identical to the values for a known sample of 2-*O*-methyl-D-xylose. *Anal.* Calcd. for $C_6H_{12}O_5$: C, 43.9; H, 7.36. Found: C, 44.0; H, 7.36.

D-Xylose.—Fraction Va was chromatographically identical to D-xylose on chromatograms developed with ethyl acetate–pyridine–water (10:4:3 v./v.) and with irrigant A. The material was allowed to react with benzaldehyde in methanolic hydrogen chloride[20] to produce D-xylose dibenzylidine dimethyl acetal, m.p.[21] 210°, having an X-ray diffraction pattern identical to the known material.

Uronic Acids.—Fraction Vb (350 mg.) was shown to be a mixture on paper chromatography. Papers developed in ethyl acetate–acetic acid–formic acid–water (18:3:1:4 v./v.) show three major zones at R_g 0.78, 0.58 and 0.15 with a trace of material at 0.09. Conversion to the methyl glycoside–methyl ester by refluxing with anhydrous methanol containing 5% hydrogen chloride for 4 hr. was followed by neutralization with silver carbonate, filtration and evaporation to a sirup. The mixture was dissolved in anhydrous ether and reduced with 100 mg. of lithium aluminum hydride[22] dissolved in ether. The excess reagent was decomposed with ethyl acetate and water and then extracted continuously with chloroform for 16 hr. A sirup remaining after reduction (250 mg.), $[\alpha]^{25}D$ +40.3° (*c* 1.2 in water), was hydrolyzed for 4 hr. in 1 *N* sulfuric acid at 98°. The hydrolyzate was neutralized with barium carbonate, filtered and deionized with resins IR-120 (H) and IR-45 (OH). Paper chromatograms in irrigant A showed a major elongated zone of R_g 0.70 to 0.80 which corresponded to a mixture of known 2,3,4-tri-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-xylose. A second zone at R_g 0.22 corresponded to 2-*O*-methyl-D-xylose, and a trace of material was present at R_g 0.03. Ionophoresis[10] in borate buffer gave four zones having M_G values of 0.96, 0.88 (trace), 0.32, and 00.0. M_G is the ratio of movement compared to that of D-glucose corrected for electroendosmotic flow.

Quantitative Paper Chromatography.—The methylated polysaccharides (100 mg.) were hydrolyzed in sealed tubes and chromatographed with irrigant A in essentially the same way as described in an earlier report,[23] except that the hydrolyzate was deionized with resins IR-120 (H) and IR-45 (OH) before concentrating and evaporating. Whatman No. 1 filter paper extracted with water and ethanol was used for the chromatograms. The sugar zones were eluted from the paper chromatogram with methanol and the molar ratios determined by alkaline hypoiodite[24] after correction for paper blanks. The results are shown in Table I.

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[CONTRIBUTION NO. 334 FROM THE DEPARTMENT OF ORGANIC CHEMISTRY AND ENZYMOLOGY, FORDHAM UNIVERSITY]

Investigations on Lignins and Lignification. XIX.* The Mode of Incorporation of *p*-Hydroxyphenylpyruvic Acid into Lignin

BY SAMUEL N. ACERBO, WALTER J. SCHUBERT AND F. F. NORD

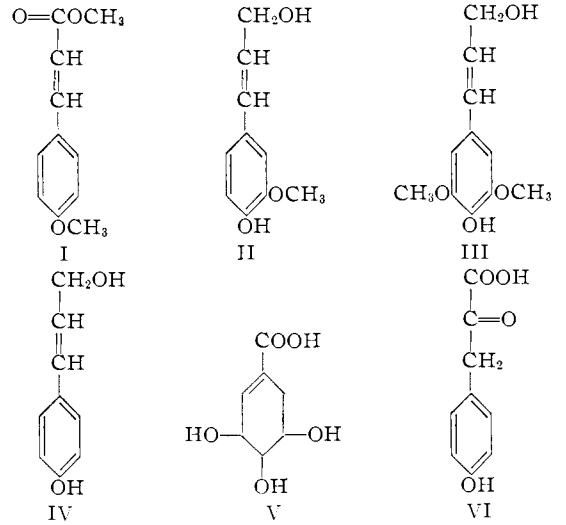
RECEIVED SEPTEMBER 9, 1957

Carboxyl-labeled *p*-hydroxyphenylpyruvic acid was incorporated into a living sugar cane plant. Seventy-one per cent. of the introduced radioactivity was located in the isolated lignin. Alkaline nitrobenzene oxidation of the lignin to (nonradioactive) vanillin and alkaline fusion degradation to (radioactive) oxalic acid indicated that the *p*-hydroxyphenylpyruvic acid was utilized by the plant as a unit in the course of its conversion to lignin.

Introduction

The mechanism of the formation *via* shikimic acid of methyl *p*-methoxycinnamate (I) by *Lentinus lepideus* has been found to be related to that of the biogenesis of certain aromatic amino acids.[1] Due to the structural similarity of I with the postulated building stones of lignin, namely, coniferyl alcohol (II), sinapyl alcohol (III) and *p*-hydroxycinnamyl alcohol (IV), this finding also establishes a relationship with the biogenesis of lignin.

During the course of investigations of the metabolism of *Lentinus lepideus*, shikimic acid and five keto-acids, including *p*-hydroxyphenylpyruvic acid, were detected in the culture medium. Shikimic acid (V) is now regarded as a direct precursor of the aromatic rings of methyl *p*-methoxycinnamate[2] and of lignin.[3] Detection in the medium of *p*-hydroxyphenylpyruvic acid (VI), which is structurally related to the postulated building stones of lignin (II, III, IV), prompted an investigation of the possible



role of this acid in the mechanism of lignification.[4] The results to be reported here present evidence

* For the previous paper of this series see W. J. Schubert, S. N. Acerbo and F. F. Nord, THIS JOURNAL, **79**, 251 (1957). For a factual and critical review of the chemistry of lignins and the mechanism of lignification the reader is referred to: (a) F. F. Nord and W. J. Schubert, *Tappi*, **40**, 285 (1957); (b) W. J. Schubert and F. F. Nord, *Advances in Enzymology*, **18**, 349 (1957); (c) F. F. Nord and Geo. de Stevens, *Handbuch d. Pflanzenphysiologie*, **10**, 389 (1958), Springer Verlag, Heidelberg.

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that *p*-hydroxyphenylpyruvic acid is also to be regarded as a precursor of the lignin building stones.

Experimental

Synthesis of *p*-Hydroxyphenylpyruvic Acid-$C^{14}OOH$.—0.65 gram of glycine-1-C^{14} (Tracerlab, Inc., Boston, Mass.; specific activity, 0.5 mc.) was dissolved in 2.9 ml. of water. Immediately upon dissolution, 1.7 ml. of 95% acetic anhydride was added. After 20 minutes of vigorous stirring, crystals of acetylglycine precipitated, and the stirring was stopped. To assure complete precipitation, the solution was kept at 5° for 6 hr. The crystals were collected by filtration, washed with cold water and dried at 100–110°; yield 0.74 g., m.p. 206–207°.

0.74 gram of acetylglycine was mixed with 0.77 g. of *p*-hydroxybenzaldehyde, and 1.7 g. of acetic anhydride and 0.4 g. of fused sodium acetate was added under strictly anhydrous conditions.[5] The mixture was refluxed in a boiling water-bath for 10 minutes until dissolution was completed, and then a heating mantle was applied to provide a higher temperature for 8 min. During this interval, gases were evolved, and a brown solution of the unsaturated azlactone of 4-acetoxy-α-acetamidocinnamic acid was formed. The solution was kept at 0° for 6 hr., and a mass of yellow-brown crystals was obtained. Water (4 ml.) was used to suspend the crystals, which were filtered, washed with ice-water and dried *in vacuo* over KOH; yield 1.18 g., m.p. 127–128°.

1.18 grams of the crude azlactone was hydrolyzed with 50 ml. of boiling water for 10 min. to *p*-acetoxy-α-acetamidocinnamic acid. The hot solution was filtered and the resulting crystals washed with water and dried at 110°; yield 0.41 g., m.p. 223–224°.

0.41 gram of *p*-acetoxy-α-acetamidocinnamic acid was further hydrolyzed by refluxing for 4 hr. with 3 g. of cation-exchange resin 50-X8 (Dow Chemical Co.), which contained 5.0 milliequiv. of hydrogen per dry gram, and 10 ml. of water. The resin was filtered off and, upon cooling, *p*-hydroxyphenylpyruvic acid crystallized; m.p. 218–220°. The yield was 76 mg., which was diluted to 130 mg. with the non-radioactive acid, yielding a specific activity of 9,123 c.p.m. per mg. carbon. The compound was identified by its ultraviolet spectrum and analysis.

Anal. Calcd. for: C, 60.00; H, 4.44. Found: C, 59.44; H, 4.53.

Incorporation of *p*-Hydroxyphenylpyruvic Acid into a Growing Sugar Cane Plant and Isolation of its Lignin.—The tagged *p*-hydroxyphenylpyruvic acid (130 mg.) was dissolved in 50 ml. of aqueous buffer containing 98.2 mg. of KH_2PO_4, maintaining a *p*H of 6.5.

This solution was absorbed by the youngest leaves of a mature, growing sugar cane plant (*Saccharum officinarum*), by immersing cut ends of the leaves into the solution. After 15 days of metabolism, the plant was cut, and the stalk was dried and ground to 60 mesh.

The lignin was isolated as Klason lignin,[6] and its content in the plant was 6.1%. It was devoid of nitrogen. The lignin was then oxidized to $BaCO_3$.[7] The total activities of the *p*-hydroxyphenylpyruvic acid and the lignin-derived barium carbonate are recorded in Table I.

TABLE I

COMPARISON OF ACTIVITIES OF *p*-HYDROXYPHENYLPYRUVIC ACID AND BARIUM CARBONATE FROM LIGNIN

	Activity c.p.m./mg. C	Total c.p.m.
p-Hydroxyphenylpyruvic acid	9100	750,200
$BaCO_3$ (from lignin)	250	533,700

Degradation of the Isolated Klason Lignin.—Four hundred and fifty mg. of the isolated radioactive Klason lignin was subjected to oxidative degradation[8] in a bomb containing 3 ml. of nitrobenzene and 45 ml. of 8% NaOH. The bomb was shaken for 2.5 hr. at a temperature of 160°. The resulting mixture contained carbonyl compounds which were isolated from a benzene extract by formation of their sodium bisulfite addition compounds. After acidic cleavage of the complexes, the freed carbonyl compounds were sublimed at a pressure of 1.5 mm. and a temperature of 61°, yielding a crystalline compound which was identified by its melting point, mixed melting point, ultraviolet spectrum and paper chromatogram[9] as vanillin.

Examination of the isolated vanillin revealed that it was devoid of any radioactivity.

One-half g. of the lignin was further subjected to alkaline fusion,[10] employing a solution of 3 g. of KOH in 4 ml. of water at a temperature of 280° for 5 minutes. After the "lignin acids" were removed by acidification with dilute HCl and filtration, an aqueous solution of $CaCl_2$ was added, causing precipitation of calcium oxalate. This was dissolved in dilute HCl and extracted with ether. Recrystallization gave oxalic acid, m.p. 187–189°. A mixed melting point with an authentic sample showed no depression.

The radioactive lignin and the isolated oxalic acid were individually oxidized to $BaCO_3$, and the radioactivities of each were determined. Activity measurements were made on infinitely thick layers of $BaCO_3$ as previously,[2,3] employing a counter of 5–10% efficiency. The following results were obtained: for lignin, 188 c.p.m./mg. C; for oxalic acid, 244 c.p.m./mg. C.

Results and Discussion

In a previous experiment[3] 2,6-C^{14}-shikimic acid was incorporated into a living sugar cane plant. The lignin isolated from this plant contained radioactivity. Vanillin, obtained by oxidative degradation of this lignin, showed a distribution of radioactivity which was comparable to the distribution of C^{14} in the originally incorporated shikimic acid. Thus, this acid is now considered as a precursor of the aromatic rings of lignin building stones.[3]

The present investigation revealed that 71% of the activity of the introduced *p*-hydroxyphenylpyruvic acid-$C^{14}OOH$ was incorporated into the lignin.

C^{14}-analysis of the vanillin obtained by degradation revealed no activity, indicating that the carboxyl carbon of the *p*-hydroxyphenylpyruvic acid was not randomized with the C_6-C_1 moiety of the lignin building units, thereby implying that the aromatic ring of the acid may be converted into the aromatic rings of lignin, also without randomization.

The data obtained from the degradation of lignin to oxalic acid show that the specific activity of the oxalic acid was greater than the specific activity of the lignin. Therefore, the side chain of the introduced *p*-hydroxyphenylpyruvic acid, which contained the radioactivity, may not be involved in the aromatization process but, rather, it too might be retained as a unit, presumably affording a "connecting link" between the several aromatic rings of the lignin polymer.

Accordingly, it may be concluded that *p*-hydroxyphenylpyruvic acid is a precursor of lignin building stones and may therefore function as an intermediate on the pathway between shikimic acid, derived from carbohydrates, and the lignin building stones, in the biogenesis of lignin itself.

Thus, the process of lignification may be formulated as carbohydrate, photosynthetically derived from atmospheric carbon dioxide, is cyclized to shikimic acid, which in turn is aromatized to an intermedi-

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ate of the type of *p*-hydroxyphenylpyruvic acid. Suitable methoxylation of such an intermediate then produces the lignin building stones, polymerization of which results in lignin.

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[CONTRIBUTION NO. 336 FROM THE DEPARTMENT OF ORGANIC CHEMISTRY AND ENZYMOLOGY, FORDHAM UNIVERSITY]

Investigations on Lignins and Lignification. XX.[1a] The Biosynthesis of Methyl *p*-Methoxycinnamate from Specifically Labeled D-Glucose by *Lentinus lepideus*

BY H. SHIMAZONO,[1b] WALTER J. SCHUBERT AND F. F. NORD

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The fungus *Lentinus lepideus* was grown in media containing 1-C^{14}-D-glucose and 6-C^{14}-D-glucose. The activities of both were significantly incorporated into methyl *p*-methoxycinnamate, a normal metabolic product. The comparative distribution of activity in the ester from 6-C^{14}-D-glucose indicates that this compound may be synthesized *via* shikimic and prephenic acids. The distribution of the activity in the ester produced from 1-C^{14}-D-glucose, when compared with that from the 6-C^{14}-compound, shows that in the metabolism of glucose by this fungus, under these conditions, a pathway other than glycolysis is operative.

Introduction

The metabolism of the mold *Lentinus lepideus*, which is a member of the group of wood-destroying fungi, is of great interest in view of the enzyme system present, which is capable of decomposing the cellulose of wood. Several aromatic compounds are formed by wood-destroying fungi from the carbohydrates of wood, and some of these metabolic products are structurally very similar to the building units of lignin, which is also susceptible to attack by certain of these fungi. *Lentinus lepideus* is known to tolerate comparatively high concentrations of creosote. This mold is also reported to give rise to methyl *p*-methoxycinnamate, methyl cinnamate and methyl anisate, while growing on wood.[2] When this fungus is cultivated on a medium containing glucose, xylose, glycerol or ethanol as sole carbon source, methyl *p*-methoxycinnamate accumulates in each case under the same conditions.[3] The mechanism of formation of this ester from the above carbohydrates and alcohols is not yet known completely. Previous investigations of this Laboratory revealed[4] that acetate was not significantly incorporated into the ester. On the other hand, in the course of studies on the metabolism of amino acids, aromatization[5] of carbohydrates and the formation of phenolic acids have been reported. The present communication reveals that the ester formed by *Lentinus lepideus* is significantly derived from carbons 1 and 6 of glucose, and describes how the activities of these positions of glucose are distributed.

(1) (a) For paper XIX of this series see the preceding communication. (b) Postdoctorate fellow from the Forest Experiment Station, Dept. of Agriculture and Forestry, Tokyo.

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Experimental

Isolation of Methyl *p*-Methoxycinnamate.—*Lentinus lepideus* was grown for approximately 40 days at 22–25° in 500-ml. erlenmeyer flasks containing 200 ml. of a medium composed of: 1-C^{14} or 6-C^{14}-D-glucose, 2%; KH_2PO_4, 0.15%; Neopeptone (Difco-Bacto), 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%, and thiamine hydrochloride 2 mg./l. in tap water.

1-C^{14}-D-glucose was obtained from New England Nuclear Corporation, Boston, Mass., 6-C^{14}-D-glucose was synthesized from $KC^{14}N$, which was purchased from Tracerlab, Inc., Boston, Mass.

Upon termination of growth, the mycelium and culture medium were filtered, and the air-dried mycelium was extracted with ethanol in a Soxhlet apparatus for 8 hr. The ethanol extract was concentrated to dryness, and the residue was sublimed twice *in vacuo* at 75°. This was recrystallized from ethanol and water and gave a m.p. of 87–88°. The melting point showed no depression when mixed with an authentic sample.

Degradation of Methyl *p*-Methoxycinnamate and Determination of its Activity.—The scheme of degradation is shown in Fig. 1. One hundred mg. of methyl *p*-methoxycinnamate was saponified with methanolic alkali to *p*-methoxycinnamic acid, which was precipitated from water by adding hydrochloric acid; yield 70 mg., m.p. 172–173°. The activity of carbon-10 was calculated by subtracting the activity of this acid from that of the ester from which it was derived.

One hundred mg. of methyl *p*-methoxycinnamate was dissolved in 20 ml. of acetone, and about 250 mg. of finely powdered $KMnO_4$ was added in small portions with shaking and cooling. The solution was filtered and the precipitate washed with acetone. This precipitate was extracted with water on the steam-bath, and the clear filtrate was acidified with hydrochloric acid. The crude crystals were purified by precipitation from water by the addition of HCl solution; yield 50 mg. (65%), m.p. 184°. The m.p. after mixing with an authentic sample of anisic acid did not show a depression.

The presence of oxalic acid in this filtrate also was detected. It is believed that during the oxidation of the ester with $KMnO_4$ in acetone, oxalate is formed from the side chain of the methyl *p*-methoxycinnamate and is precipitated in acetone. Accordingly, oxalate is accumulated in the reaction mixture without further decomposition. The filtrate was concentrated *in vacuo* and the residue dissolved in *M*/4 potassium citrate buffer (*p*H 3.0). Hereupon, oxalic acid decarboxylase, prepared[6] from the mold *Collyvia veltipes*, was added, and the CO_2 evolved was absorbed in Ba$(OH)_2$ solution. Thirty-nine mg. of $BaCO_3$ was obtained. (25 mg. as oxalic acid; yield from ester 38%.) A wet

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