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Investigations on Lignins and Lignification. XXVI.* Studies on the Utilization of Pyruvate in Lignification†

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Uniformly labeled sodium pyruvate-C^{14} was incorporated into a birch sapling by a forced feeding technique. After isolation of the milled-wood lignin, the distribution of radioactivity in the propyl side-chain of its building units was studied by hydrogenation, hydrogenolysis, and vapor phase chromatography. The results indicate that pyruvate is not a direct precursor of the propyl moiety of lignin. Together with specific activities of other wood and leaf constituents, the data reveal certain aspects of the metabolic fate of pyruvate in this plant.

The biogenesis of aromatic compounds is known to occur *via* at least two synthetic routes, *i.e.*, the acetate (Birch, 1960) or shikimate (Davis, 1955) pathways. That the latter reaction sequence is responsible for the formation of phenylpropanoid units is generally acknowledged, and it has been demonstrated (Eberhardt and Schubert, 1956; Acerbo *et al.*, 1958, 1960) that the initial stages of lignification, which also involve the genesis of C_6-C_3 intermediates, follow this same course.

The direct precursor of the C_3 side-chain of the phenylpropanoid type aromatic amino acids, phenylalanine and tyrosine, has recently been reported to be phosphoenolpyruvic acid (Levin and Sprinson, 1960), a compound also proposed as an intermediate in shikimic acid formation (Srinivasan and Sprinson, 1959). While it would be of interest to determine whether phosphoenolpyruvic acid is similarly operative in lignin biogenesis, previously observed difficulties in the permeability of many phosphorylated compounds (Weiss and Mingioli, 1956) prompted the substitution of a closely related compound. The reversibility of the reaction between phosphoenolpyruvic acid and pyruvate (Meyerhof and Oesper, 1949), despite the fact that the equilibrium constant is greatly in favor of formation of the latter, suggested the utilization of pyruvate. In addition, it has been reported (Thomas *et al.*, 1953, 1955) that yeast, when grown on labeled pyruvate as sole carbon source, synthesized tyrosine and phenylalanine with side-chains derived from this α-keto acid as an intact unit. However, the distribution of radioactivity in the aromatic rings of these amino acids indicated that the latter were not formed directly from pyruvate.

Phosphoenolpyruvic acid is also believed to play a key role in the reversal of glycolysis (Krebs, 1954). Hence, the manner in which pyruvate is incorporated into carbohydrates could be analogous to the initial steps of the process under consideration here. Recent studies in this area have shown that the nature of the tissue greatly affects the metabolic route. For example, the fact that C^{14}-labeled pyruvate is converted into rat liver glycogen with randomization of the isotopic carbon suggests that here pyruvate is not a direct precursor (Topper and Hastings, 1949), (Landau *et al.*, 1955). On the other hand, rat diaphragm tissue less efficiently converts radioactive pyruvate directly into glycogen, as exhibited by the localization of activity in the isolated carbohydrate (Hiatt *et al.*, 1958). In addition, extensive studies on various plant tissues (Neal and Beevers, 1960; Brummond and Burris, 1953) have provided evidence for the predominance of an oxidative decarboxylation of this α-keto acid.

Thus, when sodium pyruvate-3-C^{14} was incorporated into a living Norway spruce tree and the lignin was isolated and degraded to vanillin

* For the previous papers of this series see Coscia *et al.*, 1961, and Olcay, 1962.

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(Acerbo *et al.*, 1960), activity measurements indicated that although the lignin was radioactive, the vanillin was not. If direct incorporation of pyruvate into lignin had occurred, the methyl carbon of pyruvate should have been converted into the carbonyl carbon of this aldehyde. The absence of radioactivity in the vanillin was an indication that pyruvate was not a direct side-chain precursor in lignification.

However, since vanillin is a phenylmethane derivative, while lignin itself is a phenylpropane-derived polymer, we have devised and reported a method to determine the specific activities of the two terminal side-chain carbon atoms of the lignin monomer (Coscia *et al.*, 1961b; Olcay, 1962).

In the present experiments, uniformly labeled sodium pyruvate-C^{14} was incorporated into a living birch tree whose lignin was then isolated and degraded by hydrogenation and hydrogenolysis. Vapor phase chromatographic separation of the products permitted a study of the relative distribution of the radioactivity of the pyruvate in the two terminal carbon atoms of the propyl side-chain of the monomeric lignin hydrogenation products.

Experimental

Incorporation of C^{14}-Labeled Pyruvate.—Under climatic and seasonal conditions considered most favorable for lignification (Brauns and Brauns, 1960), an aqueous solution of 50 mg (1.5 mc) of uniformly labeled sodium pyruvate-C^{14} (prepared by mixing 0.5 mc each of specifically labeled sodium pyruvate-1-C^{14}, -2-C^{14}, and -3-C^{14} purchased from Nuclear-Chicago) was incorporated into a living birch sapling (*Betula pendula*) by a forced-feeding technique. Five branches were cleaved at internodes having at least a 3 mm diameter, and the severed ends were quickly immersed in 9 ml of the radioactive solution contained in conical test tubes. After complete absorption (5–10 hours), three 3-ml portions of water were used to wash the walls of the test tubes and the bark, and these were also absorbed. After 1 week the tree was felled, defoliated, and debarked, and the leaves, bark, and certain parts of the wood and roots were ground to 40 mesh. Samples of the different sections of the plant were combusted and their radioactivity counted. The total activity was determined by multiplying the total weight of carbon in the plant part by the specific activity of that section and adding them. For the lower portion of the sapling (trunk and roots), only one sample of the wood was ground and counted.

Isolation and Degradation of Milled-Wood Lignin.—Milled-wood lignin was isolated from the most highly radioactive material (*i.e.*, the twigs) in a 46% yield (Bjørkman, 1956) and was subjected to high-pressure hydrogenation and hydrogenolysis according to previously reported procedures (Coscia *et al.*, 1961a). With the aid of vapor phase chromatography, three reaction products were isolated: 4-*n*-propyl, 4-ethyl, and 4-methyl guaiacol. Since a compound collected from a vapor phase chromatographic column may be contaminated by the stationary phase (Coscia *et al.*, 1961b; Olcay, 1962), the following purification procedure was developed and found satisfactory. After collection, the phenol was removed from its trap with ether, and the solvent was evaporated. Water (in which the packing is soluble) was added, and the mixture was extracted with ether. The ether washings were transferred to a combustion chamber, and, after removal of the solvent, the pure hydrogenation product was oxidized to carbon dioxide.

Isolation of Other Wood and Leaf Constituents.—Various wood constituents were removed during the isolation and purification of milled-wood lignin by extraction with benzene-ethanol, ethanol, water, and ether (Bjørkman, 1956). The first two solvents were used for the preextraction of the wood. Included in the benzene-ethanol solubles were fats, waxes, and resins, while the ethanol extract contained tannins (Brauns, 1952). After the wood had been milled, the lignin was isolated, dissolved in 90% acetic acid, and precipitated into water. The water dissolves tannins and a part of the "lignin-carbohydrate complex" (Bjørkman, 1956). The lignin was centrifuged, and the water extract was obtained by separation of the aqueous phase. After drying, the lignin was dissolved in ethanol–ethylene chloride (1:2) and precipitated into ether. The ether-soluble materials were then obtained in a similar manner. The "delignified wood" represented the material from which milled-wood lignin had been removed, and it retained, along with cellulose and other carbohydrates, about 50% of the original lignin. The holocellulose was obtained from this fraction by the ethanolamine method (Wise *et al.*, 1939).

Fatty acids were isolated from the leaves by a procedure outlined previously (Bonner, 1950). The plant material was extracted with ether, and the ether solution was washed with 10% aqueous sodium carbonate to remove the free acids. Upon evaporation of the solvent, the residue was saponified, and the alkaline solution was extracted with ether to remove sterols, carotenoids, and other hydrocarbons. The aqueous alkaline solution was acidified, and the fatty acids were isolated by another ether extraction.

The *free acids* were obtained by acidifying the sodium carbonate soluble fraction and extracting with ether. To isolate any free acids which may have been present in the dissociated state, the leaves were acidified with 4 N sulfuric acid before ether extraction (Pucher *et al.*, 1934). The acids were then removed from the ether solution with aqueous sodium carbonate, and, after acidification, they were back-extracted.

Leaf proteins were isolated with aqueous trichloroacetic acid (Schneider, 1945).

Measurement of Radioactivity.—Estimation of the isotopic carbon was achieved by first employing the wet combustion technique, after which the

resulting carbon dioxide was collected and counted as barium carbonate (Van Slyke and Folch, 1940). Corrections for background and self-absorption were made on all samples.

Results and Discussion

A major portion of the radioactivity of the incorporated pyruvate was found in the leaves and their stems and in the twigs of the tree (Table I). Calculation of the total activity present in the sapling demonstrated relatively high recovery of C^{14}. Milled-wood lignin was prepared from the most active wood tissue, and in the process, other wood constituents were also isolated. The distribution of radioactivity among these components, recorded in Table II, indicates that the lignin, although radioactive, incorporated a relatively small fraction of the C^{14}. This was to be expected, due to the ability of pyruvate to enter a variety of metabolic pathways (Barron, 1943).

Table I

Distribution of Radioactivity in Various Parts of a Birch Sapling Fed Radioactive Pyruvate

	Specific Activity (cpm/mg C)	% of Total Weight	% of Total Activity
Leaves	13,998	5	50
Leaf stems	10,983	1	8
Twigs	3,255	9	30
Bark	403	19	8
Branches	311	11	2
Upper trunk	64	18	0.6
Lower trunk	52	14	0.4
Roots	42	22	1

Table II

Distribution of Radioactivity Among the Chemical Constituents of the Twigs

	Specific Activity (cpm/mg C)	% of Total Weight	% of Total Activity
Benzene-ethanol extract	23,437	4	42
Ethanol extract	16,479	1	6
Water-soluble materials	1,427	7	3
Ether-soluble materials	1,990	0.2	0.2
Milled-wood lignin	1,520	9	8
"Delignified wood"	2,119	77	41
(*a*) Holocellulose	676	50	16

Of greater significance, however, was the question whether pyruvate was utilized as an intact unit[1] for the derivation of the propyl side-chains of the lignin monomeric units. To investigate this possibility, the milled-wood lignin was subjected to high pressure hydrogenation, and, from the reaction products, a homologous series of alkyl guaiacol derivatives was separated by vapor phase chromatography.

As seen in Table III, the specific activities of 4-methyl, 4-ethyl, and 4-*n*-propyl guaiacol were determined, and from these values the radioactivity of the two terminal carbons of the side-chain and of the guaiacyl methane moiety were calculated. The predominance of activity in the C-3′ position indicates that pyruvate was not incorporated as an intact unit into the propyl groups of the lignin monomers. Further, these results are in agreement with those of an earlier experiment (Acerbo *et al.*, 1960) wherein, after sodium pyruvate-3-C^{14} was fed to a Norway spruce tree, the lignin was found to be radioactive but the vanillin obtained from it was not.

Table III

Distribution of Radioactivity in Lignin Hydrogenation Products

	Specific Activity (cpm/mg C)		% of Total Activity
Lignin	1520		
4-*n*-Propyl guaiacol	1568	C-3′	87.6
4-Ethyl guaiacol	218	C-2′	0.88
4-Methyl guaiacol	228	C_6-C_1	11.6

To help determine the fate of the pyruvate incorporated into the birch sapling and thereby perhaps explain the distribution of isotopic carbon in the lignin, certain leaf constituents were isolated and their activities determined (Table IV). The C^{14} level in the leaf proteins suggests that some of the α-keto acid possibly underwent transamination to alanine, although other amino acids can also be derived from pyruvate indirectly, *e.g.*, *via* the Krebs cycle (Neal and Beevers, 1960). The high activity of the fatty acids in this tissue is also significant, since the precursor of these compounds is acetyl coenzyme A. Furthermore, in Table II it is shown that the benzene-ethanol extract contains a major portion of the radioactivity. This fraction is known to contain fats, waxes, and resins (Brauns, 1952), which are also derived from acetyl coenzyme A. The low radioactivity of the wood holocellulose suggests that pyruvate plays but a minor role in carbohydrate formation (Neal and Beevers, 1960).

These observations, together with numerous reports (Neal and Beevers, 1960; Bonner and Millerd, 1953) of the ubiquity of pyruvate in a variety of plant tissues, suggest that the principal

[1] The overemphasis placed on the efficiency of conversion of incorporated labeled compounds into lignin is illustrated in this phase of the studies (*e.g.*, Brown and Neish, 1959). The fact that certain lignin precursors are also intermediates in other biosynthetic pathways renders it virtually impossible to obtain a high utilization in lignification during "*in vivo*" experiments. By the determination of the distribution of activity in the several positions of the phenyl propane moiety of lignin (Eberhardt and Schubert, 1956; Acerbo *et al.*, 1958, 1960) we can arrive at conclusions concerning the early stages of lignification (Nord and Schubert, 1957).

TABLE IV
DISTRIBUTION OF RADIOACTIVITY AMONG CHEMICAL CONSTITUENTS OF THE LEAVES

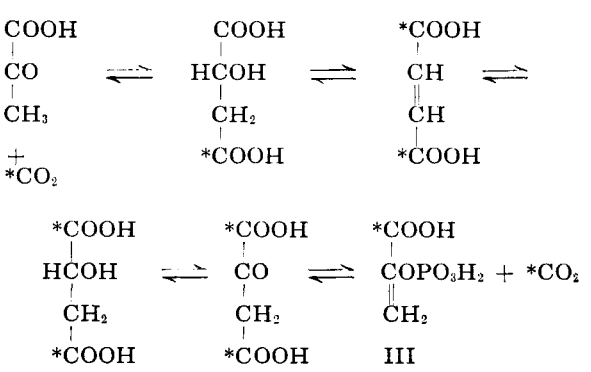
	Specific Activity (cpm/mg C)	% of Total Weight	% of Total Activity
Leaves	13,988	100	100
Fatty acids	12,507	10	14
Free acids			
(*a*) Undissociated	4,930	5	2
(*b*) Total	8,613	—	—
Protein	12,432	19	15
Carotenoids, steroids, and hydrocarbons	5,607	3	2

metabolic fate of the incorporated pyruvate (I) is oxidative decarboxylation. The derived acetyl CoA (II) can then enter into the biosynthesis of fatty

$$\underset{\text{I}}{CH_3COCOOH} + CoA + DPN^+ \longrightarrow \underset{\text{II}}{CH_3COCoA} + DPNH + H^+ + CO_2$$

acids, steroids, carotenoids, and related hydrocarbons (see Table IV). Earlier investigations[1] with labeled acetic acid have shown that this compound is not incorporated into lignin. However, the acetyl CoA can also enter the tricarboxylic acid cycle (Brummond and Burris, 1953), as indicated by the radioactivity of the free acids and of the proteins in the leaves. Although the activity of the free acids is not as great as that of the proteins or of fatty acids, the rapid turnover in the Krebs cycle would reduce the amount of C^{14} available in this pool. Thus it would be expected, in agreement with previous studies on plant tissues (Neal and Beevers, 1960), that some pyruvate would be oxidized completely to carbon dioxide.

On the basis of these considerations and in view of the relatively high recovery of incorporated C^{14} and the distribution of activity in the lignin, it appears that the C^{14} in the terminal position of the propyl side-chain of the lignin monomer is derived from isotopic carbon dioxide *via* the "dicarboxylate shuttle" (Utter and Kurahashi, 1954) as shown in Scheme I. As shown in Scheme

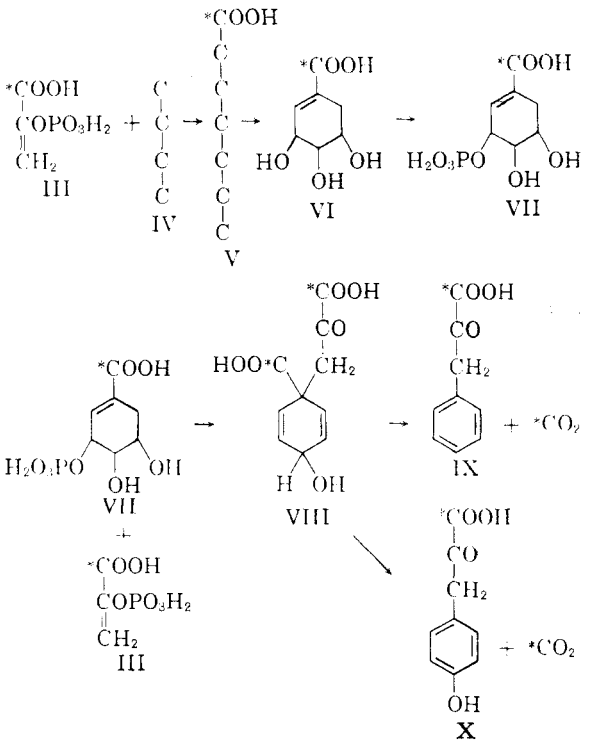


Scheme I

II, the resulting phosphoenolpyruvate-1-C^{14} (III) can combine with 5-phosphoshikimic acid (VII) to form prephenic acid (VIII) (Levin and Sprinson, 1960), which then undergoes decarboxylation to either *p*-hydroxyphenylpyruvate (X) or to phenylpyruvate (IX) and is thereby converted to the lignin monomeric units. All the enzymes, coenzymes, and cofactors operative in the dicarboxylate shuttle have been found to be widely distributed in the tissues (*i.e.*, leaves, roots, and seeds) of higher plants (Vishniac *et al.*, 1957; Conn *et al.*, 1949). The reversal of this sequence is believed to represent the dark fixation of carbon dioxide by plants (Kunitake *et al.*, 1959; Saltman *et al.*, 1956). In the presence of $C^{14}O_2$, such metabolites as alanine, malic acid, and fructose become radioactive, and the reported distribution of activity in them (Gibbs, 1951) is in agreement with the labeling found in our lignin hydrogenation products. In addition, this "shuttle" has been employed to explain the incorporation of activity from pyruvate-2-C^{14} into liver glycogen (Topper and Hastings, 1949), and from pyruvate-1-C^{14} into glucose by the endosperm tissue of castor bean (Neal and Beevers, 1960). In both cases, a major fraction of the isotopic carbon was located in the 3 and 4 positions of glucose. These locations correspond to the carboxyl carbon of phosphoenolpyruvate, which is believed to be the carbohydrate precursor in glycolysis reversal (Krebs, 1954; Benedict and Beevers, 1961). Furthermore, in both instances direct incorporation is considered unlikely.

The low percentage of radioactivity in the ring carbons of the lignin hydrogenation products (Table III) also supports this interpretation. It has been suggested (Schubert and Nord, 1961) that in the earlier stages of lignification, a 3-carbon unit and a tetrose (IV) combine to form a 7-carbon sugar (V) which then cyclizes to shikimic acid (VI). There is evidence (Srinivasan and Sprinson, 1959) that the 3-carbon compound is phosphoenolpyruvic acid (III), and, according to the reaction scheme, the carboxyl group of phosphoenolypyruvic acid would be converted into the carboxyl of shikimic acid. In this experiment, then, the shikimate carboxyl would be as highly labeled as the terminal carbon of the monomer side-chain. However, this carboxyl is lost during the conversion of prephenic acid (VIII) to either *p*-hydroxy phenylpyruvate (X) or phenylpyruvate (IX); this fact explains the low activity of the guaiacyl methane moiety.

The low activity in the 2-position of the side-chain can be explained in several ways. Despite the fact that the equilibrium between pyruvic acid and phosphoenolpyruvate overwhelmingly favors pyruvate (Meyerhof and Oesper, 1949), it is possible for a minute fraction of the α-keto acid to be directly phosphorylated to phosphoenolpyruvate. Alternatively, the radioactive pyruvate might condense with carbon dioxide, as in the first reaction of the dicarboxylate shuttle. A third possibility involves the introduction of



Scheme II

radioactive acetyl CoA into the Krebs cycle, whereupon the resulting oxaloacetate would be labeled in its 2- and 3-positions. This compound could be converted to phosphoenolpyruvate-1,2-C^{14}. Of course, for every molecule that reacted in any of the above ways, at least 100 molecules of labeled carbon dioxide would pass through the shuttle and be utilized in lignification.

Thus, the evidence indicates that pyruvate is not a direct precursor of the C_3 units of the lignin monomers of *Betula pendula*. Rather, it would appear that the fate of the C^{14} from incorporated radioactive pyruvate is varied, and that C-atoms which find their way into the propyl moieties of lignin do so predominantly by carbon dioxide fixation *via* the "dicarboxylate shuttle."

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