DL-1(=3)-O-Mesyl-N-acetyl-*myo*-inosamine-2 (IX, R' = CH_3).—Two hundred mg. of the mesyl ester of isomer A was treated with 40 ml. of absolute ethanol saturated with ammonia at 4°. After 24 hours in the refrigerator the solvent was evacuated and the acetamide sublimed in vacuum (0.05–0.1 mm., bath temperature 90°). The clear sirup was crystallized from 4 ml. of methanol with a few drops of ether; yield 110 mg. (84%), m.p. 208° dec. Three further crystallizations from ethanol with a trace of water failed to alter the melting point.

Anal. Calcd. for $C_9H_{17}O_8NS$ (229.31): C, 36.11; H, 5.73; S, 10.71. Found: C, 36.20; H, 5.98; S, 10.76.

Periodate Analyses.—The crystalline deacetylated mesyl ester of isomer A and the corresponding sirupy ester of isomer B were analyzed by the method of Fleury and Lange.[14] In a typical run 0.5 mmole of the sample was dissolved in *ca.* 2 ml. of water and 22.5 ml. of 0.321 *M* sodium metaperiodate added at 4°. The solution was diluted to exactly 25 ml. with water, mixed, and placed in the refrigerator (4°). Initial molarity of sample, 0.02; of periodate, 0.28. At various time intervals 2.0-ml. aliquots were removed and discharged into a solution of 1.5 g. of $NaHCO_3$ in 25 ml. of water. Excess periodate was titrated by the arsenite method.

For formate analyses, similar aliquots were removed and mixed with several drops of ethylene glycol in 10-ml. erlenmeyer flasks. After one hour the solution was titrated to a methyl red end-point with 0.01 *N* NaOH. A blank was simultaneously prepared and titrated.

(14) P. Fleury and J. Lange, *J. pharm. chim.*, **17**, 196 (1933).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY, THE UNIVERSITY OF ROCHESTER]

The Biosynthesis of Lignin: Evidence for the Participation of Celluloses as Sites for Oxidative Polymerization of Eugenol

BY S. M. SIEGEL

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A study of the oxidation of eugenol by peroxidase–hydrogen peroxide has been extended in relation to previous work demonstrating formation of lignins from *p*-hydroxyphenylpropanes *via* a peroxidative pathway. Model systems supplied only with eugenol, enzyme and peroxide form water-insoluble oxidation products, which, however, are fully soluble in chloroform, and fail to yield lignin color tests. In the presence either of filter paper or a methylcellulose, however, chloroform-insoluble products are formed either in paper, if present, or as a coprecipitate with methylcellulose. Included in such products are ethanol-insoluble fractions giving positive color tests for lignin, in quantities of 12–25 mg./g. of paper. Relative to the amount of cellulose supplied, a methylcellulose solution supplies more polymerization sites than does filter paper. Experimental conditions and prospects for this line of investigation are considered.

Starting with the generally accepted concept that lignins contain, as fundamental units, one or more types of phenylpropanes,[1] the author has been able to establish a pathway for conversion of eugenol and other *p*-hydroxyphenylpropanes to lignins *via* the peroxidase–hydrogen peroxide system. Original experimentation showed that slices of peroxidase-containing tissues incubated with eugenol yield products capable of isolation by the usual methods of lignin work.[2] Among the identifying characteristics and properties of synthetic products studied were the following: solubility in a wide range of solvents, evaluated on a comparative basis together with natural lignins from oak, spruce, bean and other sources[3]; analysis which gave C 63%, H 7%, and OCH_3 15%, in good agreement with known lignins[4]; and ultraviolet absorption, which showed spectra of eugenol–synthetic lignin to be similar to a variety of lignins and essentially identical with those for spruce lignin.[4] The usual lignin color tests were also given by synthetic products.

The lignin-forming system described here may be contrasted with that described by Freudenberg, *et al.*,[5] and Freudenberg and Heel,[6] who started with coniferyl or hydroxycinnamyl alcohols rather than with eugenol. The present system possesses a higher rate of polymerization and is also completely dependent on the enzyme peroxidase. Freudenberg's system also involves strictly soluble materials. When eugenol is used as a substrate, lignin polymers are not formed by a cell- or particle-free system or in the presence of crystalline enzyme and peroxide alone.

The mechanism of enzymatic peroxidation, as suggested by such work as that of Westerfield and Lowe,[7] and Herzog and Meier,[8] and through the author's own observations probably entails formation of highly reactive intermediary semiquinones which may condense to yield a variety of reaction products, but which are incapable of forming the lignin polymer under the influence of enzyme alone. Resolution of the problem thus presented came with the observation that washed cell-wall material (with a substantial quantity of peroxidase tightly bound, however) could effect polymerization at nearly the same rate as that measured for entire tissue slices consisting largely of intact cells. This result was interpreted as meaning either that a specific polymerase resided in the wall, or that cellulose or other polysaccharides in the wall substance exerted an accessory catalytic effect on eugenol such that only those molecules in intimate association with such macromolecules were polymerized to lignins.

It is the object of this communication to describe the establishment of successful model systems, in which eugenol is peroxidatively converted into

(1) F. E. Brauns, "Chemistry of Lignin," Academic Press, Inc., New York, N. Y., 1952.

(2) S. M. Siegel, *Physiol. Plant*, **6**, 134 (1953).

(3) S. M. Siegel, *ibid.*, **7**, 41 (1954).

(4) S. M. Siegel, *ibid.*, **8**, 20 (1955).

(5) K. Freudenberg, H. Reznik, H. Boesenberg and D. Ranenack, *Chem. Ber.*, **85**, 641 (1952).

(6) K. Freudenberg and W. Heel, *ibid.*, **86**, 1955 (1953).

(7) W. W. Westerfield and C. J. Lowe, *J. Biol. Chem.*, **145**, 463 (1942).

(8) O. Herzog and A. Meier, *Z. physiol. Chem.*, **73**, 258 (1911).

lignin or lignin-like products in the presence of filter paper or methylcellulose solutions.

Experimental

Filter Paper as a Polymerization Site.—Whatman No. 1 papers were infiltrated with crystalline peroxidase[9] to give a final enzyme concentration of 1.9×10^{-5} mole/1000 g. of paper. The papers were then dried *in vacuo* at 23–25°, stored at 0° until use. Filter papers of measured dry weight (0.5–1.0 g.) were placed in reaction mixtures of at least 25-ml. volume with 0.001 *M* Sörensen phosphate buffer, *p*H 5.1, 8×10^{-3} *M* eugenol, and 1.6×10^{-2} *M* H_2O_2. Controls consisted of reaction mixtures lacking peroxide, peroxidase, eugenol or all three. In addition, mixtures were set up in which the peroxidase had been dissolved in the aqueous phase, rather than being deposited in the paper; in such cases, a filter paper which had been moistened with buffer only and dried replaced the peroxidase-impregnated material. A parallel series lacking only filter paper was set up to serve as an additional control group. Reactions were allowed to proceed for 25 hr. at 23 ± 1°, the papers dried and weighed and insoluble precipitates separated by centrifugation. Papers, or sediments formed in paperless controls, were then tested for the presence of lignin. Similar experiments were also carried out substituting ordinary commercial grades of cellophanes for filter paper. Peroxidase-infiltrated cellophane possessed about one-half the peroxidase concentration of treated paper.

Hydroxypropyl Methylcellulose as a Polymerization Site.—Commercial "Methocel HG" powder[10] was prepared in 0.013% solution (calculated viscosity 2–2.5 centipoise) by wetting the powder with hot water, completing solution with chilled buffer, and clarifying by storage at 0° for 15–25 hr. To 30 ml. of this preparation was added 3×10^{-4} mmole of peroxidase, 1.0 mmole of eugenol and 2.0 mmoles of H_2O_2. Controls consisted of reaction mixtures lacking one or all of the stated constituents save for the buffer. The reaction proceeded for 25 hr. at 23 ± 1°, and all insoluble material was collected by centrifugation at 10,000 × *g* for 20 min. Sediments were then dried, weighed and tested for lignin. It should be noted, both for its theoretical and technical significance, that eugenol, and other phenols as well, are capable of flocculating methylcellulose, even from dilute solutions. The flocculation, essentially complete in the present case, necessitated that a weight correction of 3.9 mg. corresponding to the weight of precipitated methylcellulose be applied in determining amounts of insoluble products formed.

Tests for Lignin.—Elementary and methoxyl group analyses have not, as yet, been carried out. However, the present report is based on a sufficient background of experimental data to justify provisional identification of lignins by means of solubility tests and color reactions. Solubility tests were based on initial extraction of papers or precipitates with boiling chloroform, with consequent removal of unreacted eugenol, and simpler oxidation products. Following chloroform treatment, residues were extracted with 95% ethanol, and finally, *p*-dioxane. After each extraction, residues were dried, weighed and lignin color reagents applied to samples both of soluble and residual fractions. The phloroglucinol reagent was applied as a saturated solution of the phenol in 20% HCl. The chlorine–sodium sulfite test, as has been previously described,[3] involved mild chlorination of reaction products in commercial sodium hypochlorite acidified with HCl, followed by washing of the products, and transfer to saturated, aqueous sodium sulfite for color development. Both reagents yield red products with lignins within a few minutes at room temperature. Controls, consisting of various wood and paper samples were run in parallel in the color reagents to ensure adequate chlorination and other treatment. Isolations of products were also made after chloroform extraction by digesting paper samples with 72% sulfuric acid at 0° for 12–25 hr., diluting the acid to 3%, and autoclaving the digest until residues were carbohydrate-free. Small quantities of residue were then recovered by centrifugation, and dried sediments tested for lignin.

Current knowledge suggests that the ethanol-soluble fraction found in these studies may correspond to a native, unaltered,[11] or protolignin[1]; however, pending further chemical examination, only the chloroform- and ethanol-insoluble residues will be reported as a presumptive lignin.

Results and Discussion

In general, yields of chloroform- and ethanol-insoluble products in filter paper ranged from 12–25 mg./g. of dry paper. In the absence either of eugenol or peroxidase, no lignin-like products were detected, under the moderately acidic experimental conditions. Further, peroxidase could not be replaced by the enzyme tyrosinase, Cu(II), Fe(II) or Fe(III) compounds, nor did elevation of the *p*H in graded steps from 5 to 12 permit formation of lignin, although oxidation of the eugenol did occur in alkaline media, even without added catalysts.

Cellophane, when substituted for filter paper, deposited traces of materials giving the lignin color reactions after preliminary solvent treatment, but yields were only one-tenth to one-twentieth of those obtained with paper.

It was noted that with fair regularity systems lacking only peroxide would also form traces of lignin-like products, presumably utilizing peroxide formed during autoxidation of eugenol itself. Peroxide formation under such circumstances, which is not unknown,[12] is also under investigation in this Laboratory.

Representative experimental results obtained with a filter paper reaction mixture are summarized in Table I. Omitted are those parts of the experiment in which one or more reactants were absent, yielding only traces of products, or none at all. The lignin fraction both in dioxane-soluble and insoluble form totalled 12 mg./g. of dry paper, or 1.2%. The papers were a faint yellow-brown in color, and showed resistance to wetting by the color reagents, as well as water. Droplets of reagent placed on the paper would retain an essentially spheroidal form for as much as five minutes with little spread of the liquid, in contrast with the virtually instantaneous spread of reagent applied to untreated filter paper, or papers from incomplete reaction mixtures. Resistance to wetting was markedly reduced by extraction of papers with all

TABLE I

EFFECT OF FILTER PAPER ON THE PEROXIDATION OF EUGENOL[a]

	Control		With filter paper	
Product soluble in	Wt. (mg.)	Lignin test	Wt. (mg.)	Lignin test[b]
Chloroform	73	−	60	−
Ethanol	0		8	±
Dioxane	0		8	+
Residual	0		4	+
Total	73		80	
Total as % initial eugenol supplied	60.0		66.1	

[a] Products deposited in solution (control), or on 1 g. of filter paper; initial eugenol 0.8 mmole, H_2O_2 1.6 mmoles in 100 ml. reaction volume. [b] Color reactions employing both phloroglucinol–HCl and chlorine–sodium sulfite reagents. Color tests are definite (+), weak (±), negative (−).

(9) Prepared from a Worthington horseradish peroxidase of high activity.

(10) Generously provided by the Dow Chemical Company, Midland, Michigan.

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

(12) A. C. Maehly in D. Glick, ed., "Methods of Biochemical Analysis," Vol. I, Interscience Publ. Co., New York, N. Y., 1954.

three solvents, but could not be removed entirely. It should be noted that, of the lignin fraction, approximately $^2/_3$ is dioxane-soluble, an extremely high figure, even for eugenol lignins as formed in tissue slices.

Isolation of Klason (72% sulfuric acid) lignin after final dioxane extraction yielded 1–3 mg./g. of paper of a dark brown material, insoluble in all solvents employed, and giving positive color tests for lignin. The characteristic reversible darkening of lignin-containing tissues in the acid was observed also in papers yielding positive color tests, but not in control samples.

In the extension of this study, methylcellulose was selected as the first of a large series of substances, principally polysaccharides, but also intended to include synthetic polymers, mucoproteins and fibers. It will be the aim of this series to relate efficacy in the formation of lignin and other aromatic polymers to composition, configuration and molecular size. Comparison of the data in Table I with those obtained with methylcellulose (Table II) shows that in proportion to the amount of cellulosic material present, methylcellulose provides a far more efficient framework for polymerization than does paper. It has not been deemed advisable to attempt theoretical interpretation of these differences in results at present, although the particular distinction between paper and methylcellulose may reside in the inaccessibility of all but the most superficial fibers in the filter paper mat. The failure thus far met in replacing celluloses with alumina, or silica gel, does indicate, however, that the phenomena associated with polymer formation involve a more or less specific association between the eugenol, enzyme and a macromolecule. In experiments with methylcellulose, the dioxane-soluble fraction of lignin approximated 40%, in agreement with findings for eugenol synthetic lignin formed in tissue slices or cell wall material. In order to compare previous and current work it may be noted that tissue slices and cell wall fragments gave, for rate of lignin deposition, 3.46 mg./100 mg. dry matter/hr. and 2.68 mg./100 mg./hr., respectively.

TABLE II

EFFECT OF METHYLCELLULOSE ON THE PEROXIDATION OF EUGENOL[a]

Product soluble in	Control Wt. (mg.)	Control Lignin test	With methylcellulose Wt. (mg.)	With methylcellulose Lignin test[b]
Chloroform	88	−	79	−
Ethanol	0		6	±
Dioxane	0		3	+
Residual	0		5	+
Total	88		93	
Total as % initial eugenol supplied	53.6		56.7	

[a] Product deposited in solution (control) or together with 3.9 mg. of methylcellulose; initial eugenol 1.0 mmole, H_2O_2, 2.0 mmoles in 30 ml. reaction volume. [b] Color reactions employing both phloroglucinol-HCl and chlorine–sodium sulfite reagents. Color tests are definite (+), weak (±), negative (−).

On the whole, the behavior of model systems as described mimics remarkably the sequence of events which has been observed in organized tissues and cell wall preparations, suggesting that genuine advantage is to be had in using such models in the study of lignification and kindred processes.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, PURDUE UNIVERSITY]

Composition and Behavior of Soil Polysaccharides[1,2]

BY ROY L. WHISTLER AND KENNETH W. KIRBY

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A soil polysaccharide concentrate was isolated from a Brookston type agricultural soil and quantitatively analyzed. It contains D-galactose, D-glucose, D-mannose, L-arabinose, D-xylose, L-rhamnose, glucuronic acid and possibly ribose and glucosamine. Examination shows that it is likely a mixture of polysaccharides possibly derived from the cells of microörganisms. The material is found to have significant soil aggregation properties. The rate of decomposition of the polysaccharide in the soil is much slower than that of most plant polysaccharides and bacterial gums.

Polysaccharide concentrates from various British soils and a Trinidad soil have been examined chemically.[3] This Laboratory also has undertaken the examination of the soil polysaccharides. Since polysaccharides from an American soil have not been subject to close scrutiny, Brookston soil of a typical Midwest agricultural region was selected for examination. Polysaccharide material can be isolated from this soil in approximately equivalent yield by extraction with either water or 2% sodium hydroxide solution. Quantitative analyses for anhydrosugar units in the separated polysaccharide fraction are shown in Table I. By actual isolation in the form of crystalline derivatives the sugars are found to be D-galactose, D-glucose, D-mannose, L-arabinose, D-xylose and L-rhamnose. The uronic acid is undoubtedly glucuronic acid since it gives the typical color reaction[4] with thioglycolic acid, its barium salt possesses the expected optical rotation and its lactone has the expected chromatographic flow rate.

Ribose and glucosamine are present in such small

(1) Journal Paper No. 910 of the Purdue Agricultural Experiment Station, Lafayette, Indiana.

(2) Paper presented before the Division of Agricultural and Food Chemistry at the 128th Meeting of the American Chemical Society in Minneapolis, Minnesota, September, 1955.

(3) (a) W. G. C. Forsyth, *Biochem. J.*, **46**, 141 (1950); (b) R. B. Duff, *J. Sci. Food Agr.*, **3**, 140 (1952); (c) R. B. Duff, *Chemistry and Industry*, 1104 (1952); (d) 1513 (1954).

(4) Z. Dische, *J. Biol. Chem.*, **171**, 725 (1947).