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LIGNIFICATION IN *EUCALYPTUS*.

THE METABOLISM OF PHENYLALANINE AND CINNAMYL COMPOUNDS

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Abstract—Phenylalanine and cinnamyl compounds have been infused into shoots of *Eucalyptus botryoides* and transformation products identified. The principal products identified were glucose esters of cinnamic acids. Sinapic acid, besides giving its glucose ester, gave another substance which has been shown to be identical with a product given by air oxidation of sinapic acid in buffer. This product has been found to retain all its carboxyl but only about a fifth of its phenolic hydroxyl and a reduced number of its ethylenic bonds. Its chromatographic properties and its composition indicate it to be an ether-linked sinapic acid polymer. Free *p*-coumaric acid appeared as a product from a number of different precursors. Its accumulation in the free state indicates that it may not be on the main biosynthetic pathway from phenylalanine to lignin in *Eucalyptus*. A ninhydrin positive substance which gave phenylalanine by alkali hydrolysis was detected as a product from phenylalanine. The route from phenylalanine to lignin may therefore involve deamination, hydroxylation and methylation of phenylalanine combined as an ester.

INTRODUCTION

IT HAS been shown[1] that phenylalanine acts as a lignin precursor in *Eucalyptus* spp. but tyrosine was found to be a much less efficient precursor. Brown[2] found that in grasses tyrosine was utilized as a precursor as efficiently as phenylalanine. Neish[3] showed this to be made possible by the presence of tyrosine ammonia-lyase. McCalla and Neish[4] in experiments with *Salvia* found evidence that the route from phenylalanine to lignin was through the series cinnamic, *p*-coumaric, caffeic, ferulic and sinapic acids.

This appears to be the generally accepted and most obvious route from phenylalanine to lignin and is dependent on deamination of phenylalanine to cinnamic acid. However, El-Basyouni, Neish and Towers,[5] as a result of their studies of the metabolism of phenolic acids in wheat, postulated that 'active insoluble esters' occurred as intermediates between phenylalanine and lignin. Thus free cinnamic and hydroxycinnamic acids may not be natural intermediates in the formation of lignin in some plants at least. Also, Hillis and Isoi[6] were unable to detect L-phenylalanine ammonia-lyase in three *Eucalyptus* spp. nor has it been detected in the present series of experiments. The possibility exists that it cannot be detected by the standard test because of the deactivating action of phenolic substances present in the leaves of *Eucalyptus* spp.[6] Young, Towers and Neish[7] have noted the inhibition of phenylalanine ammonia-lyase activity by some acetone powders.

[1] D. E. BLAND, *Biochem. J.* **88**, 523 (1963).
[2] S. A. BROWN, *Can. J. Botany* **39**, 253 (1961).
[3] A. C. NEISH, *Phytochem.* **1**, 1 (1961).
[4] R. D. MCCALLA and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 537 (1959).
[5] S. Z. EL-BASYOUNI, A. C. NEISH and G. H. N. TOWERS, *Phytochem.* **3**, 627 (1964).
[6] W. E. HILLIS and K. ISOI, *Phytochem.* **4**, 905 (1965).
[7] M. R. YOUNG, G. H. N. TOWERS and A. C. NEISH, *Can. J. Botany* **44**, 341 (1966).

In the absence of direct evidence for the presence of the enzyme, the route from phenylalanine to lignin in *Eucalyptus* remains uncertain. Harborne and Corner[8] observed the formation of sugar derivatives when hydroxycinnamic acids were fed to a number of plants and suggested that hydroxylation of glucose esters of cinnamic acids might take place. Glennie and Bohm[9] observed glucose ester formation in *Pinus* and other genera. Levy and Zucker[10] found evidence that cinnamic acid, while combined as its quinic ester, was hydroxylated to give the caffeoyl-quinic ester (chlorogenic acid).

Transformation products of a number of cinnamyl compounds fed to shoots of *Eucalyptus botryoides* Sm. have now been identified and their possible relation to the lignin biosynthetic pathway is considered.

RESULTS

The compounds fed to the shoots and the products identified after metabolism for 24 hr are shown in Table 1. The new products were detected by comparison of paper chromatograms of extracts prepared as described in "Experimental" with similar chromatograms from shoots infused with buffer or water only. The occurrence of *p*-coumaric acid as a transformation product of cinnamic acid is as expected. Its formation also from the alcohol and aldehyde is noteworthy as this involves oxidation at the terminal carbon of the side chain as well as *p*-hydroxylation. Hydroxylation in the *para* position of *m*-methoxycinnamic acid would have given rise to ferulic acid and of 3,5-dimethoxycinnamic acid to sinapic acid but neither of these products were detected although they are readily recognizable on chromatograms. A phenolic substance detected by its reaction with the diazotized sulphanilic acid spray was observed from the *m*-methoxy- and 3,4-dimethoxycinnamic acids and in trace amounts from *m*-coumaric and sinapic acids. A trace quantity of caffeic acid was detected from the *m*-coumaric acid and also in a long-term test with *p*-coumaric acid. Phenylalanine and tyrosine both gave rise to *p*-coumaric acid. Free *p*-coumaric acid also occurred as a product of glucosido-*p*-coumaric acid and in addition to this, *p*-coumaroylglucose was produced.

As shown in Table 1, the formation of glucose esters from the free acids took place in several cases. Glucose esters were formed also when the glucosides of ferulic and *p*-coumaric acid were fed to the shoots but this was accompanied by the liberation of some free acid. The esters could be detected by observing their colours in u.v. light and the changes of these colours brought about by exposure to ammonia vapour. The *m*-coumaric ester was visible only under short wave length u.v. light, 254 nm. *p*-Coumaroylglucose, feruloylglucose and sinapoylglucose were identified by co-chromatography in three solvents with authentic specimens and by alkali hydrolysis to give the original acids. Both methods were applied also to the identification of the glucose esters formed from the corresponding glucosides of *p*-coumaric and ferulic acids.

The identity of all seven glucose esters formed was confirmed by enzymatic hydrolysis to the original acid and glucose. This follows the demonstration by Harborne and Corner[8] and Runeckles and Woolrich[11] that these esters are hydrolysed by β-glucosidase to the acid and glucose.

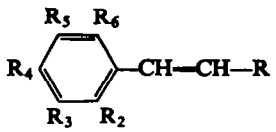
Sinapic acid, besides giving its glucose ester, gave rise to a derivative which showed on a paper chromatogram as a blue–green fluorescence in u.v. light changing to strong green with

[8] J. B. HARBORNE and J. J. CORNER, *Biochem. J.* **81**, 242 (1961).
[9] C. W. GLENNIE and B. A. BOHM, *Can. J. Biochem. Physiol.* **44**, 281 (1966).
[10] C. C. LEVY and M. ZUCKER, *J. Biol. Chem.* **235**, 2418 (1960).
[11] V. C. RUNECKLES and K. WOOLRICH, *Phytochem.* **2**, 1 (1963).

TABLE 1. TRANSFORMATIONS OF CINNAMYL COMPOUNDS IN *E. botryoides* SHOOTS

Substance fed	Solvent	Concentration (%)	Products identified
Cinnamic acid II	Aq	0.03	*p*-Coumaric acid
	B	0·5	*p*-Coumaric acid
Cinnamylalcohol III	Aq	0·5 approx	*p*-Coumaric acid
Cinnamaldehyde IV	B	0·5	*p*-Coumaric acid
m-Methoxycinnamic acid V	Aq	0·03	Phenolic substance†
p-Methoxycinnamic acid VI	B	0·5	None
3,4-Dimethoxycinnamic acid VII	Aq	0·03	Phenolic substance† (tr)
	B	0·5	3,4-Dimethoxycinnamoyl-glucose XVIII
3,5-Dimethoxycinnamic acid VIII	Aq	0·03	None
	B		None
o-Coumaric acid IX	Aq	0·03	*o*-Coumaroylglucose XIX
	B	0·5	*o*-Coumaroylglucose
m-Coumaric acid X	Aq	0·03	Phenolic substance† (tr)
	B	0·5	Caffeic acid (tr), *m*-Coumaroyl-glucose XX
p-Coumaric acid XI	Aq	0·03	*p*-Coumaroylglucose (tr.). XXI, caffeic acid (tr)*
	B	0·5	*p*-Coumaroylglucose
Glucosido-*p*-coumaric acid XII	Aq	0·5	*p*-Coumaric acid, *p*-coumaroyl-glucose
Caffeic acid XIII	Aq	0·03	Caffeoylglucose XXII
	B	0·5	Caffeoylglucose
Ferulic acid XIV	Aq	0·03	None
	B	0·5	Feruloylglucose XXIII
Glucosidoferulic acid XV	Aq	0·03	Ferulic acid
	Aq	0·5	Ferulic acid, feruloylglucose
5-Hydroxyferulic acid XVI	Aq	0·03	Sinapic acid (tr)
	B	0·5	Sinapic acid (tr)
Sinapic acid XVII	Aq	0·03	Sinapic polymer
	B	0·5	Sinapic polymer, sinapoylglucose XXIV, phenolic substance+ (tr), unidentified substance
Phenylalanine	Aq	0·03	*p*-Coumaric acid
	B, Aq	0·5	*p*-Coumaric acid, ninhydrin positive substance
Tyrosine	Aq	0·03	None
	B	0·5	*p*-Coumaric acid

B = Aqueous 0·01 M Na_3PO_4.
* After 1 week.
† A substance at R_f 0·5 in BAW (12:3:5), red with diazotized sulphanilic acid spray.



R_2, R_3, R_4, R_5, R_6 = H where not specified.
II: R = COOH; III: R = CH_2OH; IV: R = CHO; V: R = COOH, R_3 = OCH_3; VI: R = COOH, R_4 = OCH_3; VII: R = COOH, R_3 = OCH_3, R_4 = OCH_3; VIII: R = COOH, R_3 = OCH_3, R_5 = OCH_3; IX: R = COOH, R_2 = OH; X: R = COOH, R_3 = OH; XI: R = COOH, R_4 = OH; XII: R = COOH, R_4 = OGlc; XIII: R = COOH, R_3 = OH, R_4 = OH; XIV: R = COOH, R_3 = OCH_3, R_4 = OH; XV: R = COOH, R_3 = OCH_3, R_4 = OGlc; XVI: R = COOH, R_3 = OCH_3, R_4 = OH, R_5 = OH; XVII: R = COOH, R_3 = OCH_3, R_4 = OH, R_5 = OCH_3; XVIII: R = COOGlc, R_3 = OCH_3, R_4 = OCH_3; XIX: R = COOGlc, R_2 = OH; XX: R = COOGlc, R_3 = OH; XXI: R = COOGlc, R_4 = OH; XXII: R = COOGlc, R_3 = OH, R_4 = OH; XXIII: R = COOGlc, R_3 = OCH_3, R_4 = OH; XXIV: R = COOGlc, R_3 = OCH_3, R_4 = OH, R_5 = OCH_3.

ammonia and as a dark red spot with diazotized sulphanilic acid spray. Another product, blue in u.v., of different R_f value and giving a purple colour with the diazotized spray was noticed in some experiments, but this had only a transient existence and was therefore thought to be an intermediate in formation of the above derivative.

Isolation of the sinapic derivative from shoots in sufficient quantity for detailed examination was not achieved. However, it was observed that when sinapic acid in borate buffer was subjected to oxidation in a slow air stream it was converted to the derivative. It could be identified with the product from the shoots by co-chromatography, its blue–green colour in u.v., red colour with diazotized sulphanilic acid spray and by its u.v. absorption spectrum. Analysis of the sinapic acid derivative showed that it retained all of its methoxyl groups. Potentiometric titration gave a curve which showed that the carboxyl content of the derivative was the same as that of the original acid but that the number of phenolic groups was greatly reduced. This is evident from the titration curves shown in Fig. 1. The equivalent weight

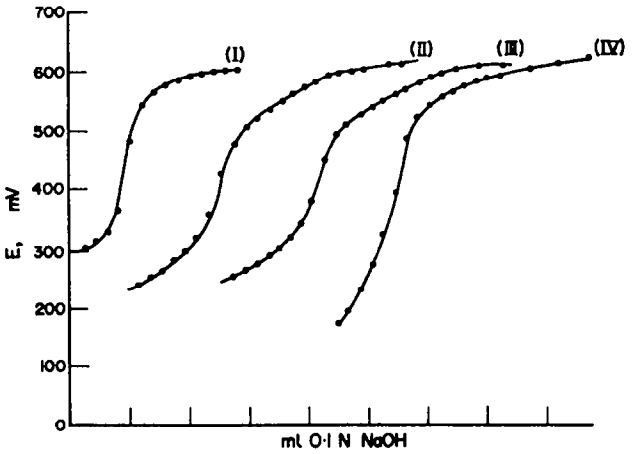


FIG. 1. POTENTIOMETRIC TITRATION CURVES OF CINNAMIC ACID (I), *p*-COUMARIC ACID (II), SINAPIC ACID (III) AND THE SINAPIC ACID DERIVATIVE (IV). THE CURVES ARE ARBITRARILY DISPLACED TO FACILITATE COMPARISON OF THEIR SHAPES.

calculated from the curve was the same for the derivative (222) as for sinapic acid (214, Theor. 214). The upper portion of the curve for cinnamic acid shows no straight part corresponding to phenolic groups as shown by *p*-coumaric acid and sinapic acid but the curve for the sinapic derivative shows only a limited straight section. Comparison with 3,5-dimethoxycinnamic acid was not possible because of the limited solubility of this acid.

Reaction of the derivative with potassium nitrosodisulphonate (Fremy's salt) gave a colour similar to that given by sinapic acid but the extinction at 351 nm of the quinone formed was only 13 per cent of that formed from sinapic acid. It has been shown by Adler and Lundquist[12] that this reagent yields 3-methoxy-1,2-benzoquinones with uncondensed guaiacyl lignin model compounds but in some cases *p*-oxidative cleavage occurs. The reaction gives no information about non-phenolic units. The expected product from syringyl units would be 3,5-dimethoxy-1,4-benzoquinone and the weak reaction of the derivative is in agreement with the low number of phenolic units indicated by potentiometric titration.

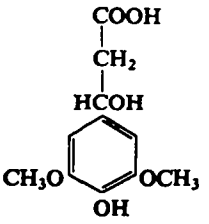
The u.v. absorption curve of the derivative exhibited a shift of the principal maximum from

[12] E. ADLER and K. LUNDQUIST, *Acta Chem. Scand.* **15**, 223 (1961).

308 nm in aqueous methanol to 358 nm when made alkaline compared with a shift from 307 to 354 nm for sinapic acid under the same conditions. This clearly indicates the presence of phenolic groups. The alkali-neutral $\Delta\epsilon$ curve for the derivative had a clear maximum at 350 nm, (sinapic acid 357 nm) but the $\Delta\epsilon$ value, 3470 compared with 19,100 for sinapic acid, shows that only about one-fifth of the original phenolic groups belonging to nuclei with unsaturated side-chains remained free.

The i.r. spectrum determined in a KCl disc showed bands near 1704 and 1688 cm^{-1}; in solution in CH_2Cl_2, there were three bands near 1742, 1712 and 1675 cm^{-1}. Sinapic acid showed bands at 1685 (sh) and 1660 cm^{-1} in the disc and 1715 and 1684 cm^{-1} in solution. The new band in the derivative corresponds to an arylaliphatic acid or ester.[13] According to Hergert,[14] absorption from C=C stretching vibration occurs at 1608–1626 cm^{-1} and the =CH out of plane deformation is at 960–988 cm^{-1}. The intensity of the latter absorption was much lower in the spectrum of the derivative than in sinapic acid, but bands near 1615 cm^{-1} were of similar intensity in the spectra of both substances. However, adjacent bands arising from stretching vibrations of the benzene ring may also contribute to the intensity of the 1615 cm^{-1} band.

During the preparation of the derivative a definite reaction with *N*-chloro-*p*-quinoneimine was obtained but in the final product this reaction had disappeared. As this reagent is believed to be specific for *p*-hydroxybenzyl alcohol groups this points to the presence of (XXV) as an intermediate.



(XXV)

That the derivative is a head-to-tail-linked ester of either sinapic acid itself or of (XXV) is excluded because the substance was resistant to alkaline hydrolysis. Two methods of coupling to form a polymer remain possible, condensation of the phenolic hydroxyl of one unit with the benzyl alcohol group of another and coupling by oxidative dehydrogenation of the phenolic hydroxyl with the methylene group of another. However, coupling exclusively one way or the other would give a product not conforming to the observed elementary composition. The observed properties of the derivative are accounted for by coupling of four molecules of (XXV) with one of sinapic acid, the sinapic acid phenolic group remaining free.

If two of the four couplings were by condensation and two by oxidative coupling, each with the elimination of two hydrogen atoms, the process could be summarized as:

$$4C_{11}H_{12}O_5 + 4H_2O \rightarrow 4C_{11}H_{14}O_6$$
$$4C_{11}H_{14}O_5 + C_{11}H_{12}O_5 - 2H_2O - 4H \rightarrow C_{55}H_{60}O_{27}.$$

This corresponds to the composition found (see Experimental) and accounts for the decrease in ethylenic links and in phenolic hydroxyl. The derivative was too insoluble to permit determination of molecular weight. However, it is not believed that the substance isolated

[13] A. J. Michell, *Australian J. Chem.* **19**, 2285 (1966).
[14] H. L. Hergert, *J. Org. Chem.* **25**, 405 (1960).

represents a polymer of exactly five units but rather that it is a mixture of various polymers whose average composition is represented by the above scheme. This is borne out by the fact that it always appeared as an elongated spot on paper chromatograms and as a series of spots on thin-layer chromatograms.

A new ninhydrin positive substance at R_f 0·76 in BAW was detected from the infusion of *Eucalpytus* shoots with 0·5% phenylalanine in both water and buffer. Alkaline hydrolysis of this substance under the same conditions as employed in the identification of the esters gave back phenylalanine.

DISCUSSION

It can be seen from Table 1 that *p*-coumaric acid was produced from several different precursors and accumulated in sufficient quantity for ready detection. Caffeic acid and sinapic acid were detected only in small quantities from their most probable precursors *p*-coumaric and 5-hydroxyferulic acid respectively. Ferulic and 5-hydroxyferulic acid were not detected as products from their most likely precursors caffeic and ferulic acids respectively. The significance of these observations is difficult to assess from these experiments but there are two possible interpretations. The first is that the hydroxylation of *p*-coumaric acid is the slowest step in the metabolic pathway which causes it to accumulate. Note, however, that free *p*-coumaric acid was not detectable in normal shoots. The second interpretation is that *p*-coumaric acid does not lie on the main route from phenylalanine to the methoxylated cinnamic acids and it therefore accumulates as a side product.

Phenylalanine ammonia-lyase was not detected in the present tests. Higuchi and Barnoud[15] have detected it in *Eucalyptus globulus*, but Hillis and Isoi[6] found none in *E. sideroxylon*. Bland[1] detected tyrosine ammonia-lyase in a variant of *E. sideroxylon* but none in other eucalypts; Hillis and Isoi detected none in the variant of *E. sideroxylon* and weak activity in two other eucalypts and Higuchi and Barnoud detected none in *E. globulus*. It must be remembered that exactly the right conditions must be found for detecting these enzymes,[16] and as pointed out by Hillis and Isoi the possibility of deactivation of the enzyme by eucalypt polyphenols during preparation of the acetone-dried powder is a real one. Such inhibition has been noted by Young, Towers and Neish,[7] although there was no indication of the nature of the inhibitor. However, such evidence as is now available is more in accordance with the second interpretation of the fact that *p*-coumaric acid appears to accumulate, i.e. that it does not lie on the principal route from phenylalanine to lignin in *Eucalyptus*.

It appears to be well established that, except in grasses,[2] tyrosine is a less efficient lignin precursor than phenylalanine. This has been shown to be so for three eucalypts.[1] Despite this, as shown in Table 1, some *p*-coumaric acid accumulated from tyrosine when fed in the higher concentration as well as from phenylalanine. This suggests that the utilization of *p*-coumaric acid is a slow step which would limit the utilization of phenylalanine also.

Indications of an alternate pathway are shown by the formation of a new ninhydrin positive substance during phenylalanine infusion (Table 1). As this gave phenylalanine on hydrolysis it shows that combination of phenylalanine before hydroxylation and methylation does occur. Thus a biosynthetic pathway from phenylalanine to lignin other than that through free cinnamic and coumaric acids may exist. A similar mechanism is known in the hydroxylation of proline after incorporation into peptides (Udenfriend[17]).

[15] T. HIGUCHI and F. BARNOUD, *J. Japan Wood Res. Soc.* **12**, 36 (1966).
[16] E. E. CONN, Personal communication.
[17] S. UDENFRIEND, *Science* **152**, 1335 (1966).

It is fairly clear from the results discussed above that esterification of cinnamic acids occurred readily in *E. botryoides*. The formation of glucose esters also when the glucosides were fed is of importance. This result cannot be taken to prove rearrangement of the glucoside to the ester but rather formation via the free acids, as these were observed to be present at the same time. Conclusive evidence for direct deamination of phenylalanine to cinnamic acid has not yet been obtained but there are experimental difficulties which need to be overcome before any final conclusion can be made on this point. *p*-Hydroxylation of aromatic nuclei clearly takes place in *Eucalyptus* shoots but there was no evidence of *o*-hydroxylation. In experiments on the non-enzymatic hydroxylation of cinnamic acid under the conditions specified by Dewhurst and Calcutt[18] it was found that *o*- and *p*-hydroxylation occurred to roughly comparable extents. This appears to support the concept that *p*-hydroxylation in the shoots is enzymatic. Hydroxylation of glucose esters could occur as suggested by Harborne and Corner.[8] Indications of a similar mechanism in the biosynthesis of chlorogenic acid by hydroxylation of cinnamoylquinic acid were observed by Levy and Zucker.[10]

Direct evidence of the order in which guaiacyl and syringyl units are united in angiosperm lignin is lacking. Freudenberg's schematic formula[19] which takes account of the most important data on gymnosperm lignin shows the guaiacyl units to be linked by aryl–alkyl ether bonds, by carbon–carbon bonds between the side chain of one phenylpropane unit and the aromatic ring of another and also by biphenyl linkage of aromatic rings. The formation of carbon–carbon bonds between syringyl and guaiacyl units would be limited to bonding of the side chain of the syringyl unit to position 5 of the nucleus of a guaiacyl unit since this position is occupied by a methoxyl in the syringyl unit. There is no evidence of linkage at the 6-position of syringyl units, no appreciable amount of trimethoxy-*o*-phthalic acid having been found in oxidation products from methylated lignin[20] or of 4-hydroxy-3,5-dimethoxyphthalic acid from sinapic polymer.[21] There is however, no apparent barrier to the formation of aryl–alkyl ether bonds between syringyl units and the properties of the sinapic derivative discovered during the present studies indicate that it is formed in this way. Its formation by non-enzymatic oxidation in buffer as well as in shoots and the failure of ferulic acid to yield a similar derivative, show that sinapyl units have a strong tendency to polymerize in this way. There is no evidence of the existence of a syringyl lignin in nature although a lignin-like syringyl polymer has been prepared artificially.[21] In view of the present studies, the occurrence of aryl–alkyl ether linkages between syringyl units in angiosperm lignins now appears probable.

EXPERIMENTAL

Materials

The glucosidoferulic acid was prepared by the method of Hann.[22] Glucosido-*p*-coumaric acid was prepared by the same method but not obtained crystalline; its identity was established by treatment with β-glucosidase to give *p*-coumaric acid and glucose. 1-Feruloylglucose was prepared according to Birkhofer *et al.*[23] Reference specimens of 1-*p*-coumaroyl- and 1-sinapoyl-glucose were kindly supplied by Dr. J. B. Harborne. The 5-hydroxyferulic acid was prepared by the method of Nakamura *et al.*[24]

[18] F. Dewhurst and G. Calcutt, *Nature* **191**, 808 (1961).
[19] K. Freudenberg, *Holzforschung* **18**, 3 (1964).
[20] K. Freudenberg, Chen-Loung Chen and Giampietro Cardinale, *Chem. Ber.* **95**, 2814 (1962).
[21] D. E. Bland and A. F. Logan, *Biochem. J.* **95**, 515 (1965).
[22] R. M. Hann, *J. Am. Chem. Soc.* **56**, 1631 (1934).
[23] L. Birkhofer, C. Kaiser, W. Nouvertné and U. Thomas, *Z. Naturforsch.* **166**, 251 (1961).
[24] Takahiro Nakamura, Yashuhiro Murase, Ryozo Hayashi and Yonekichi Endo, *Chem. Pharm. Bull. (Tokyo)* **10**, 281 (1962).

Chromatographic Solvents

The following three were employed: BzAW, Benzene–acetic acid–water (4:2:1); BAW, *n*-Butanol–acetic acid–water (12:3:5); and 6HA, Acetic acid, 6% aqueous. Thin-layer chromatography was carried out using chloroform–acetic acid (8:2).

Infusion of Compounds and Preparation of Extracts

Freshly cut growing shoots of *Eucalyptus botyroides* with about eight leaves were placed in a solution of the compound in water (usually 0·03%) or in 0·01 M Na_3PO_4 buffer (0·5%) and allowed to stand in direct sunlight until the solution was taken up. About 10 ml of buffer solution or 20 ml of water solution were usually absorbed in 24 hr. The presence of transformation products was first noted by extraction of chopped shoots with boiling ethanol for 3 min. The presence of chlorophyll in this extract made paper chromatographic examination of this extract difficult. In order to obtain complete extraction of these products in a form amenable to chromatographic examination the following procedure was adopted. The entire shoot was chopped and crushed in a mortar with ethanol, transferred with ethanol to a Soxhlet extractor and extracted for 24 hr. The ethanol extract was concentrated to about 10 ml and added dropwise with stirring to 500 ml of water. The aqueous suspension was extracted for 24 hr with light petroleum (b.p. 60–80°) to remove chlorophyll. The aqueous layer was then extracted with ether for 48 hr. The ether extract was concentrated to dryness, taken up into about 10 ml ethanol and amounts of 100–300 μl applied to paper chromatograms. Examination of the extracts consisted of development in the first instance with BzAW. The BzAW chromatograms were viewed under u.v. (365 nm) and short wave length (254 nm) u.v. lamps and the sheets were then sprayed with diazotized sulphanilic acid according to Billek and Kindl.[25] Extracts were also examined by developing first with BzAW as mentioned above and then on the same sheet after drying, with BAW in the same direction. In all cases the above extracts were compared with a blank extract from a shoot stood in water or buffer only.

Preparation of Sinapic Acid Derivative

Sinapic acid (0·5 g) was dissolved in borate buffer (0·05 M, 200 ml) and the solution maintained at 40° with a slow stream of air bubbling through it. At intervals, tests were made by withdrawing 0·5 ml, acidifying with HCl and evaporating to dryness. The dry residue was stirred with a few drops of ethanol and a drop of the ethanol solution applied to a paper chromatogram, which was developed in BzAW, viewed in u.v. and sprayed with diazotized sulphanilic acid. In this way the disappearance of the sinapic acid and the appearance of a new compound giving a red colour at R_f 0·1–0·2 could be followed. This change was complete in about 16 hr. The solution was acidified with hydrochloric acid and extracted with ether continuously for 48 hr. The ether was shaken with water in a separating funnel; all of the new compound passed into the water from which it was again extracted in a continuous extractor by ether. It was again taken into water, extracted by ether, retransferred to water and finally recovered by evaporation to a small volume under reduced pressure, cooling and collecting the compound by filtration. This procedure was necessary in order to obtain the product free from boric acid. Yield 0·2 g, composition: (Found: C, 57·2; H, 5·4; O, 37·4; OCH_3, 26·6% corrected for 0·5% ash). (Calc. for $C_{55}H_{60}O_{27}$: C, 57·3; H, 5·2; O, 37·5; 10 OCH_3, 26·9%).

Examination of Sinapic Acid Derivative

Potentiometric titrations of the sinapic acid derivative, also of sinapic acid, cinnamic acid and 4-hydroxycinnamic (*p*-coumaric) acid were carried out by titrating about 0·03 g of the acid in aqueous solution with 0·1 N NaOH using an antimony electrode. The u.v. absorption spectrum of the substance in neutral and alkaline solution was determined on an "Optica" double beam recording spectrophotometer. The i.r. absorption spectrum was determined in a potassium chloride disc and in methylene chloride. The *N*-chloro-*p*-quinoneimine reaction was carried out according to Gierer[26] on the sinapic acid solution during the preparation of the derivative.

Tests for Enzymes

Tests for tyrosine ammonia-lyase were carried out according to Neish.[3] Phenylalanine deaminase was tested for by incubation of acetone-dried powder with L-phenylalanine according to Koukol and Conn.[27] The solution was acidified, extracted with ether, and the extract transferred to a paper chromatogram which was developed in BzAW. Reference spots of cinnamic acid were located by spraying with 1% $KMnO_4$ solution. Areas corresponding to the cinnamic acid reference spots were extracted and the u.v. absorption spectrum determined.

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Identification of Esters

Sufficient ether extract was resolved on a paper chromatogram using BzAW followed by BAW to give six clearly visible spots of the substance to be examined. These spots were cut out, and the paper pieces refluxed with 20 ml 0·5 N NaOH for 30 min. Blanks on the same amount of paper were also run. The solution was acidified, filtered and extracted with ether, the ether washings taken to dryness and the residue taken up in ethanol and transferred to a chromatogram. The acid present was identified by co-chromatography. For enzymatic hydrolysis the dried ethanol extract was dissolved in 0·5 ml water and *ca.* 1 mg β-glucosidase added. After standing 48 hr at 20° the solution was heated in a boiling water bath to destroy the enzyme and transferred to a paper chromatogram on which reference spots of the suspected acids and sugars had been placed. After development in BAW the chromatogram could conveniently be cut in half and the top half sprayed with diazotized sulphanilic acid to confirm the identity of the phenolic acid and the lower half with silver nitrate reagent[28] to confirm the identity of the sugar. Where specimens of known glucose esters were available, identity was confirmed by co-chromatography in the three solvent systems.

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