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LIGNIFICATION IN *EUCALYPTUS*: METABOLISM OF 3-METHOXY-4-HYDROXYPHENYLALANINE

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Abstract—Tritiated 3-methoxy-4-hydroxyphenylalanine was fed to shoots of *Eucalyptus botryoides* and its metabolic products identified. No free ferulic acid was found but alkaline hydrolysis produced labelled ferulic acid from both the ethanol-soluble material and the residue. Rapid disappearance of the precursor in the shoot and the absence of free ferulic acid point to deamination of an esterified 3-methoxy-4-hydroxyphenylalanine. Radioactive vanillin was recovered from nitrobenzene oxidation products of the residue even after alkaline hydrolysis. Syringaldehyde from oxidation of the alkali-soluble material was weakly radioactive. The results show that the 3-methoxy-4-hydroxyphenyl units were built into the lignin but very little 5 methoxylation of these took place in 24 hr.

INTRODUCTION

THE AMMONIA lyase activity of acetone powder of *Eucalyptus* species has been studied by Bland[1] and also Hillis and Isoi.[2] These workers found only a weak tyrosine ammonia lyase activity compared with that in oat shoots and phenylalanine ammonia lyase was absent. However, it was found that phenylalanine acted as an efficient lignin[1] and polyphenol[2] precursor in *Eucalyptus* spp. Bland and Logan[3] found no evidence for conversion of phenylalanine to tyrosine in *Eucalyptus*. This is in accordance with the findings of Brown[4] that tyrosine is not utilized as a lignin precursor in dicotyledons. Bland and Logan noted also the conversion of phenylalanine to *p*-coumaric acid in *E. botryoides* but its accumulation in the free state indicated that it is probably not on the main biosynthetic pathway to lignin.

Another possible pathway from phenylalanine to lignin is the hydroxylation and methylation of phenylalanine to 3-methoxy-4-hydroxyphenylalanine (methoxytyrosine). This compound with its 3-methoxy-4-hydroxyphenyl nucleus appears to be one which could readily be assimilated into the lignin. Young and Neish[5] found that wheat shoot acetone powder deaminated this compound much less readily than phenylalanine itself. El-Basyouni and Neish[6] found that wheat and barley shoots contained metabolically active bound forms of cinnamic, *p*-coumaric, caffeic and ferulic acids. We now report the results of experiments on the metabolism of 3-methoxy-4-hydroxyphenylalanine in shoots of *E. botryoides*.

RESULTS AND DISCUSSION

The infused shoot was chopped and extracted with ethanol. Alkaline hydrolysis of the ethanol extract and residue both gave ferulic acid and oxidation of the residue gave vanillin

[1] D. E. BLAND, *Biochem. J.* **88**, 523 (1963).
[2] W. E. HILLIS and K. ISOI, *Phytochem.* **4**, 905 (1965).
[3] D. E. BLAND and A. F. LOGAN, *Phytochem.* **6**, 1075 (1967).
[4] S. A. BROWN, *Can. J. Botany* **39**, 253 (1961).
[5] M. R. YOUNG and A. C. NEISH, *Phytochem.* **5**, 1211 (1966).
[6] SAID Z. EL-BASYOUNI and A. C. NEISH, *Phytochem.* **5**, 683 (1966).

and syringaldehyde. The activities of fractions isolated are shown in Table 1. All these results refer to the count under identical conditions for "infinitely" thick films either by direct measurement as such or, when the amount of material was less than the amount necessary to constitute a thick film, by correction from a calibration curve.

It can be seen from the table that the ethanol extract from the chopped shoot and the residue after the extraction showed activity. This could of course be activity of unchanged precursor; however, in experiments in which unlabelled 3-methoxy-4-hydroxyphenylalanine was fed to shoots, and in the present experiment, none of the unchanged precursor was detected either in the extract or residue. This alone indicates a high rate of metabolism of the 3-methoxy-4-hydroxyphenylalanine. None of the amino acids in the acid hydrolysate of the residue was labelled.

No free ferulic acid was detected in control shoots of *Eucalyptus botryoides* but alkaline hydrolysis gave ferulic acid from both ethanol extract and residue. Similarly, no free ferulic

TABLE 1. ACTIVITY OF TRITIATED 3-METHOXY-4-HYDROXYPHENYLALANINE AND OF FRACTIONS FROM INFUSED SHOOTS OF *E. botryoides*

Fraction	Counts per 1000 sec
T 3-methoxy-4-hydroxyphenylalanine	267,000
Ethanol extract I	1,361
Ferulic acid from NaOH hydrolysis of I	11,130
Residue II after ethanol extraction	767
Ferulic acid from NaOH hydrolysis of II	12,330
Residue III after NaOH hydrolysis of II	91
Klason lignin from residue II	310
Syringaldehyde from II	0
Vanillin from II	181
Syringaldehyde from III	0
Vanillin from III	50
Syringaldehyde from NaOH hydrolysate of II	52
Vanillin from NaOH hydrolysate of II	257

acid was detected in the ethanol extract of the infused shoot but both the ethanol extract and the residue (II) remaining after ethanol extraction gave radioactive ferulic acid by alkaline hydrolysis. The high activity, shown in Table 1, of this ferulic acid is the most striking feature of these results. It is clear from this that the acid derived from the precursor entered the combined ferulic acid pool of the shoot. As shown in the table the residue (III) after alkaline hydrolysis retained a low level of activity, it appears from this that some metabolism past the ferulic acid stage had taken place. The fact that no free ferulic acid was detected indicates that the deamination of bound rather than of the free 3-methoxy-4-hydroxyphenylalanine took place. Klason lignin prepared from the residue after ethanol extraction showed significant activity but because of the ability of Klason lignin to carry down other materials with it this is not conclusive proof of incorporation of the precursor into the lignin. It can be seen from Table 1 that vanillin isolated from nitrobenzene oxidation products of the residue (II) remaining after ethanol extraction was active; syringaldehyde isolated at the same time was not active. Similarly, as shown in the table, the vanillin, but not the syringaldehyde isolated from the nitrobenzene oxidation products of the residue (III) after alkali extraction, was active. As alkaline hydrolysis removed ferulic acid from the residue it is apparent that the

active vanillin was derived from the lignin and shows that 3-methoxy-4-hydroxyphenyl units of the precursor had been built into the lignin.

The vanillin obtained by oxidation of the alkaline hydrolysate from residue II was more active than the vanillin from the residue itself. This was after removal of the active ferulic acid, which was therefore not the source of the vanillin. This is quite explicable on the assumption that lignin extracted by alkali would be that last laid down and therefore contain a high percentage of units derived from the precursor administered to the shoot. The activity of the syringaldehyde from the alkali-soluble fraction is very low and its significance is difficult to assess, particularly in view of the fact that no activity of syringaldehyde from the residue before alkali extraction could be detected. However, the results show that the 3-methoxy-4-hydroxyphenyl units of the precursor were incorporated into the lignin, and at the stage when metabolism was stopped there had been very little methoxylation of these units to 3,5-dimethoxy-4-hydroxyphenyl units.

Although it was necessary to use larger quantities of acetone dried powder and longer incubation times than usual in this type of test, it appears definite that an ammonia lyase capable of converting 3-methoxy-4-hydroxyphenylalanine to ferulic acid is present in *E. botryoides.* In contrast to this no phenylalanine ammonia lyase was detected under the same conditions. Admixture of acetone dried powder of *E. botryoides* leaves with that from oat shoots did not result in inhibition of the ammonia lyase activity of the latter. These results point to the absence of phenylalanine ammonia lyase from *E. botryoides* rather than to the presence of an inhibitor.

The rapid disappearance of 3-methoxy-4-hydroxyphenylalanine in the intact shoot suggests that some metabolic process other than deamination to free ferulic acid had taken place. Bland and Logan[3] observed that ferulic acid formed feruloylglucose when fed to *E. botryoides* shoots, but in their experiments some uncombined ferulic acid remained. The evidence indicates two possible initial steps in the metabolism of 3-methoxy-4-hydroxyphenylalanine. These are (1) esterification of the amino acid followed by deamination to have a combined ferulic acid, (2) deamination of the free amino acid to free ferulic acid followed by esterification of the ferulic acid. The rapid disappearance of the amino acid and absence of free ferulic acid indicates that the first route is the main one.

EXPERIMENTAL

Chromatographic Methods

Solvent systems employed were: BzAW, benzene–acetic acid–water (4:2:1 v/v); BAW, *n*-butanol–acetic acid–water (12:3:5 v/v); 6HA, acetic acid, 6% aqueous. Testing for ferulic acid and 3-methoxy-4-hydroxyphenylalanine was carried out by co-chromatography, fluorescence under u.v. and colour with the diazotized sulphanilic acid spray of Billek and Kindl.[7]

Radioactivity Measurements

Preliminary examinations of paper chromatograms were made using the Packard Radiochromatogram Scanner Model 7201. A Philips Gas-Flow Radiation Counter PW4141 was used to measure the activity of the isolated fractions.

Preparation, Labelling and Separation of 3-*Methoxy-4-Hydroxyphenylalanine*

This compound was prepared by the method of Shaw *et al.*[8] Labelling was achieved by reacting 0·15 g with tritiated water (2 ml, 10 mc) and activated PtO_2 (0·1 g) in a sealed tube for 24 hr at 100°. The mixture was evaporated to dryness on a steam bath and then extracted with aqueous acetone (1:1, 10 ml) by stirring.

[7] G. Billek and H. Kindl, *Monatsh Chem.* **93**, 85 (1962).
[8] K. N. F. Shaw, *J. Org. Chem.* **23**, 27 (1958).

This extract was streaked onto thick paper and chromatographed in BAW and the band of 3-methoxy-4-hydroxyphenylalanine eluted with Na_3PO_4 buffer (0·01 M).

Infusion of 3-Methoxy-4-Hydroxyphenylalanine and Preparation of Fractions

3-Methoxy-4-hydroxyphenylalanine (10 ml in 0·01 M Na_3PO_4 buffer) was infused into a freshly cut growing shoot of *Eucalyptus botryoides* in direct sunlight and allowed to metabolize for 24 hr. The shoot was chopped and extracted for 5 min with boiling ethanol (100 ml) which was then concentrated to 5 ml. The shoot residue remaining after ethanol extraction was dried *in vacuo* at 45° and then ground to pass 20-mesh in a Wiley mill.

Examination of Fractions

The ethanolic extract was chromatographed in the solvent systems mentioned and examined for labelled intermediates and unchanged 3-methoxy-4-hydroxyphenylalanine. Another portion of the extract (3 ml) was concentrated to dryness and hydrolysed with N NaOH (15 ml) by refluxing for 0·5 hr. The solution was acidified with HCl and extracted with diethyl ether. This extract was evaporated, the residue dissolved in ethanol and chromatographed consecutively six times in 6HA to move all of the ferulic acid (blue in u.v.) from zero R_f. As a result of this, the ferulic acid became streaked over half the chromatogram; areas containing impurities near zero R_f and the solvent front were cut off and the ferulic acid was collected into a distinct spot by chromatographing in BAW. A scan of the chromatogram revealed a peak of activity at the R_f of ferulic acid; however, two other compounds (one brown in daylight and the other brown in u.v.) were present in the same area. This area was cut out, two strips of paper attached by the method of Lakshminarayana[9] and separation of the ferulic acid from the other spots was effected by chromatographing in BzAW. A further scan of the chromatogram showed ferulic acid to be active and the other spots inactive. Finally, ferulic acid was eluted and its activity was determined using the gas flow counter.

The residue (1·0 g) was hydrolysed under reflux for 3 hr with N NaOH (50 ml) and labelled ferulic acid separated from the hydrolysate in the same manner as for the ethanolic extract. Acid hydrolysis of the residue (0·2 g) was carried out by heating in a sealed tube for 16 hr at 120° with 6 N HCl (3·0 ml). The hydrolysate was examined chromatographically for labelled 3-methoxy-4-hydroxyphenylalanine and other amino acids by developing in BAW, scanning and spraying with ninhydrin. Nitrobenzene oxidations were carried out on the original residue (II), the residue after alkaline hydrolysis (III) and on the alkaline hydrolysate after removal of ferulic acid by acidification and ether extraction, by heating in 2 N NaOH at 160° for 3 hr using stainless-steel bombs. Vanillin and syringaldehyde were separated by the paper chromatographic method of Bland and Stamp[10] and their radioactivities determined using the gas flow counter. Klason lignin was isolated from the residue according to the Tappi[11] standard procedure, and its radioactivity measured using the gas flow counter.

Test for Ammonia-lyase Activity

Acetone dried powder (0·4 g) in 10 ml of solution containing 0·15 g/100 ml of 3-methoxy-4-hydroxyphenylalanine in tris buffer at pH 8·8 was kept in a water bath at 40° for 24 hr. The mixture was filtered, the filtrate acidified and extracted with ether. The ether-soluble material was transferred to a paper chromatogram which was developed with BzAW.

[9] S. LAKSHMINARAYANA, *Lab. Prac.* **17**, 214 (1968).
[10] D. E. BLAND and C. STAMP, *Australian J. Appl. Sci.* **6**, 353 (1955).
[11] Tappi Standard T13-m54.