Lignification in *Eucalyptus*

INCORPORATION OF PHENYLALANINE, TYROSINE AND METHIONINE INTO *EUCALYPTUS SIDEROXYLON* AND *EUCALYPTUS CAMALDULENSIS*

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The fact that L-phenylalanine functioned as a lignin precursor was noted by Brown & Neish (1956). Brown (1961) found that grasses possessed the ability to utilize L-tyrosine as efficiently as L-phenylalanine, but limited ability to utilize L-tyrosine was observed in some other plants. Neish (1961) concluded that the ability to utilize L-tyrosine efficiently was dependent upon the presence of tyrase (L-tyrosine ammonia-lyase). Transmethylation of lignin from methionine in the tobacco plant was observed by Byerrum, Flokstra, Dewey & Ball (1954).

In the present work the behaviour of L-phenylalanine, L-tyrosine and L-methionine in relation to lignification in two species of *Eucalyptus* has been investigated. Acetate and glucose also have been tested.

EXPERIMENTAL

Precursors. Uniformly ^{14}C -labelled L-phenylalanine and uniformly ^{14}C -labelled L-tyrosine, $[6\text{-}^{14}\text{C}]\text{glucose}$, L-[Me- $^{14}\text{C}]\text{methionine}$, sodium $[1\text{-}^{14}\text{C}]\text{acetate}$, phenyl $[1\text{-}^{14}\text{C}]\text{alanine}$ and phenyl $[2\text{-}^{14}\text{C}]\text{alanine}$ were obtained from The Radiochemical Centre, Amersham, Bucks. Phenyl $[3\text{-}^{14}\text{C}]\text{alanine}$ was obtained from California Corp. for Biochemical Research.

Infusion. Freshly cut shoots of the desired species (about 25 cm. overall length and carrying about eight leaves) were placed with the stem in a test tube containing 0.01 mc of the compound under test in solution in water. After the solution was absorbed (usually about 24 hr.) the shoot was allowed to stand in water for 10 days to allow metabolism of the compound. The shoots were exposed to direct sunlight: no artificial illumination was employed. The shoots of *Eucalyptus sideroxylon* infused with L-phenyl- $[1\text{-}^{14}\text{C}]$ -, $[2\text{-}^{14}\text{C}]$ - and $[3\text{-}^{14}\text{C}]\text{alanine}$ were the identical specimens used by Hillis & Hasegawa (1962*a*), who allowed 24 hr. for absorption and metabolism.

Extraction. The infused shoots were dried at 45° under reduced pressure, the leaves removed and the stems ground to pass 20 mesh. The ground material was extracted three times with boiling water, filtered on paper, washed with hot water and dried under reduced pressure at 45° .

Determination of radioactivity. The specimen (0.02 g.) was oxidized by wet combustion and the carbon dioxide collected as barium carbonate according to the method of Schmid & Schmid (1953). The activity of the barium carbonate was determined as an infinitely thick layer with a Philips thin-end-window Geiger-Müller tube and electronic counter. All readings were corrected to a standard value

for a Plexiglass reference standard. The same procedure was applied to separated vanillin, syringaldehyde and triethylmethylammonium iodide.

Recovery of vanillin and syringaldehyde. A specimen of extracted stem material (about 0.4 g.) was oxidized with nitrobenzene by the small-scale method described by Bland (1960). Acidification of the reaction mixture was carried out in the apparatus of Schmid & Schmid (1953) and the carbon dioxide evolved collected as barium carbonate, the activity of which was determined. Separation of the vanillin from the syringaldehyde was carried out by counter-current distribution as described by Bland, Hillis & Williams (1952), with Shell Solvent X2-ether-water (19:1:20, by vol.). Completeness of separation was tested by placing drops of solution from every second tube of the Craig machine on a borate paper chromatogram and, after development, assessing the separation by inspection in ultraviolet light (Bland, 1955). One hundred and twenty Craig machine cycles sufficed. The vanillin and syringaldehyde were recovered by evaporation of the contents of the appropriate tubes and collection in the flask of the wet-oxidation apparatus.

Separation of methoxyl carbon. The specimen (about 0.03 g.) was decomposed by the Zeisel method and the methyl iodide collected as triethylmethylammonium iodide by the method of Byerrum *et al.* (1954). After standing overnight the absorbing solution was evaporated to dryness in the flask of the wet-oxidation apparatus.

Calculation of dilution factors. The activities of the labelled precursors were determined by dilution of specimens with an inactive carrier and converting into barium carbonate, the activity of which was measured under conditions identical with those employed in the experiments. The dilution factors were calculated as the ratio of activity of precursor (as barium carbonate) to activity of derivative (as barium carbonate) corrected to a dosage rate of $50\text{ }\mu\text{C/g. dry wt. of plant in all cases}$.

For example, 1.6 mg. of uniformly labelled L- $[^{14}\text{C}]\text{phenylalanine}$ ($64.3\text{ }\mu\text{C/mg.}$) was dissolved in 10.0 ml. of water and a drop of this solution weighing 0.0303 g. was added to 0.0190 g. of inactive phenylalanine. This mixture was oxidized to carbon dioxide and collected as barium carbonate, the activity of which, referred to the Plexiglass standard of 600 counts/min., was 788 counts/min. Thus the weight of labelled phenylalanine taken was 0.004848 mg., the total weight of phenylalanine was 19.004848 mg. and the calculated radioactivity as barium carbonate of the labelled phenylalanine was

$$788 \times 19.0048/0.004848 = 3.09 \times 10^6 \text{ counts/min.}$$

An amount ($10\text{ }\mu\text{C}$) of labelled phenylalanine infused into a specimen (*E. camaldulensis*) of dry wt. 0.9253 g. corresponds

Table 1. *Dilution factors for utilization of different substances in lignification by Eucalyptus species*

Dilution factor: radioactivity of precursor as BaCO₃/radioactivity of derivative as BaCO₃. Et₃MeNI, triethyl-methylammonium iodide from methyl iodide from the distillation of wood with HI. Carbon dioxide was from nitrobenzene oxidation of wood. U-¹⁴C, uniformly ¹⁴C-labelled.

Species	Substance fed	Specific activity (μc/mg.)	Derivative				
			Extracted wood	Vanillin	Syring-aldehyde	Et ₃ MeNI	CO ₂
<i>E. camaldulensis</i>	L-[U- ¹⁴ C]Phenylalanine	64.3	2510	1160	1300	∞	760
	L-[U- ¹⁴ C]Tyrosine	53.2	2050	15500	5380	∞	4470
	L-[Me- ¹⁴ C]Methionine	44.0	1030	570	3540	1540	∞
	D-[6- ¹⁴ C]Glucose	19.8	1240			7640	1680
<i>E. sideroxylon</i>	L-[U- ¹⁴ C]Phenylalanine	64.3	1940	10770	4270	∞	3640
	L-[U- ¹⁴ C]Tyrosine	53.2	2680	5070	4030	∞	6490
	L-[Me- ¹⁴ C]Methionine	44.0	2140	15910	7180	1250	89300
	D-[6- ¹⁴ C]Glucose	19.8	1860	15590	2670	16070	2680
	Sodium [1- ¹⁴ C]acetate	55.8	10770	72150	72150	∞	17020
	L-Phenyl[1- ¹⁴ C]alanine	23.9	4700	308000	72000	∞	3050
	L-Phenyl[2- ¹⁴ C]alanine	14.5	3260	54000	26000	∞	1220
	L-Phenyl[3- ¹⁴ C]alanine	20.0	3480	4670	5960	∞	11180

to a dosage of 10.81 μc/g. The barium carbonate from the extracted wood gave 266 counts/min. referred to the above-mentioned standard. The dilution factor corrected to a dosage of 50 μc/g. was therefore

$$3.09 \times 10^6 / 266 \times 10.81 / 50 = 2510 \text{ (to the nearer 10).}$$

Testing for tyrase (L-tyrosine ammonia-lyase). This was done by Neish's (1961) method slightly modified as follows. Acetone-dried leaf powder (0.2 g.) was incubated for 3 hr. at 20° in 3 ml. of 0.1M-disodium hydrogen phosphate (pH 9.0) containing 0.1% of L-tyrosine in suspension. The mixture was diluted to 10 ml. and filtered; the filtrate was acidified and extracted with 10 ml. of ether, the ether evaporated and the residue dissolved in a few drops of ethanol. Most of the ethanol solution was transferred to a paper chromatogram. After irrigation with butanol-acetic acid-water (12:3:5, by vol.) the *p*-coumaric acid was detected (*R_F* 0.9) by diazotized *p*-nitroaniline spray and comparison with an authentic specimen of *p*-coumaric acid on the same chromatogram.

Parallel tests were run on oat shoots and *Pinus radiata* needles.

RESULTS

The dilution factors for the extracted woods and their decomposition products are shown in Table 1. In all cases radioactivity was fixed in the stem material in an unextractable form, the lowest fixation being for the sodium acetate.

The aldehydes derived from *E. camaldulensis* infused with uniformly labelled [¹⁴C]phenylalanine were, as may be seen from Table 1, highly active. The vanillin and syringaldehyde from the phenylalanine-infused *E. sideroxylon* showed only low activity from the 1-¹⁴C- and 2-¹⁴C-labelled precursors, but the 3-¹⁴C-labelled phenylalanine gave highly active aldehydes.

The uniformly labelled [¹⁴C]tyrosine appears to have acted almost as efficiently as phenylalanine as a lignin precursor in the *E. sideroxylon*. Neish (1961) observed this in grasses and considered it to

be dependent upon the presence of tyrase. It has now been found that the leaves of this specimen of *E. sideroxylon* contained tyrase. Repetition of the test on two further samples of leaves from this specimen confirmed the presence of tyrase. None was detected in the leaves of another specimen from an area remote from the first. The enzyme was absent from *E. camaldulensis* and also from *E. regnans* and *E. bicosata*. Parallel tests gave a positive result for oat shoots and a negative one for *Pinus radiata* needles.

It is clear from the zero or negligible radioactivities of the triethylmethylammonium iodide, from specimens given phenylalanine, tyrosine or sodium acetate, that these precursors did not contribute to the formation of the methoxyl groups of the wood. Methionine, however, gave highly active triethylmethylammonium iodide from both species, and it may be assumed that transmethylation from methionine occurred. The results with the [6-¹⁴C]glucose show that C-6 of glucose did contribute to the formation of methoxyl groups in the eucalypt wood.

The activity of the carbon dioxide liberated on acidification of the nitrobenzene oxidation mixture may be seen from Table 1 to vary widely in different experiments. That from the shoots infused with 1-¹⁴C- and 2-¹⁴C-labelled phenylalanine was highly active, whereas that from the shoots infused with the 3-¹⁴C-labelled material was of low activity. Thus most of the carbonate produced during nitrobenzene oxidation of lignin is derived from the two terminal carbon atoms of the side chain.

DISCUSSION

It is apparent that the acetate pathway is not important in the biosynthesis of lignin in the specimen of *E. sideroxylon* tested. This is similar to

the findings of Kratzl & Faigle (1960) with spruce and of Hasegawa, Higuchi & Ishikawa (1960) with tissue culture of *Pinus strobus*. The high dilution factors shown by the vanillin and syringaldehyde from the 1-¹⁴C- and 2-¹⁴C-labelled phenylalanine, in contrast with the lower factors from the 3-¹⁴C-labelled precursor, show that the phenylpropane unit of this substance is incorporated into the lignin without rearrangement. This is in accord with the observations of Brown & Neish (1956) and those of Freudenberg & Niedercorn (1958) that feeding of labelled phenylalanine to spruce produced labelled lignin as well as labelled coniferin. Bardinskaya (1959) suggested that phenylalanine as well as tyrosine and dihydroxyphenylalanine were intermediates on one possible biosynthetic pathway to lignin. Kratzl (1960) also noted the efficiency of phenylalanine as a lignin precursor but questioned whether it lay on the direct route to lignin. The inactivity of the methoxyl groups even from the lignin derived from uniformly labelled phenylalanine shows the carbon of the methoxyl group to be derived from some source outside the amino acid.

Although methionine functions efficiently as a methylating agent in the growing eucalypt shoot there is no evidence from these experiments that methionine is a normal intermediate in the methylation of the lignin nucleus. The experiment with [6-¹⁴C]glucose shows that C-6 of glucose contributes to the methoxyl carbon atom of the lignin in the eucalypts, as observed by Schubert & Nord (1961) for spruce. As evidence now indicates (Neish, 1961) that amino acids may lie on the normal biosynthetic pathway to lignin it appears that methylation probably occurs after deamination of the amino acid. Koukol & Conn (1961) have demonstrated the presence of phenylalanine deaminase in a number of plants. The methoxyl is derived from the sugar pool and the possibility exists that it is transferred to the lignin nucleus via methionine. Unlike L-phenylalanine, L-tyrosine is not efficiently utilized by all plants as a lignin precursor. Following observations by Brown & Neish (1956), Brown (1961) systematically investigated the ability of different species to utilize tyrosine. It appeared that only grasses could utilize tyrosine efficiently and that this was due to the presence of tyrase. The utilization of tyrosine as efficiently as phenylalanine by the specimen of *E. sideroxylon* suggests that this specimen has something in common with the grasses and the presence of tyrase confirms this. The specimen used in this work is believed by Hillis & Hasegawa (1962b) to be a physiological variant of the species; the presence of tyrase may be connected with this and in no way indicative of its presence in the species generally or in the genus *Eucalyptus*. On the other hand, although no tyrase was detected in *E. camal-*

dulensis a relatively inefficient utilization of tyrosine did occur in this species similar to that noted by Brown (1961) in plants of the Compositae. Although utilization by some other mechanism could have occurred the presence of tyrase in minimal quantity remains a possibility.

SUMMARY

1. Sodium [1-¹⁴C]acetate, uniformly labelled L-[¹⁴C]phenylalanine, L-phenyl-[1-¹⁴C]-, [2-¹⁴C]- and [3-¹⁴C]-alanine, uniformly labelled L-[¹⁴C]tyrosine, [Me-¹⁴C]methionine and [6-¹⁴C]glucose have been infused into growing shoots of *Eucalyptus* and their contributions to the lignin assessed.

2. The acetate did not act efficiently as a lignin precursor in *E. sideroxylon*. The results with phenylalanine labelled in different positions showed that its phenylpropane skeleton was incorporated into the lignin without rearrangement and that the carbon atoms of the methoxyl groups were derived from a source other than the phenylalanine itself.

3. The methyl group of methionine was an efficient source of methoxyl carbon atoms. The C-6 atom of glucose also acted as a source of methoxyl carbon atoms. It would appear that the methoxyl carbon atom comes through the sugar pool, but whether via methionine is not shown by these experiments.

4. Tyrosine was utilized by a particular specimen of *E. sideroxylon* almost as efficiently as phenylalanine. Tyrase (L-tyrosine ammonia-lyase) was detected in this, but not in three other *Eucalyptus* species.

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