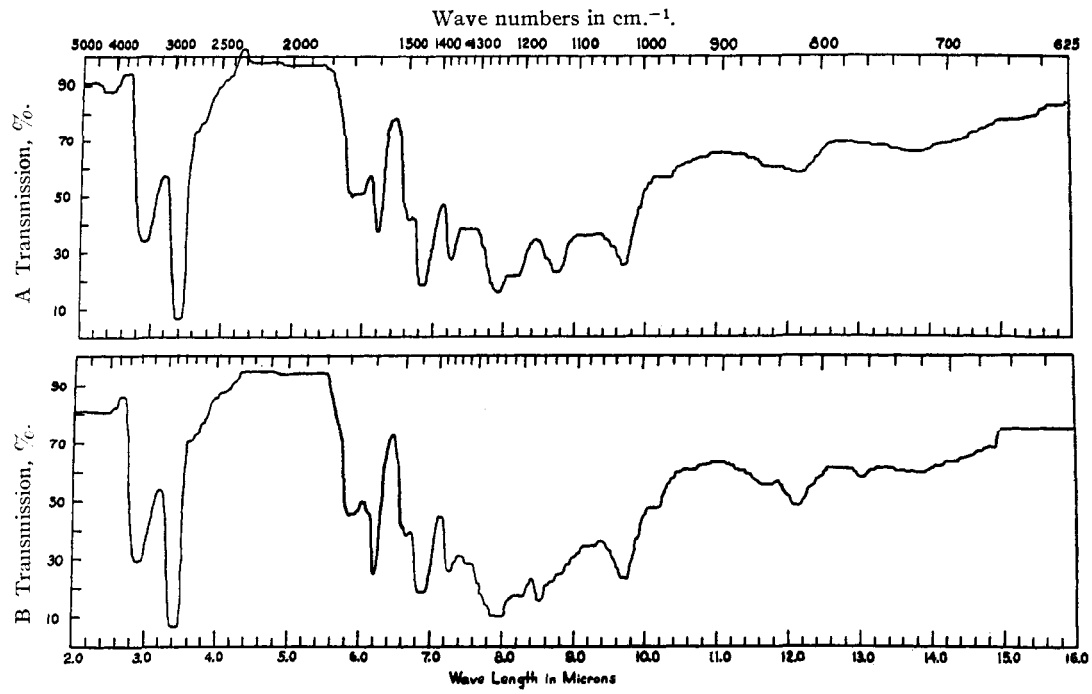

Fig. 2.—Infrared absorption spectra of lignins: A, lignin from sound white Scots pine; B, lignin from decayed white Scots pine.

lantii-liberated lignin, and moreover that both are identical with the native lignin of this wood. It therefore appears that the above properties are characteristic for the total isolatable lignin content of this species of wood.

The purpose of this phase of our research has been the isolation, in good yield, of a lignin unaltered by any chemical treatment through the selective cellulose-degrading activity of the "brown rot" molds on wood. This product was obtained by extraction of the decayed wood with alcohol at room temperature—a mild procedure. However, since there exists in wood a small amount of native lignin which can be obtained from sound wood by this procedure, native lignin was to be expected in an alcoholic extract of decayed wood, together with any lignin freed by enzymatic action. By first removing the native lignin, and then subjecting the wood to decay, we have now been able to obtain an extract which contains only that lignin liberated by the enzymatic activity of the rot. Since this product, isolated in considerable yield, appears to be identical with the native lignin of the wood, it can be concluded that all the lignin of our wood sample is a uniform chemical entity, the greater part of which is associated with the cellulose of the wood, and not extractable from sound wood by inert solvents.

Furthermore, the identity of the native and the enzymatically liberated lignins of wood makes improbable the presence of a chemical linkage between the lignin and the cellulose, for, if such were the case, one might expect some difference between the native lignin and the "extra-native" lignins. However, no such difference was observed. Accordingly, we seem to have substantiated the existence of a simple physical union between these two wood components, thereby helping to understand the resistance of the total lignin content of the wood to dissolve in organic solvents.

Finally, these findings suggest that wood represents a state of the extremely slow carbohydrate → lignin transition, which happens to prevail at the time of investigation. Moreover, the results of this and of the previous paper[3] refer to recent considerations of Freudenberg[8] regarding the formation of lignin and of linkages between this lignin and polysaccharides to the realm of speculation.

Acknowledgments.—The wood samples used in these investigations were obtained through the courtesy of Dr. L. C. Swain, of the Department of Forestry of the University, Durham, N. H., and the mold cultures from Dr. Wm. J. Robbins of the New York Botanical Garden. The infrar-red curves were obtained through the courtesy of Drs. C. C. Clark and James D. Hanly, Department of Physiology, Cornell University Medical School. The study was carried out under the auspices of the Office of Naval Research.

Summary

1. The native lignin of white Scots pine wood was isolated and identified.

2. After fifteen months of decay of this wood by *Poria vaillantii*, 22.7% of the residual lignin

(8) K. Freudenberg, *Sitzungsber. Heidelberger Akad. Wissensch.*, No. 5, 151 (1949).

was isolated by alcoholic extraction, while after thirteen months decay by *Lenzites sepiaria*, 18.3% was obtained.

3. The lignin liberated from its association in the wood by the enzymatic activity of the micro-organisms was found to be identical with the Native Lignin of the wood.

4. The nature of the linkage between lignin and cellulose in softwood is discussed.

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Experiments on the Fractionation of Isolated Wood Lignins

BY CONRAD SCHUERCH, JR.[1]

The separation of the bulk of lignin from the carbohydrate portion of wood material is invariably accomplished by the use of chemical reagents which alter the structure of the original material. The soluble lignin derivatives so obtained have usually been separated by physical methods: fractional precipitation from solution,[2] partitioning between neutral solvents,[3] or diffusion experiments.[4] The fractions isolated by a single method have usually been fairly similar in analysis, and the assumption has therefore been general that "protolignin" is a polymer system based on a single repeating structure. The following results indicate that at present such a conclusion is based on incomplete evidence.

When maple hydrol lignin[5] was subjected to a systematic fractionation by partial solution and precipitation, a series of fractions was obtained differing substantially in molecular weight but little in alkoxyl content. When, however, individual fractions were then subjected to a chemical separation, counter-current extraction against alkali and alkaline buffers,[6,7] the substrates were found to consist of mixtures of materials with widely different partition coefficients and alkoxyl contents.[8] In this case the original similarity in analysis of different molecular weight fractions obviously had little structural significance. Similar separations were also achieved using ethanol spruce and ethanol maple lignins.

The initial experiments, outlined in Table I, are in part a confirmation of the work of Brewer, Cooke and Hibbert[5] who reported that hydrol lignin was prepared in good yield, was aromatic in nature, contained both ether-soluble and ether-insoluble constituents, and was stable to ordinary laboratory manipulations. The ether-soluble portion, as these authors report, consisted largely of distillable monomers, together with some non-distillable resin, and could be separated into alkali-soluble and insoluble material.

TABLE I

RECOVERY OF HYDROL LIGNIN FROM MAPLE WOOD[a]

		G.	%[b]
A.	Ether-soluble		
1.	Alkali soluble, distillable	22.7	21.8
2.	Alkali soluble still residue	9.05	8.68
3.	Neutrals	6.35	6.1
B.	Ether-insoluble	34.0	32.6
C.	Recovered from aqueous residues	3.65	3.5
D.	Lignin in wood residue	...	20.4
	Total		93.1

[a] Weight 540 g. containing 19.3% of Klason lignin. [b] Of original Klason lignin. These values are undoubtedly high since no corrections were applied, and the lignin isolated contained the elements of ethanol derived from the hydrogenation medium.

Since dioxane was found to be a better solvent than ethanol for hydrol lignin, a second series of wood hydrogenations was carried out in which the usual solvent, 50% ethanol, was largely replaced by 50% dioxane (Method II, Experimental Section). Although the product obtained appeared identical with hydrol lignin in alkoxyl content and fractionation characteristics, the yields were erratic and poor. Ethanolysis of the protolignin appeared, therefore, to be involved in the hydrol lignin preparation, and in fact ethoxyl groups were later shown by difference[9] to be present in the product.

The ether-insoluble fraction of hydrol lignin, a light-colored powder not previously examined in detail, was found in the present research to be in part diffusible through cellophane, and to contain components of differing solubility. A systematic fractional precipitation from solvents by conventional methods, outlined in Fig. 1, separated the material into six fractions (I to VI) which were obtained as powders, and a seventh (VII), the most soluble, as a resin. The alkoxyl values

(1) Harold Hibbert Memorial Fellow 1948–1949. Present address: New York State College of Forestry at Syracuse University, Syracuse, New York.

(2) Patterson, West, Lovell, Hawkins and Hibbert, THIS JOURNAL, **63**, 2065 (1941).

(3) Lovell and Hibbert, *ibid.*, **63**, 2070 (1941).

(4) Olleman, Pennington and Ritter, *J. Colloid Science*, **3**, 185 (1948).

(5) Brewer, Cooke and Hibbert, THIS JOURNAL, **70**, 57 (1948).

(6) Craig, Golumbic, Mighton and Titus, *J. Biol. Chem.*, **161**, 321 (1945).

(7) Golumbic, THIS JOURNAL, **71**, 2627 (1949).

(8) Previous workers who have isolated hardwood lignin derivatives of varying methoxyl content include Holmberg (*Arkiv. Kemi, Mineral, Geol.*, **24A**, No. 29 (1947); *C. A.*, **43**, 2767g (1949)), Lieff, Wright and Hibbert, THIS JOURNAL, **61**, 1477 (1939)), and Lovell and Hibbert ref. 3.

(9) Cooke and Hibbert, *Ind. Eng. Chem., Anal. Ed.*, **15**, 24 (1943).