Anal. Calcd. for $C_{21}H_{43}ON$: C, 73.84; H, 12.09; N, 4.10. Found: C, 73.70; H, 12.60; N, 3.90.

Typical Emulsion Experiments.—The substances under test were dissolved in 20 ml. of water. The maximum concentration possible was generally employed since none were markedly soluble. Satisfactory results were obtained only when the solution contained 0.10–0.20 g. of emulsifier of the type investigated. This solution was mixed in an Omnimixer with 5 ml. of Nujol containing 0.2 g. of cholesterol. The emulsions were judged on the basis of stability and of particle size; in our experiments only emulsions of small particle size (1–2 μ) exhibited appreciable stability.

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Investigations on Lignin and Lignification. XVI. On the Mechanism of the Biogenesis of Methyl *p*-Methoxycinnamate and Its Possible Relation to Lignification[1]

BY G. EBERHARDT

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The mold *Lentinus lepideus* is known to produce methyl *p*-methoxycinnamate when grown on carbohydrate or ethyl alcohol as the sole carbon source. A number of metabolic products have been found and identified in the culture medium of this mold. These are 5 ketoacids, 3 sugars and shikimic acid. The results point to a relationship between the biogenesis of methyl *p*-methoxycinnamate and of tyrosine. A possible relation to the formation of lignin building stones is discussed.

Among the several varieties of wood-destroying molds, *Lentinus lepideus* (Lelep) is known to produce the so-called brown-rot in wood. It has been found that the metabolic processes associated with the decay of wood by this organism give rise to aromatic substances. These are methyl cinnamate, methyl anisate and, particularly, methyl *p*-methoxycinnamate.[2]

Studies in this Laboratory now have shown that it is possible to grow Lelep on media containing glucose, xylose or ethyl alcohol as sole carbon source, whereby methyl *p*-methoxycinnamate (I) appears as a crystalline deposit in the culture medium, after several weeks of incubation.[3] From these results it was concluded that (I) is not a product of the degradation of lignin, which might conceivably have been effected by the organism during its growth on wood. For, starting from carbohydrates or ethyl alcohol, Lelep is quite capable of synthesizing the above aromatic compound I.

Furthermore, Lelep possesses the remarkable property of being able to detoxify phenolic compounds by methylation. Therefore, we may feel quite certain that (I) is formed by methylation of *p*-hydroxycinnamic acid (Ia), an assumption which will receive support from an additional fact to be discussed later.

Recent reports seem to confirm[4] the hypothesis that hydroxy- and methoxycinnamyl alcohols, such as coniferyl alcohol (III) and sinapyl alcohol (IV), may be considered as building stones of lignin. On the other hand, from certain types of lignin, *p*-hydroxybenzaldehyde,[5] as well as vanillin and syringaldehyde, were detected as products of a mild alkaline oxidation. This would indicate that *p*-hydroxycinnamyl alcohol (II) has to be considered as a lignin building stone in certain plants. *p*-Hydroxycinnamyl alcohol differs from compound Ia only in the state of oxidation of the side chain, while coniferyl alcohol and sinapyl alcohol differ from it only by the presence of one or two additional methoxyl groups, respectively.

The investigation of the biogenesis of lignin building stones in plants meets with considerable experimental difficulty, due to the complex nature of the problem. However, studies of the biosynthesis of I by Lelep are somewhat simpler. Thus any results obtained from such experiments could have importance in theorizing on the formation of lignin building stones, if we assume similar pathways in the formation of both compounds.

This assumption of the similarity of the biogenesis of I with the formation of lignin building stones is based on the close structural relationship between the two compounds. The experiments described here were therefore undertaken as part of the investigation of the details of the biogenesis of lignin building stones.

The fact that in its natural state Lelep grows on wood and that, as a result of its metabolic activity, it forms a substance structurally similar to the lignin building stones, emphasizes this relationship.

Experimental

In the following experiments, Lelep was grown on 50-ml. portions of a nutrient medium contained in 125-ml. erlenmeyer flasks, under aseptic conditions. The medium was of the following composition: glucose, 15–20 g., or ethyl alcohol, 10 ml.; KH_2PO_4, 1.5 g.; Neopeptone (Difco), 1.0 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; thiamine hydrochloride, 2.0 mg.; water, to 1.0 liter.

The addition of 4.0 g. of sodium acetate to the glucose medium had the effect of accelerating the growth and also the appearance of methyl *p*-methoxycinnamate (I). (Lelep grows rather poorly on acetic acid–sodium acetate as the

(1) For a preliminary report see: G. Eberhardt and F. F. Nord, *Arch. Biochem. Biophys.*, **55**, 578 (1955). For communication No. XV of this series see W. J. Schubert and F. F. Nord, *Proc. Natl. Acad. Sci. U. S.*, **41**, 122 (1955).

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(4) K. Freudenberg, in L. Zechmeister, *Fortschr. Chem. org. Naturstoffe*, **9**, 43 (1954).

(5) F. F. Nord and G. De Stevens, *Naturwiss.*, **20**, 479 (1952); THIS JOURNAL, **75**, 305 (1953); H. S. Mason, in F. F. Nord, *Adv. in Enzymology*, **16**, 149 (1955).

sole carbon source.) The cultures were incubated in the dark at 27°. After about six weeks of development, the excretion of crystals of I began in the media.

Identification of Keto-Acids.—Lelep was grown on glucose as substrate, under the conditions described above. For the isolation and paper chromatography of the keto-acids, the following procedure was employed: The filtered medium was mixed with twice its volume of a cold saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl and kept at 38° for 30 min. After cooling, the solution was shaken with ethyl acetate, and the ethyl acetate layer was extracted with 2 *N* sodium carbonate. The alkaline extract was then shaken twice with small portions of ethyl acetate, and with ice-cooling, it was acidified with 2 *N* HCl. The keto-acid hydrazones were extracted with a small portion of ethyl acetate, and the extract was concentrated to a small volume (1 ml.). The yellowish solution was immediately subjected to paper chromatography. A two-phase system was used consisting of *t*-amyl alcohol, ethyl alcohol and water.[6] (Whatman No. 2 paper was used, without preliminary treatment.) The identity of the acids was determined by comparison of the R_f values with those values obtained from authentic samples of the respective acids. The resulting R_f values differ slightly from those recorded in the literature[6] (possibly due to the differences in temperature). There are listed in Table I the R_f values of the acids found and also those of the authentic samples used for comparison.

TABLE I

PAPER CHROMATOGRAPHY OF THE KETO-ACID 2,4-DINITROPHENYLHYDRAZONES

R_f values found	R_f values of comparative 2,4-dinitrophenylhydrazones
0.08	0.07, oxaloacetic acid
.35	.36, α-ketoglutaric acid
.46	.46, pyruvic acid
.66	.65, *p*-hydroxyphenylpyruvic acid
.86	.89, acetoacetic acid

The identity of the acid of R_f value of 0.66 with *p*-hydroxyphenyl pyruvic acid was confirmed by taking an ultraviolet absorption spectrum in 0.1 *N* NaOH solution from the eluate of this spot (R_f 0.66). This spectrum was identical with that of an authentic sample of *p*-hydroxyphenylpyruvic acid 2,4-dinitrophenylhydrazone. It shows an absorption maximum at 442 mμ.

Other cultures of Lelep were grown on ethyl alcohol as the sole carbon source, in the same way as described above. Paper chromatograms established the presence of the same keto-acids as were produced from glucose.

Identification of the Sugars.—For the identification of potential intermediary metabolites of carbohydrate nature, the presence of a large excess of a nutrient sugar would cause an obstacle. Therefore, in the following experiments the cultures were grown on media containing 1% ethyl alcohol, as was suggested in the original experiments.[7] For the paper chromatography of sugars the solvent system, butanol: ethanol:water, was used.[8]

Lelep was grown on 1 l. of a medium containing ethyl alcohol as substrate, distributed in 20 erlenmeyer flasks, as reported above. After eight weeks of growth, the mats were removed by filtration and washed. The combined filtrates and washings were concentrated to a volume of 25 ml. Upon the addition of 100 ml. of ethanol, the precipitated inorganic and protein constituents were removed by filtration, and the aqueous alcoholic solution was evaporated to 10 ml. A direct paper chromatogram of this solution, after spraying with aniline phthalate, revealed a positive reaction for aldoses at the zero point, suggesting the presence of phosphorylated aldoses.

The solution was then subjected to enzymatic dephosphorylation by means of "Polidase."[9] After incubation for 24 hr. at 37°, the solution was chromatographed on Whatman No. 1 paper in the solvent system butanol:ethanol: water. After 20 hr. of development, the paper was sprayed with aniline phthalate. The results are recorded in Table II.

TABLE II

COMPARISON OF R_f VALUES FOR UNKNOWN SUGARS WITH THOSE OF AUTHENTIC SAMPLES

R_f found	R_f of comparative preparations	Color of spot after application of aniline phthalate and heating
0.76[a]	Glucose, 0.76[a]	Brown
1.58[a]	Ribose, 1.57[a]	Dark red

[a] R_f value: mobility of carbohydrate/mobility of fructose (fructose = 1).

Another paper chromatogram, prepared in the same way, was subjected to the trichloroacetic acid–orcinol test. A somewhat distorted, faint blue zone appeared in the R_f region 0.12–0.26. This zone was removed from another paper chromatogram and eluted with water. The eluate was concentrated to a small volume in an atmosphere of nitrogen and was then rechromatographed in the solvent system phenol: water, on Whatman No. 1 paper.

The orcinol–TCA test developed a blue spot of R_f value 0.70 (sedoheptulosan gives 0.69).[10] It was not possible to detect sedoheptulose as such. However, based on the ability of sedoheptulose to undergo dehydration, it is probable that sedoheptulosan was formed from sedoheptulose during the procedure.

Determination of the Shikimic Acid.—Cultures of Lelep grown on a glucose-containing medium (as above) were filtered and the filtrate concentrated to one-half of its original volume. A direct bioassay of the concentrated filtrate with the test organism A 170-143 S in assays of 0.1 ml. was negative. This bioassay is selective for the detection of shikimic acid, dehydroshikimic acid (DHS) and dehydroquinic acid (DHQ) and also for the following four aromatic amino acids: tyrosine, phenylalanine, tryptophan, and *p*-aminobenzoic acid and also *p*-hydroxybenzoic acid.[11]

Phosphorylated shikimic acid is not subject to a direct bioassay.[12] Hence, 10 ml. of the filtrate was submitted to enzymatic dephosphorylation. About 100 mg. of "Polidase" was added, and the solution was incubated 24 hr. at 37°. The solution was then heated for a short time on a steam-bath to inactivate the enzymes and to precipitate the proteins. The filtrate yielded a distinctly positive bioassay with the test organism A 170-143 S, indicating the presence of shikimic acid, DHS or DHQ. As a control the Polidase preparation was tested independently for the possible presence of bioassay-positive substances, but with negative results.

The untreated filtrate (as above) was paper chromatographed and developed with the solvent system butanol: formic acid:water. The area around the region of the R_f value 0.04[12] (the R_f value of monophosphoshikimic acid) was cut out and eluted. After enzymatic dephosphorylation, the eluate gave a positive bioassay.

These results indicate the presence of phosphorylated shikimic acid (or of phosphorylated DHS or DHQ) in the filtrate of the organism.

Growth Experiments with C^{14}-Labeled Compounds.—Lelep was grown on a nutrient medium containing 1.5% glucose and 0.3% sodium acetate[13] as described above. The sodium acetate was labeled with C^{14} in the methyl group. After eight weeks of development, during which time the precipitation of methyl *p*-methoxycinnamate (I) occurs, the mats and the crystals were removed by filtration, dried and extracted with alcohol. The residue obtained upon evaporation of the alcohol was sublimed twice and then recrystallized. Its identity with I was established by its melting point (86.5°).

A portion of the compound was saponified with methanolic alkali to *p*-methoxycinnamic acid, and again a portion of the latter compound was oxidized to anisic acid.

(6) S. M. Altmann, E. M. Crook and S. P. Datta, *Biochem. J.*, **49**, LXIII (1951).

(7) F. F. Nord and J. C. Vitucci, *Arch. Biochem.*, **15**, 465 (1947).

(8) S. M. Partridge, *ibid.*, **42**, 238 (1948).

(9) Commercial sample from Schwarz Laboratories, Mount Vernon, N. Y.

(10) L. P. Zill and N. E. Tolbert, THIS JOURNAL, **76**, 2929 (1954).

(11) U. Weiss, B. D. Davis and E. S. Mingioli, *ibid.*, **75**, 5572 (1953).

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MEASUREMENT OF ACTIVITY (WINDOW COUNTING)

1 mg. of the compound per 3.8 cm.² area

	Cts./min./mg. C
Sodium acetate	900
Compound I	41
p-Methoxycinnamic acid	41
Anisic acid	33

In other experiments, ribose, shikimic acid and sodium acetate were tested for their ability to serve as competitors in the biogenesis of I. In each case, to eight Lelep cultures growing on 50 ml. of 1% glucose randomly labeled with C^{14} (see above) for two weeks, was added, under aseptic conditions, an aqueous solution of the competitor. The concentration of sodium acetate and of ribose amounted to 10% of the concentration of glucose, while that of shikimic acid was 5%. In addition, a control was grown without any added competitor.

After ten weeks of additional growth, compound I was isolated and purified, as described above. The activity of compound I in the control experiment amounted to 155 cts./min./mg. C. The competition experiments did not show any dilution of activity.

Discussion

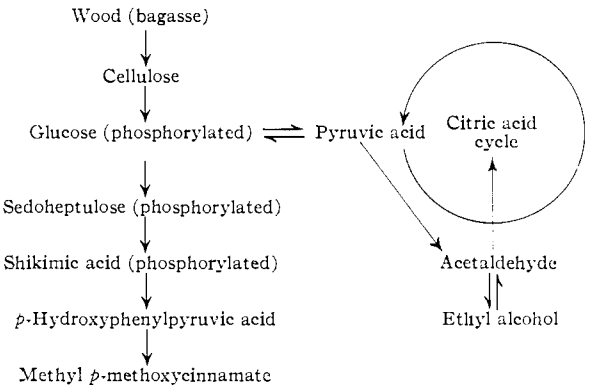
The above described experiments resulted in the detection of a number of products of the metabolism of the mold Lelep. Specifically, these are: oxaloacetic acid, α-ketoglutaric acid, pyruvic acid, *p*-hydroxyphenylpyruvic acid, acetoacetic acid, ribose, glucose, sedoheptulose, phosphoshikimic acid (or DHS, DHQ). An attempt will be made to interpret these results in terms of the biogenesis of methyl *p*-methoxycinnamate (I).

Experiments in which the organism was grown on glucose plus labeled sodium acetate and also labeled glucose plus sodium acetate as competitor show that acetyl units are unimportant in the biosynthesis of compound I. The detection of the keto-acids implies the functioning of the citric acid cycle. Acetic acid can be introduced into the citric acid cycle. The result of the experiment employing methyl C^{14}-labeled sodium acetate in addition to unlabeled glucose does not show any significant incorporation of C^{14} into compound I. This result also permits the interpretation that the keto-acids, and also acetaldehyde, which is linked with this cycle *via* pyruvic acid, are not directly involved in the formation of I.

Lelep is also an alcoholic fermenter,[14] but, after a transient accumulation, the ethyl alcohol is rapidly consumed *via* dehydrogenation to acetaldehyde.[3]

This observation has previously led to the consideration that acetaldehyde has the role of an intermediary in the formation of I.[7] However, this hypothesis requires some modification and amplification in the light of the above result and its interpretation.

Rather, the experiments indicate a more direct origin of (I) from glucose. This conclusion is also supported by the fact that the organism, when grown on 1% ethanol as substrate, resynthesizes glucose. This conversion prompts a comparison with the biogenesis of aromatic amino acids.[15] Davis assumes that the synthesis of tyrosine takes place *via* glucose and shikimic acid. Sedoheptulose, to which Davis attributes importance in the biogenesis of shikimic acid, also appears among the metabolic products of Lelep. However, in distinction to the biogenesis of aromatic amino acids it has been shown that free shikimic acid does not seem to function as a competitor in the growth of this organism on tagged glucose. Nevertheless, the results of the experiment do indicate the presence of a phosphorylated shikimic acid. If a phosphorylated, but not free, shikimic acid should be on the pathway, it is possible that the organism is unable to phosphorylate free shikimic acid directly and that, therefore, the free acid cannot enter the metabolic pathway. But it might also be that the cell wall is not permeable to the shikimic acid. This problem is still under investigation.



p-Hydroxyphenylpyruvic acid has been considered as an intermediate in the biogenesis of tyrosine.[14] This compound was identified in the medium of Lelep cultures and is to be considered a precursor of *p*-hydroxycinnamic acid. However, it is also possible that the compound is produced from a structurally related substance during the preparation of the 2,4-dinitrophenylhydrazone, just as prephenic acid is converted to phenylpyruvic acid under acidic conditions.[16] It is remarkable that, at this point, methylation of the nuclear hydroxyl groups has not yet taken place. Hence, the methylation must be one of later steps in the biosynthesis of I. This is of significance in any consideration concerning the origin of the guaiacyl and syringyl building stones in lignin.

Conclusions

The above described findings indicate a relationship between the formation of *p*-hydroxycinnamic acid and the biogenesis of tyrosine. The structural relationship existing between *p*-hydroxycinnamic acid and lignin building stones already has been discussed. Therefore, it might be possible that lignin building stones are synthesized by a similar pathway. Thus, the results presented here offer a means of contributing to the problem of the biogenesis of lignin building stones II, III and IV.

A critical experiment for the elucidation of this problem would be the incorporation of specifically C^{14}-labeled compounds like shikimic acid into living plants, followed by determination of the C^{14} distribution in lignin degradation products.

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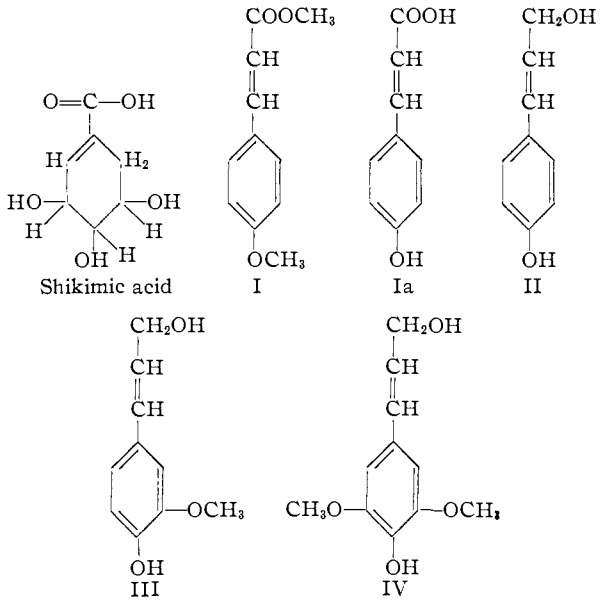

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Investigations on Lignin and Lignification. XVII. Evidence for the Mediation of Shikimic Acid in the Biogenesis of Lignin Building Stones

BY G. EBERHARDT AND WALTER J. SCHUBERT*

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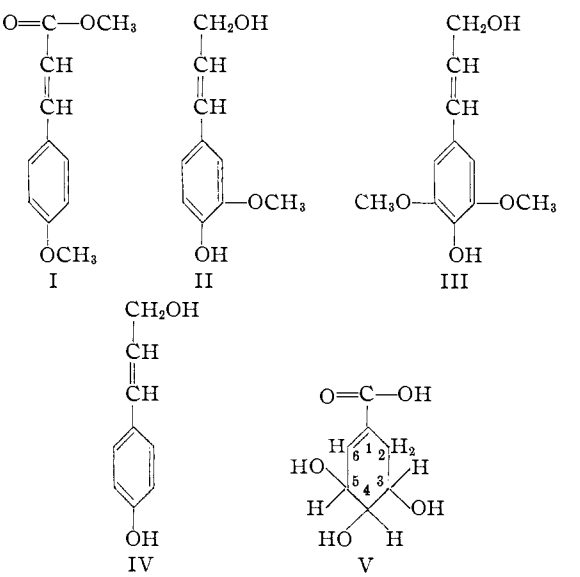
A 2,6-C^{14}-shikimic acid was incorporated into a living sugar cane plant. The lignin isolated from this plant contained radioactivity. Vanillin, obtained by oxidative degradation of the lignin, shows a distribution of radioactivity which is comparable to the distribution of C^{14} in the originally incorporated shikimic acid. Thus, this acid may be considered as a precursor of the aromatic ring of lignin building stones.

In the preceding paper of this series,[1] details of the biogenesis of methyl *p*-methoxycinnamate (I) from glucose, a metabolic product of the fungus *Lentinus lepideus* (Lelep), were studied. The mechanism of formation of this compound was found to be related to that of the biogenesis of certain aromatic amino acids. Due to the structural similarity of compound I to the postulated building stones of lignin (II, III, IV),[2] these results point to a relationship with the biogenesis of lignin.

Shikimic acid (V) is regarded as a direct precursor of the aromatic ring[3] of the amino acids phenylalanine, tyrosine, tryptophan and *p*-aminobenzoic acid. Recent experiments[4] indicate that after the introduction of radioactively labeled shikimic acid into freshly cut wheat and maple plants, there was considerable activity in the lignin portions of these plants.

The experimental results to be reported here show that shikimic acid, without any rearrangement of the carbon atoms of its six-membered ring, is also to be considered as a precursor of the aromatic rings of the lignin building stones, and, accordingly, that this transformation parallels the



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