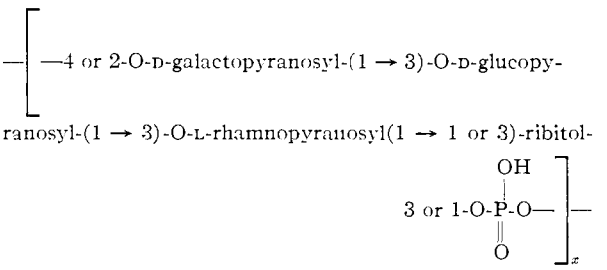
b.[43] Mild alkaline treatment renders all three substances serologically inactive and susceptible to attack by phosphatase. The action of alkali on polyribophosphate was shown to result in dialyzable, non-reducing, chromatographically homogeneous fragments. Alkali-treated S VI likewise consisted of similar fragments which no longer separated upon the addition of alcohol, failed to precipitate antiserum and showed a decreased viscosity. The titration curve and pK' of the fragments resembled those of a phosphate monoester[40] and existence of this linkage was confirmed by the action of phosphatase.

Periodate oxidation of S VI suggested that the glucose and rhamnose were joined in 1,3-like linkages. The liberation of formaldehyde indicated that a –CHOH-CHOH group was present. Since formic acid was not produced, S VI has very few end groups, while the simultaneous disappearance of the galactose indicated that this sugar is bound in 1,4- or 1,2- linkage. After alkaline hydrolysis of S VI, an additional mole of $NaIO_4$ was consumed and one mole of formic acid was released, with no change in the amount of formaldehyde. This would be expected if the 2- or 4-OH of galactose were set free by alkaline hydrolysis, as, for example, in cleavage of the phosphate diester, S VI. The production of formaldehyde might come from oxidation of a 1,3-linked ribitol.

The following formula is tentatively proposed for the structure of S VI



The ribitol phosphate residue might have either the D- or L-configuration.

Structural studies are continuing and these indicate the correctness of the order given to the glucose and rhamnose residues.

NEW BRUNSWICK, N. J.

(43) S. Zamenhof, G. Leidy, P. L. Fitzgerald, H. E. Alexander and E. Chargaff, *J. Biol. Chem.*, **203**, 695 (1953).

[CONTRIBUTION FROM THE PRAIRIE REGIONAL LABORATORY, NATIONAL RESEARCH COUNCIL OF CANADA]

Studies of Lignin Biosynthesis Using Isotopic Carbon. VIII. Isolation of Radioactive Hydrogenolysis Products of Lignin[1]

BY STEWART A. BROWN AND A. C. NEISH

RECEIVED DECEMBER 8, 1958

After the feeding of several C^{14}-labeled compounds to wheat and maple, degradation of lignin by hydrogenolysis led to the isolation of dihydroconiferyl and dihydrosinapyl alcohols. The radioactivity of these products was taken to indicate the degree of incorporation of the administered compounds into lignin. The pattern revealed was very similar to that previously found when the activity of the oxidative degradation products, vanillin and syringaldehyde, was used, reinforcing the validity of conclusions from the oxidative degradation. However, labeled sinapic acid, which is converted to lignin yielding syringaldehyde, did not form lignin degradable to dihydrosinapyl alcohol. The active syringaldehyde was shown not to arise from sinapic acid bound to lignin with alkali-labile linkages. Further evidence was obtained that vanillin can be incorporated into lignin, probably by condensation with a two-carbon metabolite. Acetic acid was converted only slightly to the lignin giving rise to hydrogenolysis products; hence the Birch–Donovan acetate pathway does not contribute significantly to lignification.

Introduction

Previous papers of this series[2] have reported comparisons of the efficiencies with which several plant species can convert numerous C^{14}-labeled compounds to lignin. Although the criterion of the conversion efficiency should, in theory, be the specific activity of an intact lignin, it is very doubtful whether such a compound has ever been isolated, and the ill-defined nature and variable composition of lignins isolated by standard methods have made this approach, in our opinion, impractical. As an alternative we have turned to lignin degradation products of low molecular weight which have known structures and either are crystalline or form crystalline derivatives. The use of these degradation products enables a direct comparison of specific radioactivities on a molar basis between the administered compound and the lignin (as represented by its derivative).

This alternative approach, too, suffers from certain disadvantages. There is no known reaction by which lignin can be degraded in anything approaching quantitative yield to products of known structure. The best reaction in this respect is nitrobenzene oxidation in alkaline medium, which yields phenolic aldehydes: vanillin, syringaldehyde and *p*-hydroxybenzaldehyde. It is this reaction which we have used in our studies to date. If one accepts the theory that the unit of the lignin polymer contains nine skeletal carbon atoms, the isolation of substituted benzaldehydes as lignin degradation products has the additional drawback that only seven-ninths of the unit is available for study, the other two carbon atoms being lost in the

(1) Presented in part at the Eighth Annual Research Conference on Plant Physiology, Hamilton, Ontario: November, 1957. Issued as N.R.C. No. 5147. For Part VII of this series see reference 2e.

(2) (a) S. A. Brown and A. C. Neish, *Nature*, **175**, 688 (1955); (b) *Can. J. Biochem. Physiol.*, **33**, 948 (1955); (c) **34**, 769 (1956); (d) D. Wright, S. A. Brown and A. C. Neish, *ibid.*, **36**, 1037 (1958); (e) S. A. Brown, D. Wright and A. C. Neish, *ibid.*, **37**, 25 (1959).

oxidation. In addition to providing an incomplete picture, the recovery of these phenolic aldehydes makes necessary the labelling of phenylpropanoid precursors in either the ring or the adjacent carbon of the side chain, normally a more difficult task than the introduction of carbon-14 into either of the other two side chain carbons.

In earlier work we have made the assumption, which appears to be generally accepted, that vanillin and syringaldehyde have a unique origin in the guaiacyl and syringyl lignin residues, respectively. If this assumption is valid, the measurement of the radioactivity of these aldehydes provides a relatively rapid and accurate way to determine the incorporation of carbon-14 into seven of the nine skeletal carbons of the lignin units. In the case of *p*-hydroxybenzaldehyde such an assumption was made only with distinct reservations, because it had been established that, under the conditions of the oxidation, tyrosine can give rise to this product.[3] Recent studies[2e] have confirmed that bound tyrosine can account for much, if not all, of the *p*-hydroxybenzaldehyde from oxidation of the cell wall residue obtained by the standard extraction procedure.

It has been pointed out[4,5] that in biosynthetic studies the use of phenolic aldehydes to represent lignin is, in itself, insufficient. Clearly, it is desirable to obtain confirmation of the results obtained by this method, and this is possible by the isolation of other types of degradation product. Freudenberg and his co-workers have obtained such confirmation by isolating phenylpropanoid ethanolysis products of lignin after the administration of C^{14}-labeled phenylalanine[6a] and ferulic acid.[6b] In addition, phenylalanine was shown[7] to be used by spruce for synthesis of coniferin, which is a lignin precursor.[5,6a,8] On the other hand, Freudenberg[6b] has reported that vanillin-C^{14} glucoside fed to spruce became incorporated into an insoluble fraction of the wood which did not, however, yield radioactive ethanolysis products. This contrasts with the finding, in this Laboratory, that free vanillin can be incorporated to an appreciable extent into the phenolic aldehyde-yielding lignin structures of wheat.[2b]

Since more complete confirmation of the earlier findings was wanted, yet another degradative reaction has been employed for comparative purposes. The present paper reports comparisons of the specific activity found for vanillin and syringaldehyde with those of the analogously substituted products of lignin hydrogenolysis: dihydroconiferyl alcohol (DHCA) and dihydrosinapyl alcohol (DHSA), isolated after the administration of a series of C^{14}-labeled compounds to maple and wheat. In several cases the specific activities of the phenolic aldehydes and isolated lignins have also been compared.

(3) J. E. Stone, M. J. Blundell and K. G. Tanner, *Can. J. Chem.*, **29**, 734 (1951).

(4) K. Freudenberg, *Ind. Eng. Chem.*, **49**, 1384 (1957).

(5) K. Kratzl and G. Billek, *Tappi*, **40**, 269 (1957).

(6) K. Freudenberg, *Angew. Chem.*, **68**, 84 (1956); (b) **68**, 508 (1956).

(7) K. Freudenberg and F. Niedercorn, *Chem. Ber.*, **91**, 591 (1958).

(8) K. Kratzl and G. Hofbauer, *Monats. Chem.*, **89**, 96 (1958).

Experimental

Cultivation of the Plants.—Wheat plants (*Triticum vulgare* Vill. var. Thatcher) were grown in gravel culture as described previously.[2b] Maple twigs (*Acer negundo* L. var. *interius* Sarg.) of the current season's growth were collected from the wild state.

C^{14}-Labeled Compounds.—L-Phenylalanine-R-C^{14} (randomly labeled) and the sodium salt of acetic acid-2-C^{14} were purchased from Atomic Energy of Canada, Ltd., Ottawa. Syntheses of other labeled compounds have been reported in previous papers.[2b,c]

Administration of Labeled Compounds.—As in the earlier experiments, the labeled compounds (as the sodium salts in the case of carboxylic acids and phenols) were administered as aqueous solutions through the cut ends of the stems and the plants allowed to metabolize 24 hr. in the light before harvesting.

Isolation and Extraction of Plant Material.—The plants were cut into small pieces and blended with a convenient volume of water in either a VirTis homogenizer or a Waring blendor. The homogenate was filtered through Nylon bolting cloth, which passed the disintegrated cytoplasmic material while retaining most of the cell wall fibers. The homogenization and filtration was repeated and the residue washed a third time by suspension in water. The residue was homogenized with 0.02 *N* sodium hydroxide, washed twice by suspension in water and then suspended successively in 80% ethanol, absolute ethanol (twice for maple) and ether. The resulting cell wall fraction from maple was nearly white but that from wheat retained a yellow color. This was removed by the peptic digestion described by Crampton and Maynard[9] to which the residue from both species was subjected.

Hydrogenolysis of the Cell Wall Residue.—The techniques used in the present work for the hydrogenolysis of lignin and isolation of the products are those described by Brewer, Cooke and Hibbert,[10] adapted to a semi-micro scale. The principal modification has been the introduction of chromatographic procedures not available to the earlier workers.

In a typical run the purified cell wall residue, weighing 5 to 7 g., was transferred to a high pressure hydrogenation bomb with about twelve times its weight of dioxane–water (1 + 1) containing 0.1% hydrogen chloride. The use of this solvent gave yields comparable to those obtained by us using ethanol–water, and possible complications arising through the formation of ethoxy groups[11] were avoided. After the addition of about 10 g. of settled Raney nickel suspension,[12] the bomb was filled with hydrogen to an initial pressure of 100 atmospheres and heated for 4 hr. at 160–165°.

Isolation of Hydrogenolysis Products.—The reaction mixture, after cooling, was filtered and the residue washed with 50% dioxane. The mixture of filtrate and washings was concentrated in a rotary film evaporator until quite turbid and then transferred to a continuous liquid–liquid extractor, where a chloroform extraction was carried out for *ca.* 7 hr. Extraction in a separatory funnel invariably led to the formation of extremely intractable emulsions. The chloroform extract was washed with a few milliliters of 5% sodium bicarbonate solution and then extracted with 0.2 *N* sodium hydroxide. The extract, containing the phenolic fraction, was acidified with hydrochloric acid and back-extracted into chloroform. Evaporation of the chloroform gave a light brown oil.

A 7.5-g. lot of acid-washed diatomaceous earth (Celite 535) was mixed with 6.2 ml. of water, the mixture was slurried with *n*-butyl ether (alkali-washed and distilled from sodium) and packed with a plunger in a column 2 cm. in diameter. Above this was packed 1.5 g. of anhydrous Celite 535 slurried in *n*-butyl ether. As much as possible of the chloroform-soluble oil was taken up in a total of 1.2 ml. of warm water, and the solution was pipetted on to the top of the column. Development was carried out with water-saturated *n*-butyl ether, and thirty 5-ml. fractions were

(9) E. W. Crampton and L. A. Maynard, *J. Nutrition*, **15**, 383 (1938).

(10) C. P. Brewer, L. M. Cooke and H. Hibbert, THIS JOURNAL, **70**, 57 (1948).

(11) J. M. Pepper and H. Hibbert, *ibid.*, **70**, 67 (1948).

(12) R. Mozingo, "Organic Syntheses," Coll. Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1955, p. 181.

TABLE I

BIOSYNTHESIS OF LIGNIN FROM C^{14}-LABELED COMPOUNDS AS MEASURED BY ISOLATION OF BOTH OXIDATION AND HYDROGENOLYSIS PRODUCTS

Species	Compound administered: Name	Specific activity (μc./mmole)	Dose (μmole/g. dry wt.)	Specific activity of lignin degradation products (μc./mmole): V[b]	S[b]	DHCA	DHSA	Dilution[a] (Cor. for dry wt. of plants): V[b]	S[b]	DHCA	DHSA
Maple	L-Phenylalanine-R-C^{14}	21.6	9.9	0.242	0.125	...	...	89	173	....	..
	Cinnamic acid-3-C^{14}	31.4	12.6	.255	.050	...	...	157	805	....	..
	Sinapic acid-3-C^{14}	31.4	12.9	.055	.875	...	...	745	47	....	..
	L-Phenylalanine-R-C^{14}	50.0	7.3	1.01	.771	...	...	49.5	64.9	....	..
	Anisic acid-C^{14} OOH	50.0	11.4	0.002	.003	..	...	39,000	23,000	....	..
	Acetic acid-2-C^{14}	50.0	12.8	.026	.037	...	...	3,420	2,350	....	..
	Vanillin C^{14} HO	50.0	12.8	.204	.195	...	...	430	450	....	..
	L-Phenylalanine-R-C^{14}	63.7	48	...	...	0.570	0.272	....	....	110	230
	Cinnamic acid-2-C^{14}	63.7	44	...	...	.340	.111	....	....	180	530
	Sinapic acid-3-C^{14}	63.7	36	...	...	.010	.011	....	....	5000	4400
	Anisic acid-C^{14} OOH	63.7	49	...	...	.003	.010	....	....	25,000	5500
	Acetic acid-2-C^{14}	63.7	44	...	...	.011	.015	....	....	5600	3800
	Vanillin-C^{14} HO	63.7	..	...	...	.025	.032	....	....	2300	1800
Wheat	L-Phenylalanine-R-C^{14}	43	33.4	0.697	.715	...	...	62	60	....	..
	Cinnamic acid-3-C^{14}	94	19.6	0.534	.564	...	...	164	159	....	..
	Ferulic acid-3-C^{14}	87	17.7	1.920	.060	...	...	41	1,330	....	..
	L-Phenylalanine-R-C^{14}	39.4	6.1	0.444	.620	...	...	89	64	....	..
	Acetic acid-2 C^{14}	55.4	6.1	0.003	.005	...	...	20,000	10,000	....	..
	L-Phenylalanine-R-C^{14}	74.0	25.5	...	...	3.38	4.97	....	....	22	15
	Cinnamic acid-2-C^{14}	74.0	26.0	...	...	1.54	...	....	....	49	..
	Ferulic acid-3-C^{14}	74.0	22.9	...	...	4.14	2.97	....	....	13.5	22.5
	Acetic acid-2-C^{14}	74.0	24.1	...	...	0.033	...	....	....	2200	..

[a] Uncorrected dilution equals value in column 4 divided by the value in column 6, 7, 8, or 9. [b] V = vanillin, S = syringaldehyde.

collected. These were tested for phenols by spotting small aliquots of each on paper and spraying with a ferric ferricyanide solution.[13] Dihydroconiferyl alcohol (DHCA) appeared between fractions 6 and 11, and dihydrosinapyl alcohol (DHSA) followed in a wider band beginning about fraction 15. The chromatographic behavior of these two compounds was checked with authentic samples. The appropriate fractions were combined and the solvent evaporated. Each residue, after brief heating *in vacuo* to remove volatile impurities, was dissolved in chloroform and the solution washed with 5% sodium bicarbonate. Extraction of the phenol into 0.2 *N* sodium hydroxide, acidification and back-extraction into chloroform followed. Removal of the solvent yielded 6 to 12 mg. of crude product in each case, with the DHCA usually predominating.

The bis-*p*-nitrobenzoates of these two compounds were prepared by the procedure of Kawai and Sugiyama[14] for DHCA. The crude product, after removal of excess *p*-nitrobenzoic acid, was taken up in a small volume of benzene and passed through a 1 × 6 cm. column of alumina which had been deactivated by washing with methanol. The progress of the rapidly moving ester bands was easily followed by their strong absorption in the near ultraviolet. Upon removal of the benzene the esters crystallized readily. They were washed with ethanol and recrystallized in microcentrifuge tubes from benzene–ethanol until a melting range no wider than 1.5° resulted. There was no depression of melting point when mixed with authentic samples. Lack of material prevented adoption of the more rigorous criterion of recrystallization to constant specific activity.

DHCA-bis-*p*-nitrobenzoate melted at 120–121° (cor.). The reported m.p. is 121–121.5°.[14]

DHSA-bis-*p*-nitrobenzoate melted at 164.5–166° (cor.).

Anal. Calcd. for $C_{25}H_{22}O_{10}N_2$: C, 58.82; H, 4.34; N, 5.49. Found: C, 59.55, 58.96; H, 4.31, 4.37; N, 5.42.

Nitrobenzene Oxidations.—The preparation of cell wall residues by extraction of plant material with ethanol–benzene and water, oxidation of these with nitrobenzene, and the isolation of derivatives of the vanillin and syringaldehyde so obtained have been described in previous publications of this series.[2b,15]

Preparation of Lignins.—Klason 72% sulfuric acid lignin was prepared as outlined by Hägglund.[16] The preparation of dioxane lignin was essentially that of Pepper, *et al.*[17] In each case the plant material was prepared as in the section above on "Isolation and extraction of plant material."

Saponification of Maple Residues.—A 2-g. sample of the extracted residue was refluxed for 1 hr. under nitrogen with a solution of 1 g. of potassium hydroxide in 60 ml. of absolute ethanol. The filtrate was evaporated *in vacuo*, the residue taken up in water and the solution acidified with hydrochloric acid. The resulting mixture was continuously extracted with ether until all suspended material had dissolved, and the ether solution extracted with 5% sodium bicarbonate solution. Acidification of this extract and back-extraction into ether gave an acid fraction, which was chromatographed on Whatman No. 1 paper with toluene–acetic acid–water.[18] The phenolic acid spots were made visible by spraying with diazotized *p*-nitroaniline reagent.[19]

Measurement of Radioactivity.—The method of determining carbon-14 in organic samples and barium carbonate has been outlined in a previous publication.[2b]

Results and Discussion

In earlier publications a variety of labeled compounds have been compared in wheat and maple as precursors of the structures giving rise to vanillin and syringaldehyde; these precursors have included both C_6,C_3 and C_6,C_1 compounds. In Table I a number of these precursors, together with acetic acid, have been listed. Here are given the dilutions of carbon-14 during conversion of a com-

(13) G. M. Barton, R. S. Evans and J. A. F. Gardner, *Nature*, **170**, 249 (1952).

(14) S. Kawai and N. Sugiyama, *Ber.*, **72**, 367 (1939).

(15) S. A. Brown, K. G. Tanner and J. E. Stone, *Can. J. Chem.*, **31**, 755 (1953).

(16) E. Hägglund, "Chemistry of Wood," Academic Press, Inc., New York, N. Y., 1951, p. 326.

(17) J. M. Pepper, P. E. T. Baylis and E. Adler, unpublished data, in preparation.

(18) E. C. Bate-Smith, *Chemistry & Industry*, 1457 (1954).

(19) T. Swain, *Biochem. J.*, **53**, 200 (1953).

TABLE II

COMPARISON OF RELATIVE DILUTIONS OF LABELED PRECURSORS DURING CONVERSION TO OXIDATIVE AND REDUCTIVE DEGRADATION PRODUCTS OF LIGNIN

Compound administered	Relative dilutions[a]					
	Oxidation products				Hydrogenolysis products	
	Vanillin		Syringaldehyde		DHCA	DHSA
			1. Maple			
L-Phenylalanine-R-C^{14}	1	1	1.9	1.3	1	2.1
Cinnamic acid-(2 or 3)-C^{14}	1.8		9.1		1.6	4.8
Sinapic acid-3-C^{14}	8.4		0.53		45	40
Anisic acid-C^{14} OOH		790		465	230	50
Vanillin-C^{14} HO		8.7		9.1	21	16
Acetic acid-2-C^{14}		69		48	51	35
			2. Wheat			
L-Phenylalanine R-C^{14}	1	1	0.97	0.72	1	0.68
Cinnamic acid-(2 or 3) C^{14}	2.6		2.6		2.2	...
Ferulic acid-3-C^{14}	0.66		21		0.61	1.0
Acetic acid-2-C^{14}		200		100	100	...

[a] The dilution of L-phenylalanine in its conversion to that part of lignin yielding vanillin and DHCA is taken as unity, and other dilutions are calculated on this basis.

pound to the structures which give rise to vanillin and syringaldehyde on oxidation and to DHCA and DHSA on hydrogenolysis. Some of the data from experiments in which vanillin and syringaldehyde were isolated have been published elsewhere and are reprinted in the interests of convenience. The dilutions calculated here are the basis of the relative dilutions shown in Table II. Because the data of several separate experiments have been pooled and compared in Table II, we have taken as unity the dilution of carbon-14 in the conversion of L-phenylalanine to the vanillin- and DHCA-yielding residues (boldfaced in Table I) and recalculated all other dilutions on this basis. This has been possible because in each experiment L-phenylalanine has been fed as a standard of comparison. Under the "V" and "S" headings of Table II, for both wheat and maple, two columns of figures have been used to group compounds which were compared in experiments done at the same time.

It was not to be expected that the results of experiments done at different times, and using different plants, would be strictly comparable, because of the well-known effects of biological variation. Nevertheless, a comparison of the "V" and "DHCA" columns and the "S" and "DHSA" columns reveals a clear parallelism in each case. This is especially striking where cinnamic acid and acetic acid were fed to both plants and in the conversion of ferulic acid to vanillin- and DHCA-yielding lignin; the comparable figures do not differ by more than a factor of 2 and in some cases are virtually identical. When anisic acid was administered, much greater differences in the dilution values resulted, but these values are so high that what little carbon-14 there is in the lignin derivatives probably represents recycling rather than direct incorporation.

Serious discrepancies are evident, however, after feeding ferulic and sinapic acids to wheat and maple, respectively. If the activity of the phenolic aldehydes is taken as the criterion, ferulic acid was not nearly so well converted to syringyl as to guaiacyl lignin, but this relationship does not hold when the activity of the hydrogenolysis products is considered. In the latter instance, the ferulic acid was efficiently utilized for forming the syringyl lignin residues as well. Sinapic acid was preferentially converted by maple to lignin yielding syringaldehyde, but little vanillin, yet when DHCA and DHSA were isolated after feeding sinapic acid, neither product contained appreciable radioactivity.

It seemed probable that at least a part of the above discrepancies could have resulted from the existence of uncontrolled variables, which led to marked quantitative variations in the metabolism of the administered compounds in experiments done at different times. To confirm or eliminate this possibility, ferulic and sinapic acids were fed to a larger number of maple twigs, and the C^{14}-contents of the products from the oxidation and hydrogenolysis of the same plant material were compared. The results of this experiment are shown in Table III. After the administration of ferulic

TABLE III

SPECIFIC ACTIVITIES OF LIGNIN DEGRADATION PRODUCTS FROM THE OXIDATION AND HYDROGENOLYSIS OF ALIQUOTS OF THE SAME PLANT MATERIAL

Compound administered	Compound isolated	
	Name	Specific activity (μc. mmole)
Ferulic acid-3-C^{14}	DHCA	0.869
	Vanillin-NBH[a]	.570
	DHSA	.238
	Syringaldehyde-NBH[a]	.144
Sinapic acid-3-C^{14}	DHCA	.010
	Vanillin-NBH[a,b]	.000
	DHSA	.010
	Syringaldehyde-NBH[a,b]	.971

[a] NBH = *m*-nitrobenzoylhydrazone. [b] These aldehydes were isolated from the residue after saponification of cell wall fraction (see text).

acid-3-C^{14} the molar specific activities of the isolated vanillin and syringaldehyde were about 35 to 40% lower than those of DHCA and DHSA, respectively. These results suggest that the lignin structures from which the oxidation products are formed do not fully correspond to those which give rise to the products of hydrogenolysis. The fact that the yields of the aldehydes are the higher may mean that a greater part of the pre-formed, inactive

lignin is attacked by the oxidant, with consequent dilution of the carbon-14. This would not be surprising in view of the fundamental difference in the two types of reaction, and the values are sufficiently alike that there is no reason to suspect either as a valid criterion of the incorporation of carbon-14 into lignin.

Such is definitely not the case in the experiments involving sinapic acid. Here, the molar specific activity of the syringaldehyde exceeded that of DHSA by nearly 100 times. The sinapic acid results are in agreement with earlier work, both as regards the preferential incorporation of sinapic acid into the syringyl-type lignin as represented by syringaldehyde, and the discrepancy in the specific activities of the oxidative and hydrogenolytic lignin degradation products. A test revealed that syringaldehyde could be formed in about 15% yield by nitrobenzene oxidation of sinapic acid itself under the standard conditions. Therefore, a possible explanation of the observed discrepancy was that the administered sinapic acid was merely being attached to lignin in such a way that oxidation, but not hydrogenolysis, could yield a radioactive product.

Conceivably, an attachment by ester linkages, such as described by Smith[20] in the cases of *p*-hydroxycinnamic, ferulic and some other acids, could explain the finding. The possibility was tested by saponification of a sample of the cell wall residue from the sinapic acid feeding and separation by chromatography of the phenolic acids liberated. Spots corresponding to ferulic acid and smaller amounts of other phenolic acids were easily detectable, but no sinapic acid spot was found. A test on an inactive sample of maple showed that at least half of one milligram of added sinapic acid was recoverable and could be detected easily on the chromatogram, by the technique used. From these results it was concluded that sinapic acid bound as the ester or in other alkali-labile form constituted at most a negligible part of lignin. As further confirmation, oxidation of the saponification residue with nitrobenzene yielded syringaldehyde with a specific activity comparable to that of syringaldehyde from the aliquot oxidized before saponification. It is the specific activities from the saponified sample which are given in Table III.

These findings leave unanswered the question of why sinapic acid, alone among the several phenylpropanoid compounds judged to be efficient precursors of lignin on the basis of oxidative degradation, is not incorporated into lignin structures which can be reduced to a substituted cinnamyl alcohol. The role of sinapic acid as a lignin precursor is evidently more complex than was hitherto believed. Although there is little doubt that it can be incorporated into a lignin polymer, more study of its function is clearly necessary.

One or two additional points deserve comment in considering the results of the hydrogenolysis experiments. After the feeding of vanillin to maple, the specific activities of DHCA and DHSA indicated a dilution of carbon-14 only 8 to 20 times greater than was the case when L-phenylalanine was fed. Although these dilutions are relatively high, they are much lower than obtained with anisic acid, or even acetate. This result shows that, in this species at least, vanillin can be incorporated into the phenylpropanoid unit and provides further support for our previous proposal[2b] that such a unit can be formed in the plant by the condensation of vanillin with a two-carbon metabolite. Freudenberg's failure to demonstrate this reaction in spruce by the isolation of Hibbert's ethanolysis products may be explicable on the basis of species differences, a number of which we have shown to exist in lignification reactions. On the other hand, it should be remembered that Freudenberg's group employed the glucoside of vanillin, and this may well exhibit very different reaction behavior from that of the free phenol. Support for this explanation comes from Freudenberg's finding[6b] that free ferulic acid-C^{14}, but not its glucoside, is incorporated into a lignin which yields radioactive ethanolysis products.

Because of the tedious purification procedures and low yields, hydrogenolysis offers a doubtful alternative to oxidation for the routine recovery of lignin degradation products after feeding experiments. The value of nitrobenzene oxidation has now been placed on an even firmer basis, and if the number of feedings to be done is large, it would appear more economical to label the precursor in such a position that the carbon-14 is recoverable by the oxidation method. When all nine skeletal carbons must be recovered, hydrogenolysis is a possible alternative to ethanolysis. But the hydrogenolysis products are less amenable to further degradation, a procedure which has not proved feasible with the amounts we have been able to isolate.

Further investigation of the possible role of acetate in lignification has involved the isolation of two forms of lignin, and the comparison of their C^{14}-content with that of vanillin and syringaldehyde from the same plant residue. L-Phenylalanine and cinnamic acid have also been studied in this way. The results are shown in Table IV. In calculating the molar specific activity of the Klason and dioxane lignins from the specific activity of the carbon dioxide obtained by their combustion, it has been necessary to assume an average size of the monomeric unit. This has been taken as $C_{10.5}$, the average of the 10-carbon coniferyl and the 11-carbon sinapyl alcohol units which are believed to make up most of lignin.

The molar specific activities of the isolated lignins from the phenylalanine and cinnamic acid feedings, if calculated in this way, are found to be somewhat lower than the average molar specific activity of the vanillin and syringaldehyde. This suggests the presence of some non-radioactive extraneous matter associated with the isolated lignins. Analysis showed the nitrogen content of the dioxane lignin to be about 0.2% in wheat and up to 0.67% in maple; the Klason lignin of maple analyzed about 1.4% nitrogen. Part of the extraneous material, therefore, likely consisted of denatured protein and humin.

In contrast, the data from the acetate feeding experiments show that the molar specific activities calculated for the Klason and dioxane lignins are much greater than those of the phenolic aldehydes; the discrepancy is especially marked in wheat.

(20) D. C. C. Smith, *Nature*, **176**, 267 (1955); *J. Chem. Soc.*, 2347 (1955).

TABLE IV

RADIOACTIVITY OF ISOLATED LIGNINS AND LIGNIN OXIDATION PRODUCTS AFTER ADMINISTRATION OF LABELED PHENYLALANINE, CINNAMIC ACID AND ACETATE TO WHEAT AND MAPLE

Compound administered[a]	Material recovered: Name	Sp. activity ($m\mu c./mmole$ CO_2): Wheat	Maple	Sp. activity of lignin monomer ($m\mu c./mmole$): Wheat	Maple
L-Phenylalanine-R-C^{14}	Klason lignin	42.1	21.1	442[b]	222[b]
	Dioxane lignin	38.9	16.3	409[b]	171[b]
	Vanillin-NBH[d]	29.6	23.0	570[c]	444[c]
	Syringaldehyde-NBH[d]	28.8	10.7	800[c]	220[c]
Cinnamic acid-3-C^{14}	Klason lignin	30.9	..	324[b]	..
	Dioxane lignin	25.5	..	268[b]	..
	Vanillin-NBH[d]	20.9	..	314	..
	Syringaldehyde-NBH[d]	28.8	..	461	..
Acetic acid 2-C^{14}	Klason lignin	38.1	7.1	400[b]	75[b]
	Dioxane lignin	5.7	12.0	60[b]	126[b]
	Vanillin-NBH[d]	0.17	0.82	2.6	12
	Syringaldehyde-NBH[d]	0.33	1.22	5.3	19.5

[a] Wheat plants (equivalent to *ca.* 4.5 g. dry weight) were fed 4.0 μc. carbon-14 in 0.079 mmole of the compound. Maple twigs (dry wt. *ca.* 7.5 g.) received 6.5 μc. carbon-14 in 0.10 mmole. [b] Calculated on the basis of the average number of carbon atoms per lignin molecule equalling 10.5 (see text). [c] Results are multiplied by 9/7 to correct for loss of two skeletal carbons during oxidation. [d] NBH ≡ *m*-nitrobenzoylhydrazone.

Two possible explanations suggest themselves for this. It could have been due to a $C_6,C_1 + C_2$ condensation incorporating carbon-14 into carbons 1 and 2 of the side chain, which were then lost in the oxidation but recovered as isolated lignin. However, after feeding acetate to both species virtually inactive DHCA and DHSA were isolated (Table I), and as these contain all nine skeletal carbons, the above possibility was eliminated. The alternative explanation is that acetate has been incorporated into some artifact associated with the isolated lignins, which does not yield vanillin and syringaldehyde. These results underline the difficulties involved if isolated lignins are employed in lignification experiments.

Caution is needed when comparing the degree of utilization of such different compounds as acetate and substituted phenylpropanes. There may be considerable difference in pool sizes, of course, and feeding of equivalent amounts of the two types of compound has been shown in this Laboratory[21] to yield data which may not be closely comparable. But even allowing for this, the present results indicate that incorporation of acetate into the isolable aromatic ring or side chain of lignin is slight

Acknowledgments.—Microanalyses reported here were done by Mr. J. A. Baignée and analyses of carbon-14 by Mr. John Dyck. The technical assistance of Mr. J. P. Shyluk throughout the investigation is gratefully acknowledged.

(21) J. E. Watkin and A. C. Neish, unpublished data.

SASKATOON, SASK., CANADA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND DEPARTMENT OF BOTANY, UNIVERSITY OF CALIFORNIA, LOS ANGELES]

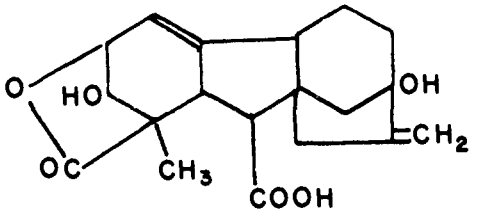
Gibberellins from Flowering Plants. I. Isolation and Properties of a Gibberellin from *Phaseolus vulgaris* L.[1]

BY CHARLES A. WEST AND BERNARD O. PHINNEY

RECEIVED OCTOBER 13, 1958

A procedure is described for the isolation of a gibberellin, bean factor II, from acetone–water extracts of immature bean seed (*Phaseolus vulgaris* L.). Bean factor II can be distinguished from the fungal gibberellins, gibberellic acid, gibberellin A_1 and gibberellin A_2, on the basis of its differential biological activity for dwarf maize mutants *dwarf-1* and *dwarf-5*, its chromatographic behavior and its infrared spectrum. Preliminary chemical studies suggest that bean factor II has a carboxylic acid and a lactone functional group, as well as sites of unsaturation not in conjugation with either of these.

The gibberellins, which are potent plant growth regulators,[2] were isolated as a crude crystalline mixture from culture filtrates of the vegetative stage of the fungus *Gibberella fujikuroi* (Saw.) Wr. by Yabuta and Sumiki in 1938.[3] Since that time four different gibberellins have been isolated in the pure state from that source, either as a free acid or methyl ester: gibberellic acid ($C_{19}H_{22}O_6$);[4] gibberellin A (gibberellin A_1)($C_{19}H_{24}O_6$)[5]; gibberellin A_2 ($C_{19}H_{26}O_6$);[6] and gibberellin A_4 ($C_{18}H_{22}O_5$) or $C_{19}H_{24}O_5$).[7] A structural formula has been assigned to gibberellic acid by Cross, *et al.*[8] Gibberellin



(1) This project was supported in part by grants from Merck and Co., Inc., National Science Foundation (Grant 3526), and Research Corporation.

(2) B. B. Stowe and T. Yamaki, *Ann. Rev. Plant Physiol.*, **8**, 181 (1957).

(3) T. Yabuta and Y. Sumiki, *J. Agr. Chem. Soc. Japan*, **14**, 1526 (1938).

(4) P. J. Curtis and B. E. Cross, *Chem. Ind. (London)*, 1066 (1954).

(5) F. H. Stodola, K. B. Raper, D. I. Fennell, H. F. Conway, V. E. Sohns, C. T. Langford and R. W. Jackson, *Arch. Biochem. Biophys.*, **54**, 240 (1954); F. H. Stodola, G. E. N. Nelson and D. J. Spence, *ibid.*, **66**, 438 (1957).

(6) N. Takahashi, H. Kitamura, A. Kawaranda, Y. Seta, M. Takai, S. Tamura and Y. Sumiki, *Bull. Agr. Chem. Soc. Japan*, **19**, 267 (1955).

(7) N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, *ibid.*, **21**, 396 (1957).

(8) B. E. Cross, J. F. Grove, J. MacMillan and T. P. C. Mulholland, *Proc. Chem. Soc.*, 221 (1958).