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ALKALINE OXIDATION OF ^{14}C-LABELLED PROTOLIGNIN, FORMED FROM CINNAMIC ACID IN SPRUCE AND ASPEN TWIGS*

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Abstract—Cinnamic acid-^{14}C was incorporated into the lignin of spruce twigs but not appreciably into proteins or carbohydrates. Advantage was taken of this fact to study the alkaline copper oxidation of spruce protolignin without separation from other insoluble plant constituents. Cinnamic acid-[COOH]-^{14}C (carboxyl-labelled) fed to spruce cuttings formed lignin which on oxidation gave about 16% of the isotopic carbon as carbon dioxide and 28% as formic acid whereas cinnamic acid-α-^{14}C lignin gave 23% as carbon dioxide and 4·5% as formic acid. Cinnamic acid-ring β-^{14}C lignin gave 4·3% of ^{14}C as carbon dioxide and 5·4% as vanillin. Similar results were obtained with aspen twigs, although they incorporated a small amount of ^{14}C into protein. The main results can be interpreted on the assumption that a guaiacyl glycerol ether unit is an important part of protolignin.

IN EXPERIMENTS on the biosynthesis of lignin from labelled precursors fed to plant cuttings it is customary to kill and extract the plant material with hot aqueous ethanol. The fibrous residue is then extracted with other neutral solvents to give an insoluble material composed mainly of lignin, polysaccharides and denatured proteins. Since only neutral solvents are used under mild conditions it may be assumed that the lignin has undergone little or no alteration and may be regarded as "protolignin."[1] There is no simple method for isolating the protolignin from this residue without serious chemical alteration. The preparation of milled wood lignin (Björkman lignin) is considered to be the best way to obtain relatively unaltered lignin.[1] However, studies on milled wood lignin have been restricted to a few woods and the procedure has not been applied to materials containing substantial amounts of protein. In addition it is a slow procedure requiring special apparatus found in only a few laboratories.

The difficulty of isolating an unaltered protolignin has been a serious handicap in structural studies. A method of circumventing this would be to label the lignin by administering to the plant a ^{14}C-labelled compound which would be incorporated into lignin, but not appreciably into carbohydrates or proteins. The extractive-free plant material obtained from such an experiment could be used to study the chemical degradation of protolignin without attempting its isolation, since the degradation products of carbohydrates and proteins would be unlabelled whereas those from lignin could be distinguished and measured by their radioactivity. Experiments of this type are reported in this paper. Cinnamic acid labelled with ^{14}C in the carboxyl group or in carbons α or β (Table 1) was fed to spruce or aspen cuttings and the

* Issued as N.R.C. No. 7996.

[1] F. A. BRAUNS and D. A. BRAUNS, *The Chemistry of Lignin*, Suppl. Vol., Chap. 5, Academic Press, New York and London (1960).

extractive-free residue subjected to alkaline cupric hydroxide oxidation in order to determine the fate of the terminal carbons of the phenylpropane units of lignin. The use of cinnamic acid for this purpose was suggested by previous experiments with wheat, which showed cinnamic acid was readily incorporated into lignin without randomization of the carbons,[2] but not into the aromatic amino acids.[3]

Table 1 shows that a considerable amount of the total activity was present in the residues of both aspen and spruce, and especially in those obtained from the latter, which contained

TABLE 1. ADMINISTRATION OF LABELLED CINNAMIC ACID TO SPRUCE AND ASPEN TWIGS

Species	Fresh wt. of twigs (g)	Position of label in cinnamic acid fed	Specific activity (μc/mM)	Amount fed (mg)	% of ^{14}C fixed in extractive-free residue*
Spruce	48	COOH	52·4	12·4	36·0
Aspen	41	COOH	52·4	13·0	19·1
Spruce	39	α-C	91·0	14·0	33·4
Aspen	39	α-C	91·0	14·3	22·5
Spruce	49	ring and β-C	92·5	15·1	34·4
Aspen	42	ring and β-C	92·5	13·8	27·4

* The insoluble material remaining after extraction with hot 80% ethanol, absolute ethanol and ether.

TABLE 2. DISTRIBUTION OF ^{14}C BETWEEN THE CONSTITUENTS OF THE EXTRACTED RESIDUES

Species*	% of total ^{14}C of the extractive-free residue found in†			
	Klason lignin	Holocellulose	Cellulose	Protein
Spruce	97	14·7	4·0	1·3
Aspen	86	10·9	2·2	13·1
Spruce	103	14·7	3·8	2·1
Aspen	76	9·4	2·9	16·0
Spruce	100	14·5	3·0	1·1
Aspen	92	11·4	2·5	4·3

* Experiments in same order as in Table 1.

† See text for details. Analyses for nitrogen showed the spruce residues contained about 6·5% protein and the aspen residues about 17%.

more than one-third of the administered isotope in each case. The amount of ^{14}C incorporated into the extractive-free residue by spruce was about the same no matter where the label was positioned as would be expected if the cinnamic acid was incorporated as a C_6–C_3 unit. Samples of these residues were subjected to various treatments (see Table 2) to establish whether or not the proteins and polysaccharides were ^{14}C-labelled. It can be seen that the Klason lignin isolated from the residues of spruce contained practically all of the ^{14}C. The

[2] S. A. BROWN and A. C. NEISH, *Canad. J. Biochem. Physiol.* **33**, 948 (1955).
[3] O. L. GAMBORG and A. C. NEISH, *Canad. J. Biochem. Physiol.* **37**, 1277 (1959).

amino acid fraction contained minor amounts of ^{14}C. The holocellulose, prepared by chlorite treatment, retained about 14% of the isotopic carbon. The cellulose prepared by mild acid hydrolysis of the holocellulose contained 3–4% of the ^{14}C, whereas a sample prepared by extraction of the holocellulose with 10% KOH retained only 0·6%. Furthermore, the hemicelluloses, precipitated by addition of alcohol to the acidified alkaline extract of

TABLE 3. DISTRIBUTION OF ^{14}C IN PRODUCTS FORMED BY ALKALINE COPPER OXIDATION OF SPRUCE EXTRACTIVE-FREE RESIDUES

	% of total ^{14}C from sample activated by		
Compound	Cinnamic acid [COOH]-^{14}C	-α-^{14}C	-ring,β-^{14}C
Carbon dioxide	15·6	22·9	4·3
Formic acid	28·0	4·5	0·7
Acetic acid	4·6	7·3	0·6
Vanillin	< 0·1	0·6	5·4
Dehydrovanillin	< 0·1	< 0·1	0·6
Total	48·2	35·3	11·6

holocellulose, on hydrolysis with 3% nitric acid yielded sugars which contained no radioactivity. It can be concluded, therefore, that the protolignin from spruce contains all of the activity isolated in the extractive-free residue.

The residues from aspen, on the other hand, have incorporated appreciable amounts of activity in the protein (see Table 2, Expt. No. 4), although no activity could be detected in the sugars from the polysaccharide fraction. The variability of the results obtained from different

TABLE 4. DISTRIBUTION OF ^{14}C IN PRODUCTS FORMED BY ALKALINE COPPER OXIDATION OF ASPEN EXTRACTIVE-FREE RESIDUES

	% of total ^{14}C from sample activated by cinnamic acid		
Compound	[COOH]-^{14}C	-α-^{14}C	-ring,β-^{14}C
Carbon dioxide	10·9	16·7	3·5
Formic acid	21·7	3·2	0·5
Acetic acid	4·3	4·9	0·2
Vanillin	< 0·1	< 0·1	7·0
Syringaldehyde	< 0·1	0·1	5·3
Total	36·9	24·9	16·5

aspen samples, however, suggests that although they were collected at the same time they may have different physiological ages, and might incorporate very little ^{14}C from labelled cinnamic acid into protein at a later stage of development. For example, sample No. 6 which incorporates the most ^{14}C into protolignin incorporates the least into protein.

Table 3 shows that when cinnamic acid ^{14}C-labelled in the carboxyl carbon or in the α-carbon is used, the carbon dioxide, formic acid, and acetic acid obtained by oxidation of the residues from spruce contained considerable amounts of ^{14}C. On the other hand when

cinnamic acid is ^{14}C-labelled on the β-carbon the corresponding oxidation products contained much less activity. Similar results were obtained with aspen (Table 4). It was established that formic acid is not oxidized to carbon dioxide, to any great extent, under the experimental conditions. A sample of formate-^{14}C (0·2 μmoles) added to an oxidation with unlabelled plant material showed only 0·8% oxidation to carbon dioxide.

It is interesting to note that the carboxyl carbon gives more formic acid than carbon dioxide whereas the reverse holds for the β-carbon. This is true for both species which gave almost identical *relative* yields of carbon dioxide and formic acid. Thus the ratio of the yields of CO_2/HCOOH from cinnamic acid-[COOH]-^{14}C was calculated from the data in Tables 3 and 4 to be 0·55 for spruce and 0·51 for aspen, and from cinnamic acid-α-^{14}C it was calculated

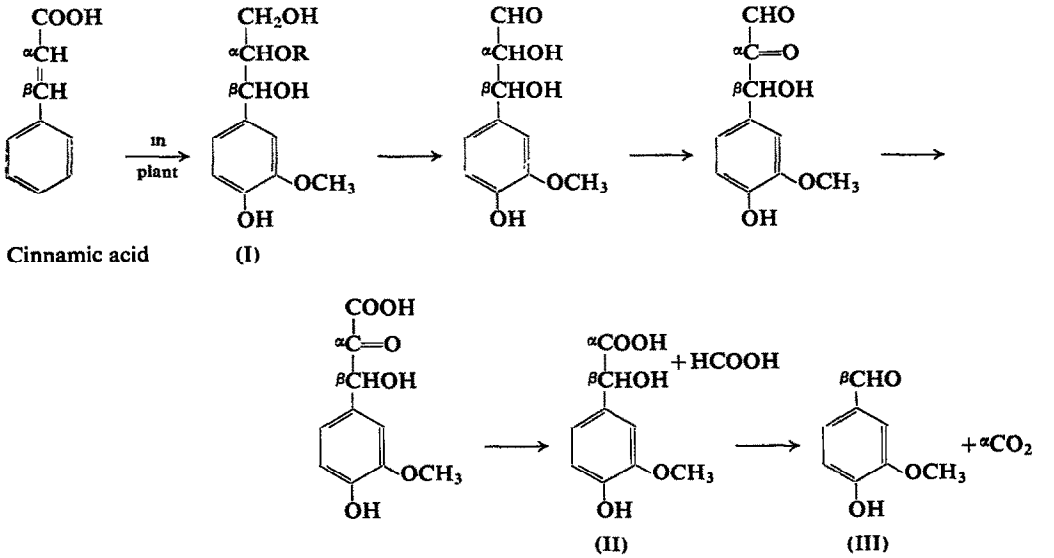

FIG. 1. POSSIBLE EXPLANATION FOR ORIGIN OF A MAJOR PART OF THE FORMIC ACID AND CARBON DIOXIDE OBTAINED BY ALKALINE OXIDATION OF PROTOLIGNIN.

to be 5·1 for spruce and 5·2 for aspen. This is a remarkable similarity considering the spruce has only guaiacyl lignin whereas the lignin of aspen contains both guaiacyl and syringyl groups. The total amount of ^{14}C recovered as CO_2 and HCOOH was greater from spruce being 43·6% from the COOH-carbon and 27·4% from the α-carbon as compared to recoveries of 32·6% and 19·9%, respectively, from aspen. These recoveries are much higher than recovery of the guaiacyl nucleus as vanillin. These results also support the belief that cinnamic acid gives C_6–C_3 units of lignin without randomization of the carbon atoms.

The main results of these experiments can be interpreted by the hypothetical scheme in Fig. 1. Cinnamic acid, fed to the plant, is converted to lignin which is represented by the guaiacyl glycerol ether unit (I). Evidence for the existence of such a unit in lignin has been provided by studies on the enzymatic formation of "DHP-lignin" by Freudenberg[4] and analytical studies on spruce milled-wood lignin by Adler.[5] Alkaline hydrolysis and oxidation

[4] K. FREUDENBERG, *Nature* **183**, 1152 (1959).
[5] E. ADLER, *Papier* **15**, 604 (1961).

would be expected to convert this to a 3-guaiacyl glyceraldehyde unit which, by analogy with alkaline oxidation of aldoses,[6] would be expected to undergo the reactions shown in Fig. 1 to give formic acid (from COOH-carbon) and 3-methoxy-4-hydroxymandelic acid (II). Further oxidation of II would be expected to give carbon dioxide from the carboxyl group (α-carbon of I) and some vanillin (III). This assumption is supported by a model experiment in which a synthetic sample of II was found to give 1 mole of carbon dioxide and 0·6 moles of vanillin.

This interpretation (Fig. 1) explains the main results of these experiments, i.e. the origin of formic acid from COOH-carbon and carbon dioxide from α-carbon. However it should not be thought that this is a unique interpretation. Although the guaiacylglycerol ether unit is probably an important part of lignin it is known that lignin is a complex condensation product of phenylpropanoid units linked by C—C and C—O bonds involving C-5 of the aromatic ring and the carbons of the side chain. Other groups could give rise to formic acid or carbon dioxide. Further experiments with model compounds would probably make possible a more sophisticated interpretation of the data presented in this paper.

EXPERIMENTAL

Preparation of Labelled Protolignins (Extractive-free Residues)

Cinnamic acid-[COOH]-^{14}C and cinnamic acid-α-^{14}C were prepared by condensation of unlabelled benzaldehyde with malonic acid-[COOH]-^{14}C or -α-^{14}C respectively. Cinnamic acid-ring, β-^{14}C was prepared by condensing generally labelled benzaldehyde with unlabelled malonic acid.

Twigs of *Picea mariana* (Mill.) BSP (black spruce) and *Populus tremuloides* Michx. (aspen) were collected near Halifax, Nova Scotia, on 18 July. The labelled compounds were fed through the cut ends of the detached twigs as described previously.[7] A period of about 30 hr was allowed for metabolism and the samples extracted with hot ethanol in a VirTis homogenizer as described in a previous paper.[8] The insoluble residue was washed with hot absolute ethanol and then with ether.

Preparation of Klason Lignins

A 300-mg sample of extractive-free plant material was mixed thoroughly with 5 ml of 72% sulphuric acid. After standing for 2 hr at 20° the mixture was diluted with 190 ml of water and refluxed for 4 hr. The Klason lignin was recovered by filtration, washed, and dried to constant weight at 105°.

Isolation of Carbohydrate Fractions

Holocellulose was prepared by successive treatments of a 300-mg sample of the extractive-free plant material with aqueous sodium chlorite[9] at pH 4. A cellulose fraction was obtained by heating the holocellulose with 2% hydrochloric acid for 3 hr at 100°. The holocellulose was also fractionated by extraction with 10% potassium hydroxide. The hemicelluloses were

[6] J. W. GREEN, Chapter 6 in *The Carbohydrates* (Edited by W. PIGMAN), Academic Press, New York and London (1957).
[7] A. C. NEISH, *Can. J. Botany* **37**, 1085 (1959).
[8] A. C. NEISH, *Can. J. Botany* **36**, 649 (1958).
[9] L. E. WISE, M. MURPHY and A. A. D'ADDIECO, *Paper Trade J.* **122**, 35 (1946).

recovered by acidifying the alkaline extract with acetic acid, and adding ethanol until precipitation was complete. The hemicellulose was hydrolysed by heating in 3% nitric acid[10] for 4 hr at 100°. The sugars in the hydrolysate were separated by thin layer chromatography.[11]

Isolation of Amino Acids

A 300-mg portion of extractive-free plant material was heated with 6 N hydrochloric acid in a sealed tube at 105° for 24 hr. The contents were filtered and the filtrate evaporated to dryness. The residue was taken up in water, and the solution put through a column of Amberlite IR-120-H ion exchange resin. The column was washed with water, and the amino acids eluted with 1 N ammonium hydroxide.

Alkaline Oxidation

A 300-mg sample of extractive-free plant material was placed in a 22-ml nickel bomb, along with 1·3 g of cupric hydroxide (City Chemical Corporation, New York) and 10 ml of 2 N sodium hydroxide. The sealed bomb was heated with occasional shaking in an oil bath at 175°. After 3¼ hr the contents were filtered, and the residue washed with hot water. The combined filtrate and washing were acidified to pH 2–3, and the carbon dioxide liberated by this reaction was absorbed in dilute sodium hydroxide.

The acidified solution was centrifuged to remove precipitated material and freeze-dried. The sublimate was neutralized with 0·1 N sodium hydroxide, and the water evaporated on a steam bath. The residue consisted of the sodium salts of acetic and formic acids. The salts were taken up in dilute hydrochloric acid, and the acids separated by chromatography on a column of silicic acid.[12] The acids were recovered as their sodium salts.

The freeze-dried residue, containing the non-volatile products of the oxidation, was redissolved in water and the solution shaken with two lots of chloroform. The combined chloroform extracts were washed with water, and then shaken with aqueous sodium bisulfite to extract the aldehydes. Vanillin and syringaldehyde were separated by chromatography and recovered as the *m*-nitrobenzoylhydrazones by the procedure reported previously.[2]

Oxidation of 3-Methoxy-4-hydroxymandelic acid

A sample of 3-methoxy-4-hydroxymandelic acid (0·28 mM), prepared by the method of Gardner and Hibbert,[13] was oxidized as described above under "alkaline oxidation". This gave 0·29 mM of carbon dioxide, 0·09 mM of formic acid and 0·16 mM of vanillin.

Measurement of Radioactivity

A Packard Tri-Carb Model 314A Liquid Scintillation Spectrometer was used. The salts of the volatile acids were dissolved in 1·0 ml of 0·5 N sodium hydroxide. An aliquot (0·5 ml) of the solution was pipetted into a counting vial previously filled with Cab–O–Sil and then with 15 ml of a liquid scintillator prepared by mixing 378 ml of ethanol with 600 ml of toluene containing 0·5% PPO (2,5-diphenyloxazole) and 0·0015% POPOP (1,4-*bis*-2[5-phenyl-oxazolyl]-benzene).

[10] M. A. JERMYN, *Modern Methods of Plant Analyses* (Edited by K. PAECH and M. V. TRACEY), Vol. II, p. 206, Springer-Verlag, Berlin (1955).
[11] E. STAHL and U. KALTENBACH, *J. Chromatog.* **5**, 351 (1961).
[12] A. C. NEISH, *Analytical Methods for Bacterial Fermentations*, National Research Council of Canada, Report No. 2952 (1952).
[13] J. A. F. GARDNER and H. HIBBERT, *J. Am. Chem. Soc.* **66**, 1607 (1944).

All other materials were converted to carbon dioxide by the wet combustion procedure of Baker *et al.*,[14] and counted as barium carbonate. The scintillator was 0·5% PPO and 0·03% POPOP in toluene, with Cab–O–Sil as the suspending agent.

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[14] N. Baker, H. Feinberg and R. Hill, *Analyt. Chem.* **26**, 1504 (1954).