The Origin of the Wood and Bark Polyphenols of *Eucalyptus* Species

BY W. E. HILLIS AND ANN CARLE

Division of Forest Products, C.S.I.R.O., Melbourne, Australia

(Received 24 July 1961)

Several workers have subscribed to the view that polyphenols, like auxins, viruses, herbicides, etc., are translocated throughout the plant. In particular, the cambium has been considered to be the origin of the bark and heartwood polyphenols. The validity of this viewpoint could be tested if a comparison was made of the composition of the extractives in different tissues. This paper reports an examination and comparison of the polyphenols in various tissues of *Eucalyptus astringens* and *Eucalyptus marginata*, the polyphenols being identified by chromatographic methods.

EXPERIMENTAL

Materials

Eucalyptus astringens. Branches from trees, grown near Melbourne, were collected during the spring seasons of 1959 and 1960. The cambial tissues were removed, with a blunt scraper, within 6 hr. of collection of the samples, dropped into a large excess of hot methanol, boiled for 5 min. and, after cooling, stored at 0–4°. The fresh bark was thinly sliced, pulped in acetone with a high-speed propellor-type disintegrator, and the pulp washed with acetone and then methanol until almost all the polyphenols were removed; the extracts were evaporated under vacuum. The wood samples were stored at 0–4° until required, and then extracted with methanol in a Soxhlet apparatus.

Eucalyptus marginata. Small branches collected in Western Australia during the spring seasons of 1959 and 1960 were sealed in polythene bags, and within 5 days the cambial tissues were removed as described above. The other tissues were stored at 0–4° and then extracted with methanol.

Chromatographic methods

The methods have been described by Hillis & Carle (1960). The reference compounds used were (+)-catechin, cyanidin and delphinidin.

Developing solvents. The solvents used were (1) 6% acetic acid; (2) butan-1-ol-acetic acid-water (6:1:2, by vol.); (3) conc. HCl-acetic acid-water (3:30:10, by vol.; Forestal solvent; Bate-Smith, 1954); (4) *m*-cresol-acetic acid-water (50:1:49, by vol.; Bate-Smith, 1954); (5) formic acid-conc. HCl-water (5:2:3, by vol.; Harborne, 1958); (6) ethyl acetate-pyridine-water (12:5:4; Isherwood & Jermyn, 1951); (7) butan-1-ol-acetic acid-water (2:1:1); (8) butyl acetate-pyridine-ethanol-water (8:2:2:1).

Colour reactions. After the chromatograms had been air-dried for 2–5 hr. the resolved constituents were detected by the colour given under the following conditions: (a) in daylight; (b) in u.v. light; (c) in u.v. light and with exposure to fumes from aq. NH₃ soln. (sp.gr. 0.88). The following

chromogenic reagents were used: (d) ferric chloride-potassium ferricyanide (Barton, Evans & Gardner, 1952); (e) Diazo Fast Red GG (diazotized *p*-nitroaniline, Rohner Ltd., Switzerland; 0.5%, w/v) in 20% sodium acetate; (f) vanillin-HCl (Bate-Smith & Swain, 1953); (g) 5% KCN soln. (Young, 1983); (h) AgNO₃-NH₃ soln. (Partridge & Westall, 1948); (i) dilute KMnO₄ soln.

Isolation of components. The components present in the various extracts from the two species have been numbered 1–33. The location of these components on a two-dimensional chromatogram developed with solvents (1) and (2) is shown in Figs. 1 and 2.

Small amounts of components 2, 3, 4, 5, 7, 8 and 10 were isolated by streaking partly purified extracts on Whatman no. 3 paper sheets and developing in water or solvent (1). Bands were identified in u.v. light or by spraying a strip cut from the side of the sheet with chromogenic reagents (d) and (e). The bands on the rest of the sheet were cut out, extracted in a Soxhlet apparatus and the vacuum-concentrated extract was streaked as above and developed in solvent (2). When necessary, part or all of the above process was repeated.

Methods of identification

Components were compared with authentic materials subjected to the same treatment at the same time; methods used to identify certain components are given below.

Chlorogenic acid. Two-dimensional co-chromatography with solvents (1) and (2); colour reactions (b)–(e); hydrolysis and chromatographic identification of caffeic acid; u.v.-absorption spectra in ethanol and 2 MN-sodium ethylate in ethanol.

Catechin and gallo catechin. Two-dimensional co-chromatography with solvents (1) and (2); colour reactions (b), (d), (e) and (f).

Ellagic acid. Co-chromatography in solvents (1), (2) and (3); colour reactions (b)–(e); u.v.-absorption spectra in ethanol.

Gallic acid. Two-dimensional co-chromatography with solvents (1) and (2); one dimensional co-chromatography with solvent (4); colour reactions (b)–(e) and (g).

Resveratrol and resveratrol-3-β-D-glucoside. Two-dimensional co-chromatography with solvents (1) and (2), one-dimensional co-chromatography with solvents (3) and (4), colour reactions (b)–(f).

Glucose. Two-dimensional co-chromatography with solvents (1) and (2); co-chromatography in solvents (6), (7) and (8); colour reaction (h).

Examination of ellagitannins

A number of components (nos. 18–26 and 32) present in different tissues were opaque in u.v. light and gave a pink or slightly yellow colour with spray (e), a strong blue with spray (d) and no colour with spray (f). Except for the

polyphenols in the bark of *E. marginata*, small amounts of the monomeric ellagitannins (see Table 1) could be obtained pure by paper chromatography.

Ellagitannins were hydrolysed with 2N-H₂SO₄ on a boiling-water bath for 30 min. and ellagic acid and gallic acid were detected in the hydrolysate by the methods indicated previously. Glucose was detected after a portion of the hydrolysate was passed through an anion-exchange resin (Duolite A4, Chemical Process Co., Redwood City, Calif., U.S.A.).

Examination of flavans

When the extract of *E. marginata* heartwood was heated with butanol-conc. HCl (100:5, v/v) and the products were chromatographed in solvents (2), (3) and (5), delphinidin and cyanidin were detected.

Three fractions (*A*, *R_F* 0.0-0.05; *B*, *R_F* 0.05-0.15; *C*, *R_F* 0.15-0.25; *R_F* values in solvent 2) were obtained from the extract of the bark of *E. astringens* by paper-chromatographic methods. The fractions were heated with butanol-conc. HCl and the products chromatographed in solvents (2), (3) and (5). Fraction *A* yielded delphinidin, cyanidin and pelargonidin, fraction *B* cyanidin, pelargonidin and traces of delphinidin, fraction *C* pelargonidin.

Components nos. 27 and 29-33 in *E. astringens* bark and components nos. 28, 29 and 33 in *E. marginata* heartwood gave strongly positive colours with sprays (*d*), (*e*) and (*f*). The relative amount of component no. 29 was greatest in *E. astringens* bark.

An aqueous solution of the extract of *E. astringens* bark was extracted with ethyl acetate in a liquid-liquid extractor for periods of 6-7 hr., and at the end of each period new solvent was used. The extracts were dried over anhydrous Na₂SO₄ for 2 days, and evaporated *in vacuo* under N₂ at less than 60°.

The monomeric components (nos. 3-30) formed a major portion of the first six extractions but the amount of polymeric material increased in the subsequent extractions. The monomers were successfully separated on cellulose columns, which were developed with water. However, components nos. 18-31 could not be recovered from the dilute eluates before the greater portion had polymerized. Attempted separations on polyamide or silica-gel columns or stepwise precipitation of dried ethyl acetate solutions with light petroleum (40-60°) were unsuccessful.

Small amounts of compound no. 29 were obtained by paper-chromatographic methods. The yields of the pale-brown amorphous powder were low and the compound could not be obtained free from polymeric impurities. Compound no. 29 was immobile when chromatographed with butanol-water with borate-impregnated paper. It gave a red-brown solution when heated with butanol-HCl, and when this solution was chromatographed in solvents (2), (3) or (5) the only anthocyanidin detected was pelargonidin; carbohydrate could not be detected in this solution.

Examination of stilbenes

Some of the components in the extract of *E. astringens* bark possessed fluorescent colours similar to those of the stilbenes in the heartwood. In addition, they gave similar colour reactions with sprays (*f*) and (*i*). Very small amounts of resveratrol-3-β-D-glucoside (no. 7) and the glucoside of 4'-methoxy-3,5,3'-trihydroxystilbene (rhapontin, no. 11) appeared to be present. The most important

components in this class were nos. 12 and 13, present in equal amounts.

A concentrate of these components was prepared by repeatedly extracting an aqueous solution of bark extract with pentan-1-ol. The alcohol extracts were evaporated under vacuum at less than 50°. Isolation of components nos. 12 and 13 by means of adsorption columns was unsuccessful as the low solubility in eluting solvents and the instability of the stilbenes resulted in partial polymerization before recovery could be completed. Small amounts of these components (accompanied by polymeric material) were obtained by paper-chromatographic methods. Hydrolysis of the latter sample with 2N-HCl gave only glucose and one stilbene. The latter, like the glucosides, possessed in u.v. light a characteristic intense light-blue fluorescence which, when brought into contact with ammonia vapour, changed to an intense green fluorescence. Both aglucone and glucosides decolorized reagent (*i*) and gave a strong blue with (*d*) and a pink with (*f*).

An aqueous solution of the pentanol extracts was hydrolysed with 2N-HCl on a boiling-water bath for 2 hr. and extracted with ether; the evaporated extract was dissolved in a small amount of water and placed on a polyamide column. After elution with aq. 30% ethanol, the stilbenes were removed with 100% ethanol. The yield was less than 0.005% of dry bark. Attempts to recrystallize and to prepare the acetate of the aglucone were unsuccessful.

The major stilbene in a methanol extract of *Vouacapaou americana* (3,5,3',4',5'-pentahydroxystilbene; King, King, Goodson & Manning, 1956) possessed the same *R_F* values in solvents (2), (3) or (4) as did the aglucone of component nos. 12 and 13 (and component no. 6 in the heartwood), when chromatographed at the same time. The *R_F* values of both were reduced by the same extent when chromatographed on borate-impregnated paper with the upper layer of butanol-water. The fluorescent colours before and after treatment with ammonia were also identical as well as the u.v.-absorption spectra.

RESULTS

Eucalyptus astringens

Catechin (no. 3) and gallic acid (no. 4) were present in all tissues but the nature of the other polyphenols was different in different tissues.

The bark contained chlorogenic acid (no. 5, Fig. 2), flavans [nos. 3, 8, 27 (trace), 29, 30, 31 and 33] and stilbene glucosides (nos. 12 and 13). The monomeric flavan component no. 29 was present in greater amounts than components nos. 27, 30 and 31. The instability of these materials prevented characterization, but sufficient amounts of a concentrate of component no. 29 were obtained to permit the collection of some data.

The colour reactions show that component no. 29 is a 5,7-dihydroxyflavan possibly containing also three vicinal hydroxy groups. The colour reactions and chromatographic properties of components nos. 27-31 indicate that they have very similar compositions. A comparison of the *R_M* values (Bradfield & Bate-Smith, 1950) of component no. 29, (+)-catechin and (+)-galocatechin in solvent

(2) indicates the presence of six phenolic hydroxyl groups, but such a compound would not possess the high R_F value in solvent (1) (Fig. 2). After completion of this work Hergert (1960) reported the R_F values of a number of flavans including uncharacterized leucodelphinidins. When the R_F values reported by Hergert and those found in the present investigation were converted into R_M values, it was found that one of his leucodelphinidins bore the same chromatographic relationship to catechin as did compound no. 29.

Chromatographic comparison of a partially purified extract (by the method of Ganguli & Seshadri, 1959) of *Myrica magi* indicated that the leucodelphinidin reported to be present (Laumas & Seshadri, 1958) is different from component no. 29. However, only small amounts of pelargonidin were detected when this latter compound was heated with butanol-HCl. As component no. 29 could not be obtained pure, it could not be determined whether the pelargonidin arose from contaminating polymeric substances or whether

the corresponding leucoanthocyanin exists as a bimolecular compound with a catechin (Forsyth, 1955; Forsyth & Roberts, 1960).

Leucodelphinidin, leucocyanidin and leucopelargonidin were present in the extracts of *E. astringens* bark (cf. Hillis, 1954) but they existed almost entirely in the polymerized form.

The stilbene glucosides (nos. 12 and 13) were conspicuous components of the bark extract. However, the particularly intense fluorescence in u.v. light of small amounts of a concentrate of these stilbenes suggested that the amount in the bark was low. The low yield obtained on extraction is probably due also to the instability of these materials. The properties of components nos. 12 and 13 are consistent with the view that they are *cis-trans* isomers of the glucoside of 3,5,3',4',5'-pentahydroxystilbene.

The major portion of the monomeric polyphenols of the cambium of *E. astringens* was composed of ellagitannins [nos. 18, 19 (trace) and 24; Fig. 1]. Catechin (no. 3), gallic acid (no. 4), gallic acid (no. 4), polymerized flavans (no. 33) and polymerized ellagitannins (no. 26) were also present.

The polyphenols of the sapwood of *E. astringens* were markedly different from those of the heartwood in that the sapwood contained the monomeric flavans [nos. 28, 29 and traces of 8 (gallic acid) and 27, Fig. 2] and the ellagitannin no. 18, whereas

Fig. 1. A composite two-dimensional chromatogram showing the major polyphenols in the cambium of *E. astringens* and the cambium and bark of *E. marginata*. The chromatogram was run first in 6% acetic acid followed by butan-1-ol-acetic acid-water (6:1:2, by vol.). Components identified: 2, resveratrol; 3, catechin; 4, gallic acid; 5, chlorogenic acid; 7, resveratrol-3- β -D-glucoside; 8, gallic acid; 10, ellagic acid. Component 6 is probably 3,5,3',4',5'-pentahydroxystilbene and 12 and 13 its glucosides; 11 is possibly the glucoside of 4'-methoxy-3,5,3'-trihydroxystilbene. Components 18-26 and 32 are ellagitannins, 27-31 and 33 are flavans; 1, 6, 9 and 11-17 have not been characterized.

Fig. 2. A composite two-dimensional chromatogram showing the major polyphenols in the bark, sapwood and heartwood of *E. astringens* and the sapwood and heartwood of *E. marginata*. The solvents used and the key to the numbers are the same as for Fig. 1.

the heartwood contained one monomeric flavan (no. 29), appreciable amounts of different stilbenes (resveratrol, no. 2, and its glucoside, no. 7, and another stilbene, no. 6), chlorogenic acid, polymerized ellagitannins (no. 32), traces of ellagic acid and the unidentified components nos. 1 and 9. Two-dimensional chromatograms of an extract of *E. astringens* heartwood were very similar to those of *E. wandoo* heartwood (Hathway & Seakins, 1959a). Our work confirms the results of Hathway & Seakins (1959b) and indicates that the third stilbene observed by them is probably 3,5,3',4',5'-pentahydroxystilbene.

Eucalyptus marginata

Catechin (no. 3) and gallic acid (no. 4) were present in all tissues. The major components of the bark [nos. 18, 20, 22, 23 (trace), 25, 26; Fig. 1] and cambium [nos. 18 (trace), 20 (trace), 21 (trace), 23, 24, 26; Fig. 1] of this species were ellagitannins, although the composition of the mixture differed (Fig. 1). Polymerized flavans (no. 33) and traces of ellagitannin no. 10 were present in both tissues. The bark contained galocatechin (no. 8) and traces of components nos. 14 and 15 which were absent in the cambium. The composition of the mixtures of polyphenols in the sapwood and heartwood of *E. marginata* was very similar. The polyphenols were mainly of a flavan type (nos. 3, 8, 28, 29 and 33; Fig. 2), although the polymerized ellagitannins (no. 32) and trace amounts of ellagitannins nos. 18, 19 and 20 present in the sapwood were absent from the heartwood and replaced by ellagic acid (no. 10). The leucoanthocyanins appeared to exist almost entirely as polymers and when treated with HCl yielded delphinidin and cyanidin. Previously, Robinson & Robinson (1933) detected only leucodelphinidin.

DISCUSSION

If the translocation of polyphenols occurs in plants, the polyphenolic extractives of the various tissues might be expected to exhibit an obvious biochemical interrelationship. This supposition is difficult to reconcile with the different types and different hydroxylation patterns of the polyphenols present in the various tissues of *Eucalyptus astringens* and *E. marginata*.

The major portion of the monomeric components in the cambium of *E. astringens* and *E. marginata* is composed of ellagitannins. The polyphenols of *E. marginata* bark are similar to those in the cambium in that they contain a number of ellagitannins. However, those in *E. astringens* bark are distinctly different and contain, instead of ellagitannins, a number of flavans, stilbenes and chlorogenic acid. On this evidence, it is unlikely that the bark polyphenols originate in the cambium.

The polyphenols of the xylem tissues of both species are different from the cambial polyphenols. In the sapwood and heartwood of *E. marginata* they are mainly of a flavan type. In addition, the polyphenols of the sapwood of *E. astringens* are markedly different from those of the heartwood, which contains one of the two flavans present in the sapwood, appreciable amounts of different stilbenes, chlorogenic acid and an unidentified component.

Recent biosynthetic studies indicate that gallic acid and ellagic acid are end products of the shikimic acid pathway and also that it is improbable that these units in the ellagitannins would enter the biosynthetic pathway again to combine with acetate units to form flavans or stilbenes (Neish, 1960). It is most unlikely that one type of polyphenol or phenolic precursor is translocated throughout *E. astringens* and *E. marginata* trees to give rise to such a wide range of other polyphenols.

Carbohydrate metabolism leads to the cyclohexanecarboxylic acids related to shikimic acid, from which originate the immediate precursors of ellagic acid and gallic acid (Conn & Swain, 1961; Haslam, Haworth & Knowles, 1961; Hathway, 1956) of the C₆-C₃ residues of chlorogenic acid and the flavonoids (e.g. Geissman & Swain, 1957; Underhill, Watkin & Neish, 1957; Watkin, Underhill & Neish, 1957) and of one of the rings of the stilbenes (Birch & Donovan, 1953; Birch, 1957; Erdtman, 1958; Hathway & Seakins, 1959a). This present work does not exclude the possibility of translocation of these alicyclic precursors.

An explanation in better accord with the above-mentioned data is that sucrose and oligosaccharides (Zimmermann, 1960) are translocated and, together with stored carbohydrate, are converted *in situ* (Hillis & Carle, 1958, 1960; Hillis, 1958) into the relevant polyphenols by the local enzyme systems. Recent work involving the injection of [¹⁴C]glucose into the phloem and cambium of *E. sieberiana* and subsequent examination of the extractives yielded results supporting this view (W. E. Hillis & M. Hasegawa, unpublished results).

SUMMARY

1. Ellagitannins are the major monomeric components in the cambium of *Eucalyptus astringens* and *E. marginata*. Flavans are the most important components of the extractives of the bark of the former and the heartwood of the latter. Also, the composition of the extractives of the sapwood and the heartwood of *E. astringens* are different from each other and from the above-mentioned tissues. It is concluded that polyphenols are formed *in situ* probably from carbohydrate.

2. A flavan present in *E. astringens* bark and *E. marginata* heartwood has the expected chromatographic properties and colour reactions of leucodelphinidin but does not yield delphinidin when heated with acid.

3. The major stilbenes in the bark of *E. astringens* appear to be the previously unreported *cis-trans* isomers of a glucoside of 3,5,3',4',5'-penta-hydroxystilbene. The aglucone of these stilbenes appears to be present in the heartwood of this species.

The authors thank Dr D. E. Hathway for samples of authentic resveratrol and its glucoside, Professor T. R. Seshadri, F.R.S., for the sample of *Myrica magi* bark, Mr G. E. Brockway of the West Australian Department of Forests for collection of samples of *Eucalyptus marginata*. Miss E. Gloss is thanked for experimental assistance.

REFERENCES

- Barton, G. S., Evans, R. S. & Gardner, J. A. F. (1952). *Nature, Lond.*, **170**, 249.
- Bate-Smith, E. C. (1954). *Biochem. J.* **58**, 122.
- Bate-Smith, E. C. & Swain, T. (1953). *Chem. & Ind.* p. 377.
- Birch, A. J. (1957). In *Progress in the Chemistry of Organic Natural Products*, vol. 14, p. 186. Ed. by Zechmeister, L. Vienna: Springer Verlag.
- Birch, A. J. & Donovan, F. W. (1953). *Aust. J. Chem.* **6**, 360.
- Bradfield, A. E. & Bate-Smith, E. C. (1950). *Biochim. biophys. Acta*, **4**, 441.
- Conn, E. E. & Swain, T. (1961). *Chem. & Ind.* p. 592.
- Erdtman, H. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **2**, 1.
- Forsyth, W. G. C. (1955). *Biochem. J.* **60**, 108.
- Forsyth, W. G. C. & Roberts, J. B. (1960). *Biochem. J.* **74**, 374.
- Ganguli, A. K. & Seshadri, T. R. (1959). *Tetrahedron*, **6**, 21.
- Geissman, T. A. & Swain, T. (1957). *Chem. & Ind.* p. 984.
- Harborne, J. B. (1958). *J. Chromat.* **1**, 473.
- Haslam, E., Haworth, R. D. & Knowles, P. F. (1961). *J. chem. Soc.* p. 1854.
- Hathway, D. E. (1956). *Biochem. J.* **63**, 380.
- Hathway, D. E. & Seakins, J. W. T. (1959a). *Biochem. J.* **72**, 369.
- Hathway, D. E. & Seakins, J. W. T. (1959b). *Biochem. J.* **72**, 37p.
- Hergert, H. L. (1960). *For. Prod. J.* **10**, 610.
- Hillis, W. E. (1954). *J. Soc. Leath. Tr. Chem.* **38**, 91.
- Hillis, W. E. (1958). *Nature, Lond.*, **182**, 1371.
- Hillis, W. E. & Carle, A. (1958). *Holzforschung*, **12**, 136.
- Hillis, W. E. & Carle, A. (1960). *Biochem. J.* **74**, 607.
- Isherwood, F. A. & Jermyn, M. A. (1951). *Biochem. J.* **48**, 515.
- King, F. E., King, T. J., Goodson, D. H. & Manning, L. C. (1956). *J. chem. Soc.* p. 4477.
- Laumas, K. R. & Seshadri, T. R. (1958). *Proc. Indian Acad. Sci.* **49A**, 47.
- Neish, A. C. (1960). *Annu. Rev. Pl. Physiol.* **11**, 55.
- Partridge, S. M. & Westall, R. G. (1948). *Biochem. J.* **42**, 238.
- Robinson, G. M. & Robinson, R. (1933). *J. chem. Soc.* p. 206.
- Underhill, E. W., Watkin, J. E. & Neish, A. C. (1957). *Canad. J. Biochem. Physiol.* **35**, 219.
- Watkin, J. E., Underhill, E. W. & Neish, A. C. (1957). *Canad. J. Biochem. Physiol.* **35**, 229.
- Young, S. (1883). *Chem. News*, **48**, 31.
- Zimmermann, M. H. (1960). *Annu. Rev. Pl. Physiol.* **11**, 167.

Biochem. J. (1962) **82**, 439

Teichoic Acid from the Walls of *Staphylococcus aureus* H

STRUCTURE OF THE N-ACETYLGLUCOSAMINYLRIBITOL RESIDUES

BY J. BADDILEY, J. G. BUCHANAN, U. L. RAJBHANDARY AND A. R. SANDERSON*

Department of Chemistry, King's College, University of Durham, Newcastle upon Tyne 1

(Received 30 August 1961)

The teichoic acid in the walls of *Staphylococcus aureus* H is a ribitol phosphate polymer containing alanine and N-acetylglucosamine residues (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). Preliminary studies suggested that this compound resembles the teichoic acids in the walls of *Lactobacillus arabinosus* 17-5 (Armstrong *et al.* 1958; Archibald, Baddiley & Buchanan, 1961b) and *Bacillus subtilis* (Armstrong, Baddiley & Buchanan,

1960, 1961), in both of which D-alanine is attached through its carboxyl group in remarkably labile ester linkages, and the sugar residues are attached as glycosides to ribitol.

The structure of this teichoic acid is of particular interest, in view of the extensive investigations on the walls of this and related strains of *S. aureus* in connexion with mucopeptide biosynthesis and the mechanism of penicillin action (Park & Strominger, 1957; Strominger, Park & Thompson, 1959). Moreover, teichoic acids are associated with the

* Salters Fellow.