3. Preparations of bacterial chondrosulphatase, purified chondroitinase and testicular hyaluronidase showed no activity.

4. Preparations of α -amylase from saliva and cereal seeds were inactive but a crystalline sample of pancreatic α -amylase showed activity. This disappeared on further recrystallization, and it is concluded that the elastolytic activity of α -amylase reported in the literature is due to contamination with pancreatic elastase.

5. Various commercial preparations of ficin all showed the same ratio of elastolytic to proteolytic activity as measured by viscosity reduction of gelatin. This same ratio was maintained throughout fractionation and purification of commercial dried fig latex. It was concluded that 'fig-latex elastase' is identical with the major proteolytic enzyme in preparations of 'ficin'.

6. The results of the survey confirm the view that elastolysis is the result of proteolytic activity. The failure of certain well-characterized proteolytic enzymes such as trypsin and chymotrypsin to dissolve elastin is attributed either to their failure to penetrate the cross-linked structure of elastin or to the very restricted peptide-bond specificity exhibited by these enzymes.

REFERENCES

- Balo, J. & Banga, I. (1950). *Biochem. J.* **46**, 384.
 Banga, I. (1951). *Z. Vitam.-Horm.u. Fermentforsch.* **4**, 49.
 Banga, I. (1952). *Acta physiol. hung.* **3**, 317.
 Banga, I. (1953). *Nature, Lond.*, **172**, 1099.
 Banga, I. & Balo, J. (1953). *Nature, Lond.*, **171**, 44.
 Banga, I. & Balo, J. (1956). *Nature, Lond.*, **178**, 310.
 Bernfeld, P. (1951). *Advanc. Enzymol.* **12**, 379.
 De La Haba, G., Cammarata, P. S. & Fruton, J. S. (1955). In *Methods in Enzymology*, vol. 2, p. 64. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
 Dixon, G. H., Neurath, H. & Pechere, J. F. (1958). *Annu. Rev. Biochem.* **27**, 489.
 Dodgson, K. S. & Lloyd, A. G. (1957). *Biochem. J.* **68**, 88.
 Dvornich, W. & Alburn, H. E. (1959). *Arch. Biochem. Biophys.* **79**, 146.
 Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
 Grant, N. H. & Robbins, K. C. (1955). *Proc. Soc. exp. Biol., N.Y.*, **90**, 264.
 Grant, N. H. & Robbins, K. C. (1957). *Arch. Biochem. Biophys.* **66**, 396.
 Hall, D. A. (1955). *Biochem. J.* **59**, 459.
 Hall, D. A. (1957). *Arch. Biochem. Biophys.* **67**, 366.
 Hall, D. A. & Gardiner, J. E. (1955). *Biochem. J.* **59**, 465.
 Hall, D. A., Reed, R. & Tunbridge, R. E. (1952). *Nature, Lond.*, **170**, 264.
 Labella, F. S. (1957). *Nature, Lond.*, **180**, 1360.
 Lansing, A. I., Rosenthal, T. B., Alex, M. & Dempsey, E. C. (1952). *Anat. Rec.* **114**, 555.
 Lewis, U. J. & Thiele, E. H. (1957). *J. Amer. chem. Soc.* **79**, 755.
 Lewis, U. J., Williams, D. E. & Brink, N. G. (1956). *J. biol. Chem.* **222**, 705.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Miyada, D. S. & Tappel, A. L. (1956). *Food Res.* **21**, 217.
 Naughton, M. A. & Sanger, F. (1958). *Biochem. J.* **70**, 4P.
 Newman, R. E. (1949). *Arch. Biochem.* **24**, 289.
 Partridge, S. M. & Davis, H. F. (1955). *Biochem. J.* **61**, 21.
 Partridge, S. M. & Davis, H. F. (1958). *Biochem. J.* **68**, 298.
 Partridge, S. M., Davis, H. F. & Adair, G. S. (1955). *Biochem. J.* **61**, 11.
 Pepler, W. J. & Brandt, F. A. (1954). *Brit. J. exp. Path.* **35**, 41.
 Petersen, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
 Sanger, F. (1945). *Biochem. J.* **39**, 507.
 Sørensen, M. & Haugaard, G. (1933). *Medd. Carlsberg Lab.* **19**, no. 12.
 Svensson, H., Hagdahl, L. & Lerner, K. D. (1957). *Sci. Tools*, **4**, 1.
 Wang, H., Weir, E., Birkner, M. L. & Ginger, B. (1958). *Food Res.* **23**, 423.
 Whitaker, J. R. (1957). *Food Res.* **22**, 468.
 Yemm, E. W. & Willis, A. J. (1954). *Biochem. J.* **57**, 508.
 Yokota, H. (1957). *Kumamoto med. J.* **10**, 131.

The Formation of Phenolic Substances in *Eucalyptus gigantea* and *Eucalyptus sieberiana*

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It has been pointed out (Hillis, 1956; Hathway, 1958) that resins and kinos produced in the cambial region contain components which are absent from heartwood of the species which produced them. For these and other reasons, it was suggested that the phenolic substances in bark and wood were produced elsewhere. It was further suggested that, in

some instances, the leucoanthocyanins were the precursors of heartwood extractives (see also Bate-Smith & Swain, 1953; King & White, 1957; Roux, 1958; Roux & Evelyn, 1958). Recent information obtained with the aid of a staining technique (Hillis, 1958) indicated that the leucoanthocyanins in some eucalypts were synthesized *in situ*. A

preliminary study of the phenolic substances from the cambial and woody tissues of other eucalypts supported this view of formation *in situ* (Hillis & Carle, 1958). The present work was undertaken to gain more information about the precursors of the phenolic substances in the heartwood of *Eucalyptus* spp. and their site of biosynthesis.

Eucalyptus gigantea and *Eucalyptus sieberiana* were investigated as their pale-coloured heartwoods contain small amounts of leucoanthocyanins but larger amounts of other phenolic substances. An examination of the latter would show whether they interfere with the staining technique (Hillis, 1958) for leucoanthocyanins, as reports (Jurd, 1957; Grassman, 1958) indicate that other substances could do so. As eucalypt kinos contain large amounts of leucoanthocyanins, the contrast in their chemical composition with that of heartwood extractives may indicate the role of leucoanthocyanins. Kino is usually formed when the cambium of *Eucalyptus* trees (and of members of some other genera) is damaged. Kino is found in veins or pockets according to the severity of the injury and the species. In veins, the accretions of kino are separated by radial bridges of woody tissue, and rarely interfere with the conversion of sapwood into heartwood.

EXPERIMENTAL

Materials

Wood and phloem samples. The samples of *E. gigantea* were taken from freshly felled logs (Table 1), wrapped in polythene bags and examined within 3 weeks of collection. Log 4 was collected from a 100-year-old *E. sieberiana* (3 ft. diam.) tree grown in north-eastern Victoria, and log 5 from a 5-year-old *E. sieberiana* (2 in. diam.) grown in Melbourne.

Kino. Viscous *E. gigantea* kino was taken from the heartwood in log 3. The *E. sieberiana* kino was collected from experimental trees (see below).

Study of kino formation

The two experimental *E. sieberiana* were 5 years old and in a vigorous state of growth. The experiments were carried out from mid-summer to early autumn, when the cambium was still active. One tree was polled at 4 ft. above ground

level, the saw cut being 3 in. above the two remaining branches. Incisions of different types and at different heights above ground level were made on the other tree. In all cases the cambium was exposed or damaged.

Epicormic shoots began to form below the highest damaged areas. The polled tree formed twenty-two vigorous shoots and the other tree formed thirteen shoots. One month after injury the epicormic shoots on the polled tree were about 5 in. long and bore three or four leaves; traces of kino were then evident in some of the cuts and the daily production increased as the shoots lengthened (up to 3.25 ft. in 9 weeks). This sequence of events had been observed in a 4-year-old tree which had been polled in the previous season.

The amount of kino produced by the different types of injuries varied; 1.2 g. (dry wt.) of kino was collected from the top of the polled tree and 2.9 g. from the other injuries. Some of the knife cuts healed over and did not exude kino, and only traces of kino were obtained from a slash wound, the periphery of which was covered by callus tissue. The kino exuded from the horizontal uppermost cut of the triangular-shaped incisions.

When freshly exuded, the kino of *E. sieberiana* was a pale tan honey-like material which dried to a glassy solid. After standing for 3–4 weeks the colour became increasingly red.

Chromatographic methods

The samples (or methanol extracts of fresh tissues) were dissolved in methanol, methanol-water (1:1, v/v) or dimethylformamide. Solutions of the samples were applied to Whatman filter papers nos. 1 or 3, by repeated applications in a warm air stream. A reference compound (phloroglucinol, gallic acid or cyanidin) was used on each one-way chromatogram and phloroglucinol added to every alternate two-way chromatogram. The chromatograms were developed at 20°.

Developing solvents. Two-way chromatograms were developed by an ascending method employing (1) 6% acetic acid followed by (2) butan-1-ol-acetic acid-water (6:1:2, by vol.) after drying in air for 4 hr. Both solvents travelled about 11.5 in. One-way chromatograms were developed with the following solvents: (3) HCl-acetic acid-water (3:30:10, by vol.) (Bate-Smith, 1954); (4) butanol-acetic acid-water (10:4:2, by vol.) (Schmidt, Hensler & Stephan, 1957); (5) butanol-acetic acid-water-glycol (4:1:5:1, by vol.) (Schmidt & Lademann, 1951a); (6) ethyl acetate-acetic acid-water-ethanol (3:1:3:0.5, by vol.) (Schmidt & Schmidt, 1952); (7) formic acid-HCl-water (5:2:3, by vol.) (Harborne, 1958a); (8) butanol-HCl-water (7:2:5, by vol.) (Abe & Hayashi, 1956).

Table 1. Description of samples of *Eucalyptus gigantea*

	Log 1	Log 2	Log 3
Age of tree (years)	300	300	100
Collected in	Northern Tasmania	Northern Tasmania	North-eastern Victoria
Diam. of log (ft.)	3.5	4.5	3
Location of heartwood samples:			
(a) Outer	Outer 5 in.	Outer 4 in.	Outer 2 in.
(b) Inner	Adjacent to pith	Adjacent to pith	—

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) under ultraviolet light; (c) under ultraviolet light whilst fuming with ammonia (sp.gr. 0.88). The chromatograms were then sprayed with the following chromogenic reagents: (d) ferric chloride–potassium ferricyanide (White, Kirby & Knowles, 1952), for phenols; (e) diazotized *p*-nitroaniline–sodium acetate (Swain, 1952), for phenols; (f) vanillin–HCl (Bate-Smith & Swain, 1953), for flavans with phloroglucinol nucleus; (g) 5% potassium cyanide solution (Young, 1883), for gallic acid; (h) sodium periodate and aniline (Yoshida & Hasegawa, 1957), for shikimic acid; (i) bromocresol green (Lugg & Overell, 1947), for acidic compounds; (j) aniline–phthalic acid (Partridge, 1949), for aldose sugars; (k) phloroglucinol–HCl (Wiesner, 1878), for lignin.

Estimation of ellagic acid

Portions (20 g.) of the fresh samples were split and soaked in methanol for 48 hr. with two changes of solvent. The samples were then air-dried, ground in a small Wiley mill to 60-mesh and extracted in a Soxhlet apparatus for 20 hr. The two extracts were combined. Measured portions were hydrolysed by heating with 2*N*-HCl under reflux on a boiling-water bath for 6 hr. Known fractions of the unhydrolysed and hydrolysed extracts were suitably diluted with dimethylformamide. The ellagic acid in these samples was estimated by comparing the area and the intensity of fluorescence of the spot with those of standards of pure ellagic acid after chromatography in solvent 3 (Hillis & Carle, 1959).

Examination of leucoanthocyanins

The anthocyanidin produced by heating a solution of the kinos in methanol–water (1:1, v/v) with butanol–HCl (100:5, v/v) (Swain & Hillis, 1959) was estimated as delphinidin from the absorption at the maximum wavelength with the constants of Schou (1927). The R_F values of the anthocyanidins in solvents 3, 7 and 8 were obtained on examination of the product given by heating kino (0.7 g.) with butanol–HCl (30 ml.) on a boiling-water bath for 30 min. The R_F values of the major anthocyanidin were identical with those of delphinidin, and traces of cyanidin (less than one-tenth of delphinidin) were present. Purified delphinidin was obtained for spectral examination by streaking this solution on no. 3 paper, developing in solvent 3, and extracting the band at R_F 0.3 at room temperature in darkness with methanol containing 0.01% of conc. HCl. An absorption maximum of 561 m μ was obtained (see Harborne, 1958b). The colour reactions were observed on material obtained by displacing the above reaction products in butanol (5 ml.) into 1% (v/v) HCl (2 ml.) by means of benzene (20 ml.) and extracting impurities in the aqueous layer with ethyl acetate (3 $\times$ 5 ml.). After extraction of the remaining aqueous layer repeatedly with benzene, the colour reactions with pentanol–sodium acetate and the cyanidin reagent (Robinson & Robinson, 1931) were observed and found to be those of delphinidin.

The products obtained by heating woody tissues or their extracts with butanol–HCl were purified as above and the red was extracted with pentanol. Portions of the fractions from the methanol extract of heartwood were heated with butanol–HCl as above and the colours observed.

Degradation of kino

Samples of kino (1 g.) were heated with 3*N*-HCl (20 ml.) on a boiling-water bath for 40 min. and the aqueous layer was extracted with ether in a liquid–liquid extractor for 16 hr. The extract was examined by means of two-way cochromatography in solvents 1 and 2, and colour reactions (b)–(g) and (j), and ellagic acid, gallic acid and phloroglucinol were shown to be present.

Extraction of constituents from wood

Fresh samples of outer (1.5 kg. dry wt.) and inner (2.0 kg. dry wt.) heartwood of *E. gigantea* were taken in the form of 1 in. cubes from log 1. They were processed separately in the same manner as that used in the estimation of ellagic acid (q.v.) except that the extraction periods were longer.

Fractionation of extracts

The combined methanol extracts from each sample were vacuum-concentrated (to approx. 500 ml.), water (500 ml.) was added and they were centrifuged to remove the insoluble materials. The liquor was extracted with light petroleum (b.p. 40–60°) for 6 hr. in a liquid–liquid extractor, then with ethyl ether for 4 $\times$ 8 hr. and finally with ethyl acetate for 6 $\times$ 8 hr. with regular changes of solvent. A further amount of buff-coloured insoluble material was centrifuged from the remaining black liquor.

A portion of the methanol-extracted outer heartwood was extracted ten times with 2*N*-NaOH at 70° and the filtrate was immediately neutralized.

Examination of fractions

Ethyl ether-soluble fraction. Two-way chromatograms were run (solvents 1 and 2, colour reactions a–e). Only about one-third of this fraction had R_F values > 0.5 in solvent 2. It was further fractionated by passing the material which was soluble in ether saturated with water through a column (prepared by the method of Bradfield & Penney, 1947) of silica gel (L. Light and Co. Ltd.; 'chromatographic grade'; purified by the method of Hathway, 1958). The column was developed with ether saturated with water, and the eluent after drying was washed five times with small amounts of ether; the insoluble material recrystallized three times from water to yield pale-brown needles. This component (no. 10, Fig. 1) was identified as gallic acid by its mixed m.p. (215°) with authentic material, its R_F values in solvents (1)–(4) and colour reactions (d), (e) and (g), when compared with authentic material, and its ultraviolet-absorption spectra in ethanol and ethanol–sodium acetate (Jurd, 1956).

The mother liquor contained several components (2–7) with similar R_F values (Fig. 1); these reacted weakly with colour reagent (d), their R_F in butanol–water was greatly reduced when borate paper was used, and components 2–7 and 13 were unchanged after boiling with 2*N*-H₂SO₄ for 1.5 hr. Attempts to separate the components on cellulose columns with different solvents failed to purify them to permit identification. Chromatographic examination showed that the more common polyphenols and aromatic acids were absent.

Ethyl acetate-soluble fraction. The components present had R_F values less than 0.5 in solvent 2. A portion of the vacuum-evaporated mixed extract (5.0 g.) was mixed with

dry cellulose powder (0.5 g.), then water (5 ml.) and a few drops of ethanol were added and the mass was stirred until a slurry was obtained. The slurry was carefully added to the top of a 1.25 in. diam. $\times$ 10 in. cellulose column. The column had previously been prepared by pouring a thick slurry of cellulose (Solka Floc, Brown Co., Mass., U.S.A.; purified by the method of Campbell, Work & Mellanby, 1951) and dry acetone into the chromatography tube with its tap open. When the cellulose had settled to a height of 10 in., the supernatant acetone and suspended cellulose were removed from the top and water (about 2 l.) was allowed to percolate through the column. After the addition of the sample to the column, water was added to maintain a head of 3 ft., 120 $\times$ 10 ml. fractions were collected over a period of 7 hr. and each fraction was chromatographed in 6% acetic acid. The contents of the tubes were mixed as follows: group A, tubes 1 and 2; B, tubes 3-7; C, tubes 9-13; D, tubes 17-30; E, tubes 33-53; F, tubes 57-120; NaCl was added to saturation. Then they were completely extracted (for periods varying from 2 days for group F to 2 weeks for group A) with ethyl acetate in a liquid-liquid extractor. After drying (Na_2SO_4) and concentration, the extract was added to five times its volume of warm hexane, and the precipitate washed with hexane and

dried at 100°. Groups A and F yielded almost pure components 28 and 18 (Fig. 1) respectively whereas groups B-E contained components 20-26 in different stages of purity.

These components gave a strong blue with iron salts and a pink to brown with 2N-NaOH, indicating the presence of vicinal trihydroxyl groups. As no colour change took place when the two-way chromatogram was sprayed with bromocresol green it is evident that they were not acidic. Samples of groups B-F were separately hydrolysed with 2N- H_2SO_4 for 2 hr. and the samples taken at intervals of 20 min. were chromatographed in solvents 1 and 2. Ellagic acid and gallic acid and glucose, apart from traces of a phenolic substance R_F 0.85/0.35 (solvents 1 and 2), were the only substances shown to be present by means of the colour tests (a)-(e), (g) and (j). They were identified by their R_F values when compared with authentic materials. When the ethyl acetate-soluble fraction (components 18-28) was hydrolysed with aq. 5% H_2SO_4 , ellagic acid (34% yield) and glucose were separated by the method of Jurd (1958). The identity of the glucose was confirmed by the separate and mixed m.p. (205°) of the glucosazone.

Components 22 and 25 were identified as corilagin (Schmidt & Lademann, 1951b; Schmidt, Schmidt & Herok, 1954) and juglanin (Jurd, 1958) respectively, by cochromatography on two-way chromatograms with solvents 1 and 2, comparison with authentic samples in solvents 4, 5 and 6, together with colour reactions (b)-(e). Traces only of corilagin were present. The R_F values of corilagin and juglanin reported by Jurd (1958) are higher than those in Fig. 1. However, we have found that under our conditions the R_F values in solvents 1 and 2 of these phenols from the walnut were very similar to those of components 18-28.

Insoluble material. Chromatographic examination showed that the insoluble fraction consisted almost entirely of ellagic acid (see also Hillis, 1953). It was identified as described above by its R_F values and colour reactions when compared with authentic material, and also by the separate and mixed m.p. (245-246°) of its tetracarboxy derivative (Feist & Bestehorn, 1925; Reichel & Schwab, 1942). Also present were traces of a phenolic material immobile in all the solvents tried and with a bright emerald-green ultra-violet fluorescence.

RESULTS

Phenolic substances in the heartwood of Eucalyptus gigantea

The percentage (on a dry-weight basis) of the fractions of the methanol extractives from the outer and inner heartwoods respectively of log 1 were as follows: petroleum-soluble, —, 0.06; ethyl ether-soluble, 0.61, 0.52; ethyl acetate-soluble, 3.15, 1.78; insoluble, 1.00, 0.66. A large portion of the methanol extractives (see Table 2) was a dark-brown material (30) which could not be resolved chromatographically.

Petroleum-soluble fraction. When chromatographed this fraction occupied the position of component 1 (Fig. 1, Table 3). In earlier work on *E. sieberiana* (Hillis & Carle, 1958) the purple reaction (with spray e) was given only by the extracts of cambium, particularly when it was very active.

Fig. 1. Chromatogram showing major phenolic substances in the woody tissues of *E. gigantea*. The chromatogram was run first in 6% acetic acid followed by butanol-acetic acid-water (6:1:2, by vol.). On the diagram the relative intensity is shown by: ---, weak; —, medium; ———, strong. Substances identified are: spot 10, gallic acid; 11, (+)-catechin (sawwood only); 14, ellagic acid; 22, corilagin (traces); 25, juglanin. Spots 10, 12 and 14-30 gave a blue with FeCl_3 - $\text{K}_3\text{Fe}(\text{CN})_6$; spots 6 and 13 gave a straw-yellow, spots 4, 5 and 7 an orange, spots 3 and 10 a yellow changing to black and spot 1 a purple colour with diazotized *p*-nitroaniline; spot 2 had a water-green, spot 9 a yellow u.v. fluorescence, and spots 4, 5 and 8 a light-blue u.v. fluorescence after fuming with ammonia.

Table 2. *Extractive and ellagic acid contents of Eucalyptus gigantea*

Results are expressed as percentages from oven-dry wood.

Sample	Log 1			Log 2			Log 3		
	Extrac- tive	Ellagic acid		Extrac- tive	Ellagic acid		Extrac- tive	Ellagic acid	
		Free	Combined		Free	Combined		Free	Combined
Phloem	—	—	—	20.1	0.36	1.26	—	—	—
Sapwood	—	—	—	10.7	0.042	0.90	3.1	0.017	0.38
Outer heartwood	11.7	1.10	2.77	18.3	1.50	3.26	6.1	0.23	0.98
Inner heartwood	6.3	0.71	1.48	10.1	0.78	1.05	—	—	—

Ether-soluble fractions. Gallic acid was the major single component (no. 10, Fig. 1, Table 3). A chromatographic estimation (spot size and intensity of colour with colour reagent (*d*) Hillis & Carle, 1959) indicated that 0.17 and 0.08 % of gallic acid was present in these samples of outer and inner heartwood respectively.

Ethyl acetate-soluble fractions. Components 18–28 (Fig. 1, Table 3) were present. In view of the hydrolytic products and the absence of acidic properties in the components, it is evident that the chebulinic acids, the chebulagic acids and chebulic acid were absent. This view was supported by comparison with the R_F values of the components when two-way chromatograms were prepared with the solvents of Hathway (1956) and White (1956). The properties of components 18–21, 23, 24 and 26–28 suggest that they are isomers of corilagin and juglanin. The chemistry of some of these components is being studied.

Insoluble material. Almost pure ellagic acid separated from the methanol extracts during extraction with other solvents. The other substance present appeared to be identical with a material found in the pipes of pulp digesters when *E. gigantea* is processed, and which had the properties of either a polymer of ellagic acid or a complex of it with some other substance (Hillis & Carle, 1959).

Residual methanol extracts. These materials were immobile in solvent 2, formed a dark-brown streak along the solvent 1 axis and became weak red (stronger at R_F 0) when sprayed with vanillin-HCl. This colour reaction indicates that some of the materials present possess a phloroglucinol nucleus with a vacant position *para* to a free hydroxyl group (Hillis & Urbach, 1959). Some of these materials could be either polymerized catechin or leucoanthocyanin. The red produced when they were heated with butanol-HCl indicated that flavandiol nuclei were present. Chromatography (in solvent 3) of the product suggested that cyanidin and delphinidin were formed but the spots were not discrete. Other products present are probably similar to the oxidation products of tea (Roberts, 1958), as leucodelphinidin could be oxidized in the

presence of gallic acid to form tropolones in a manner analogous to that of gallocatechin. When the black residual methanol extracts were hydrolysed the amounts of ellagic acid produced were greater than that expected from the traces of components 18, 20 and 24 still present. The ellagic acid could arise from the oxidation products of these components or from ellagitannins which were immobile in solvent 2.

Extracted wood. When the completely methanol-extracted ground wood was extracted with warm 2N-NaOH a further amount of ellagic acid was removed (0.035 % of the outer heartwood, Hillis & Carle, 1959). This ellagic acid was probably in the cell wall and other evidence (D. E. Bland, personal communication) exists which supports this view. Also, when this wood was heated with butanol-HCl, a reddish-brown solution was obtained but the wood became red. Even after the solution was purified a red streak was obtained on the chromatograms (solvents 3 and 7) although it appeared to be strongest at the R_F of delphinidin. The colour of the treated wood could not be removed with alcohol, indicating that the substance containing the flavandiol nucleus (or the red-forming material) was incorporated into the cell wall. As only a very faint red colour was formed when the extracted wood was treated with vanillin-HCl, the phloroglucinol nucleus of the leucodelphinidin (if present) must be linked through the C_8 position to lignin or to the cell wall.

General. When samples of the petroleum ether and ethyl acetate fractions were heated with butanol-HCl, either no colour or a faint brown was obtained. Therefore the materials contained in these fractions do not interfere with the butanol-HCl staining technique (Hillis, 1958).

Chromatographic comparison of phloem and woody tissues of Eucalyptus gigantea and Eucalyptus sieberiana

When two-dimensional chromatograms (solvents 1 and 2) of the extracts of phloem, sapwood and heartwood were prepared with the same spot area of ellagic acid and compared, the following observations were made. (The compounds were identified

Table 3. Major phenolic substances of *Eucalyptus gigantea* and *Eucalyptus sieberiana*

Component	No. on Fig. 1	100 × R _F (solvents 1 and 2)	Material examined									
			<i>E. gigantea</i>					<i>E. sieberiana</i>				
			Heartwood			Sapwood	Phloem	Kino	Sapwood	Phloem	Kino	
			Inner	Outer								
Lignan (?)*	1	00/97	+	+		a	a	a	a	a	a	
Unidentified	2-5	—	tr†	tr†		a	a	a	a	a	a	
Unidentified	6	60/80	++	++		a	a	a	tr	a	a	
Unidentified	7-9	—	tr†	tr†		a	a	a	a	a	a	
Gallic acid	10	40/59	+	+		tr	tr	a	+	tr	a	
(+)-Catechin	11	40/59	a	a		+	+	a	+	+	a	
Unidentified	12	50/46	tr	tr		a	a	a	+	+	a	
Unidentified	13	74/53	a	++		a	a	a	a	+	a	
(-)-epiCatechin	—	38/41	a	a		a	a	a	tr	tr	a	
Ellagic acid	14	00/35	+	+		tr	+	a	+	+	tr	
Unidentified	15	01/23	+	+		+	+	a	+	+	a	
Unidentified	16	26/31	+	+		a	a	a	a	a	a	
Unidentified	17	22/26	+	+		a	a	a	a	a	a	
Ellagitannins;	18	19/8	+	+		+	tr	a	+	+	a	
Unidentified	19	22/18	+	+		+	+	a	tr	tr	a	
Unidentified	20	32/05	+	+		+	+	a	+	+	a	
Unidentified	21	36/15	+	+		+	+	a	tr	tr	a	
Corilagin	22	39/20	tr	tr		tr	tr	a	tr	+	a	
Unidentified	23	45/25	+	+		+	+	a	+	+	a	
Unidentified	24	47/06	+	+		+	+	a	+	+	a	
Juglanin	25	50/14	+	+		+	+	a	+	+	a	
Unidentified	26	55/20	+	+		+	+	a	+	+	a	
Unidentified	27	66/10	+	+		+	+	a	+	+	a	
Unidentified	28	66/14	+	+		+	+	a	+	+	a	
1-Galloyl-β-glucose	—	68/27	a	a		a	a	a	a	+	a	
1:3:6-Trigalloylglucose	—	12/32	a	a		a	a	a	a	a	a	
Shikimic acid	—	42/77	a	a		a	a	a	a	+	a	
Leucodelphinidin	—	45/20	a	a		a	a	tr	a	a	tr	

* For properties see Hillis & Carle (1958); alkoxy groups (Zeisel, 1885) present, lignin absent.

† Much smaller amounts were in logs 2 and 3.

‡ Absent in logs 2 and 3.

by two-dimensional cochromatography and colour reactions.)

Eucalyptus gigantea. The ellagitannins (18–28) and ellagic acid (14) were the main components resolved (see Table 3). The phloem and sapwood contained more ellagitannins in relation to ellagic acid than did the heartwood (see also Table 1). The ratio of the amounts of components 18–28 to each other was about the same in all tissues. Polymerized leucoanthocyanin was present in all tissues.

Eucalyptus sieberiana. The composition (Table 3) of the extracts from the phloem of a 5-year-old tree (log 5) closely resembled that of cambium from other trees of the same species (Hillis & Carle, 1958). The phloem contained a higher ratio of resolved ellagitannins to unresolved material than did the sapwood, in particular β -glucogallin (1-galloyl- β -glucose; Fischer & Bergmann, 1918), juglanin and component 24. The phloem resembled sapwood in that it contained larger amounts of free ellagic acid than did cambium.

The heartwood from a 100-year-old specimen (log 4) did not contain (+)-catechin, in contrast with that from a younger tree (about 25 years old, Hillis & Carle, 1958).

Examination of kino

When a sample of *E. sieberiana* kino, which had been exuded in the preceding 24 hr., was chromatographed only very small traces of substances were resolved (see Table 3). The streaks which formed on the axes of solvents 1 and 2 gave deep blue with reagent (d) and an intense red with reagent (f); they also had a light-blue ultraviolet fluorescence when fumed with ammonia. The kino of *E. gigantea* behaved identically.

Delphinidin and traces of cyanidin were produced when the kinos of *E. sieberiana* and *E. gigantea* were heated with butanol-HCl. On analysis (Swain & Hillis, 1959) the kino of *E. sieberiana* gave an absorption at 560 m μ equivalent to a production of 2.8% delphinidin. This low yield indicates that some oxidative polymerization involving direct linkage between aromatic nuclei has taken place. After acid-treatment, 9% (w/w) of ether-soluble material was obtained from *E. sieberiana*. This material contained large amounts of phloroglucinol and gallic acid, traces of ellagic acid (less than 1%) and four other substances, but sugars were absent. The amount of ellagic acid present was greater than that in the untreated kino. Acid-treated *E. gigantea* kino gave an identical two-way chromatogram.

DISCUSSION

Kino. The kinos of *E. sieberiana* and *E. gigantea* could have been formed *in situ* or originated else-

where. If the kino had been translocated, evidence of a discoloured zone around the kino vein would be expected. However, the phloem adjacent to the freshly formed kino vein of *E. sieberiana* was identical to that diametrically opposite, when examined visually or when the extractives were examined chromatographically. Examination of kino veins in woody tissue (Hillis, 1958) also showed that there was no concentration of leucoanthocyanin in the rays of the woody tissue leading to the vein. The technique used in that study showed that only a few of the rows of cells surrounding the vein contained large amounts of leucoanthocyanins and this suggests that the leucoanthocyanins and the kino are formed *in situ*. In addition the kinos yielded on acid treatment some components which were not observed in the hydrolysed phloem and woody tissues; if translocation of kino had occurred these components would be found in those tissues.

The kino could be formed *in situ* from translocated shikimic acid, the contents of the cambial cells in the injured region or from translocated carbohydrate. Although shikimic acid has been found in the cambium (Hillis & Carle, 1958), this acid was absent in freshly exuded *E. sieberiana* kino. As the volume occupied by the cambial cells is usually less than that of the kino (particularly in the kino pockets), it is unlikely that the cambial cells can provide all the material necessary for the formation of kino. The recent investigations of Zimmermann (1957a, b) showed that the sieve tubes of the phloem of several species (of genera other than *Eucalyptus*) contain a solution consisting almost entirely of sugars of the raffinose family and mannitol, and that polyphenols were present in only very small amounts. This evidence suggests that kino is formed *in situ* from translocated sugars (light is not essential for the formation of the C₁₅ nucleus; Kursanov & Zaprometov, 1958). This view is supported by the observation that kino did not begin to exude from the injuries until the epicormic shoots had a number of leaves which could synthesize sugars. The epicormic shoots possessed chlorotic leaves as did the injured branches of *Myoporum platycarpum*, which exuded mannitol from the cambial region (Hatt & Hillis, 1947).

Cambium. The amount of leucoanthocyanins in the cambium is high during the period of its greatest activity (Pigman, Anderson, Fischer, Buchanan & Browning, 1953; Hillis, 1956). The decrease in amount with decrease in activity of the cambium could be due to translocation of leucoanthocyanins to other tissues, although there is evidence against this view (Hillis, 1956; Hathway, 1958). In view of the association between the presence of leucoanthocyanins and the process of

lignification in many plants (Bate-Smith & Lerner, 1954; Hillis & Swain, 1957), it would seem that the decrease of leucoanthocyanins in the cambium could be due to conversion into lignin, either by combination with other lignin precursors or by methylation. In support of the latter view other work has shown that the methoxyl content of Klason 'lignin' in the cambium and developing sapwood is less than that of true lignin (Stewart, Amos & Harvey, 1953), and that both the 'lignin' and the methoxyl content of it increase as the growing season progresses (Hillis & Carle, 1958). It is not possible with these species to determine whether the leucoanthocyanin has combined with the cellulose independently of lignin.

In addition to polymerized leucoanthocyanins, the major components in active *E. sieberiana* cambium have been shown to be catechin, epicatechin, shikimic acid and ellagic acid-gallic acid-glucose derivatives ('ellagitannins'); the latter were the most important components (Hillis & Carle, 1958).

Phloem and sapwood. The significant differences between the constituents of phloem and sapwood and those of cambium were the increase of free ellagic acid, the presence of gallic acid, a pronounced increase of unresolvable material and the relative decrease of ellagitannins. Hathway (1957) has suggested that free ellagic acid may be formed, under certain conditions, from glucogallin and ellagitannins (see also Schmidt, 1954). Similarly this work suggests that during metabolism in the ray parenchyma cells of the phloem and sapwood the free ellagic acid was formed from the glucosides (ellagitannins) and not by direct synthesis. The glucosides could be formed in the cambial cells and remain there during transformation into phloem or sapwood cells, and in addition be formed *in situ*. As expected from the work of Roberts (1957), the gallic acid was oxidized to unidentifiable materials before the catechin, which was still present.

Heartwood. In view of the low solubility of ellagic acid it is unlikely that significant quantities of this compound could be translocated to the heartwood periphery. The free ellagic acid in this region is probably formed as a result of hydrolysis of ellagitannins in the cells, at the time of the intense metabolism (Chattaway, 1952) preceding the death of the living horizontal and vertical parenchyma cells of the sapwood when heartwood is formed. King & White (1957) have also reported that the galloylglucose derivatives in the sapwood cells of quebracho disappeared at the periphery of the heartwood. The amount of combined ellagic acid in the sapwood could give rise to the free ellagic acid in the heartwood but the amount of combined ellagic acid in the latter region has also increased (Table 2). The ratio of free ellagic acid to

combined ellagic acid is higher in the heartwood than in the sapwood, confirming that there is a region of greater metabolism at the heartwood periphery.

By means of studies involving a staining technique for leucoanthocyanins, it was found (Hillis, 1958) that the amount of leucoanthocyanins in *E. camaldulensis* increased gradually from the cambium through the sapwood to the sapwood-heartwood boundary. At the boundary, the amount of leucoanthocyanins suddenly increased in the horizontal and then the vertical parenchyma cells. This analogy suggests that the increase in combined ellagic acid in the heartwood of *E. gigantea* took place at the heartwood periphery.

The amounts of ellagic acid and extractives in the inner heartwood were smaller than those in the outer heartwood (Table 1). The ratio of free to combined ellagic acid in the inner heartwood is a little higher than the ratio in the outer heartwood, so that apparently some change in the ellagitannins takes place as the heartwood of *E. gigantea* ages. It has also been observed that the amounts of catechin and monomeric leucoanthocyanins decrease from the heartwood boundary to the centre of the tree (Hillis, 1956; King & White, 1957; Hasegawa, 1958; Roux, 1958; Roux & Evelyn, 1958). Occasionally these changes are delayed (Hillis, 1956), which enabled the detection of catechin in the heartwood of a young *E. sieberiana* tree (Hillis & Carle, 1958), whereas it was absent in the heartwood of a larger tree.

General. The above data support the view that leucoanthocyanins (Bate-Smith & Metcalfe, 1957; Hillis & Swain, 1959) and other phenolic substances (Hillis & Carle, 1958) are formed within the living cells of the cambium, sapwood and phloem. Differences in the activities of the enzyme systems in different tissues could be responsible for the differences in the composition of the phenolic constituents found in those tissues (see also Hillis & Swain, 1959). As some evidence indicates that translocation of phenolic substances does not take place to a significant extent, they must be synthesized from alicyclic acids or carbohydrates. Shikimic acid has been detected in the phloem from a young tree, cambium and newly formed sapwood of *E. sieberiana* (and in other trees, Hasegawa, Nakagawa & Yoshida, 1957), but as it can be formed in shaded *E. sieberiana* leaves (W. E. Hillis, unpublished work) the acid in the woody tissues could be formed *in situ*.

The evidence presented indicates that most of the phenolic constituents in the heartwood are formed at the heartwood boundary from the starch and glycosides stored in the sapwood cells (Hillis & Carle, 1958) and also from translocated carbohydrate.

SUMMARY

1. The distribution of phenolic substances in the woody tissues and kino of *Eucalyptus gigantea* and *E. sieberiana* is described.

2. Ellagic acid is present to a varying degree in all woody and phloem tissues of these species.

3. The ellagitannins present include juglanin, corilagin and, in the phloem of young *E. sieberiana*, β -glucogallin also. The ratio of free to combined ellagic acid is low in the sapwood, higher in the phloem and higher still in the heartwood. The amount of free and combined ellagic acid is higher in the heartwood than in the sapwood.

4. Catechin is present in the bark and sapwood of both species and in the cambium of *E. sieberiana*. Shikimic acid and β -glucogallin is present in the cambium and phloem of young *E. sieberiana*. Traces of polymerized leucodelphinidin and leucocyanidin were present in all woody tissues examined.

5. The substances that are removed from the methanol extract of heartwood with light petroleum, ether and ethyl acetate do not give a red colour when heated with butanol-hydrochloric acid.

6. The kinos from these species contain polymerized leucodelphinidin and traces of leucocyanidin and ellagic acid. On acid-treatment gallic acid and further amounts of ellagic acid are liberated, and, in addition, phloroglucinol and other substances not present in the woody tissues.

7. Present evidence indicates that kino and the phenolic constituents in woody tissues are formed *in situ* from starch, glycosides and translocated carbohydrate.

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REFERENCES

- Abe, Y. & Hayashi, K. (1956). *Bot. Mag., Tokyo*, **69**, 577.
 Bate-Smith, E. C. (1954). *Biochem. J.* **58**, 122.
 Bate-Smith, E. C. & Lerner, N. H. (1954). *Biochem. J.* **58**, 126.
 Bate-Smith, E. C. & Metcalfe, C. R. (1957). *J. Linn. Soc. (Bot.)*, **55**, 669.
 Bate-Smith, E. C. & Swain, T. (1953). *Chem. & Ind.* p. 377.
 Bradfield, A. E. & Penney, M. (1947). *J. chem. Soc.* p. 32.
 Campbell, P. N., Work, J. S. & Mellanby, E. (1951). *Biochem. J.* **48**, 109.
 Chattaway, M. M. (1952). *Aust. For.* **16**, 25.
 Feist, K. & Bestehorn, H. (1925). *Arch. Pharm., Berl.*, **263**, 16.
 Fischer, E. & Bergmann, M. (1918). *Ber. dtsch. chem. Ges.* **51**, 1760.
 Grassman, W. (1958). *Das Leder*, **9**, 193.
 Harborne, J. B. (1958a). *J. Chromat.* **1**, 473.
 Harborne, J. B. (1958b). *Biochem. J.* **70**, 22.
 Hasegawa, M. (1958). *J. Jap. For. Soc.* **40**, 111.
 Hasegawa, M., Nakagawa, T. & Yoshida, S. (1957). *J. Jap. For. Soc.* **39**, 159.
 Hathway, D. E. (1956). *Biochem. J.* **63**, 380.
 Hathway, D. E. (1957). *Biochem. J.* **67**, 445.
 Hathway, D. E. (1958). *Biochem. J.* **70**, 34.
 Hatt, H. H. & Hillis, W. E. (1947). *J. Coun. sci. industr. Res. Aust.* **20**, 207.
 Hillis, W. E. (1953). *J. Sci. Fd Agric.* **4**, 135.
 Hillis, W. E. (1956). *Aust. J. biol. Sci.* **9**, 263.
 Hillis, W. E. (1958). *Nature, Lond.*, **182**, 1371.
 Hillis, W. E. & Carle, A. (1958). *Holzforschung*, **12**, 136.
 Hillis, W. E. & Carle, A. (1959). *APPITA*, **13**, 74.
 Hillis, W. E. & Swain, T. (1957). *Nature, Lond.*, **179**, 586.
 Hillis, W. E. & Swain, T. (1959). *J. Sci. Fd Agric.* **10**, 135.
 Hillis, W. E. & Urbach, G. (1959). *J. appl. Chem.* **9**, 474.
 Jurd, L. (1956). *J. Amer. chem. Soc.* **78**, 3445.
 Jurd, L. (1957). *J. Amer. chem. Soc.* **79**, 6043.
 Jurd, L. (1958). *J. Amer. chem. Soc.* **80**, 2249.
 King, H. G. C. & White, T. (1957). *J. Soc. Leath. Tr. Chem.* **41**, 368.
 Kursanov, A. L. & Zaprometov, M. N. (1958). *Atompraxis*, **1**, 280.
 Lugg, J. W. H. & Overell, B. T. (1947). *Nature, Lond.*, **160**, 87.
 Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
 Pigman, W., Anderson, E., Fischer, R., Buchanan, M. A. & Browning, B. L. (1953). *T.A.P.P.I.* **36**, 4.
 Reichel, L. & Schwab, A. (1942). *Liebigs Ann.* **550**, 152.
 Roberts, E. A. H. (1957). *Chem. & Ind.* p. 1354.
 Roberts, E. A. H. (1958). *J. Sci. Fd Agric.* **9**, 381.
 Robinson, G. M. & Robinson, R. (1931). *J. chem. Soc.* **25**, 1687.
 Roux, D. G. (1958). *Nature, Lond.*, **181**, 1454.
 Roux, D. G. & Evelyn, S. R. (1958). *Biochem. J.* **69**, 530.
 Schmidt, O. T. (1954). *Das Leder*, **5**, 129.
 Schmidt, O. T., Hensler, R. H. & Stephan, P. (1957). *Liebigs Ann.* **609**, 186.
 Schmidt, O. T. & Lademann, R. (1951a). *Liebigs Ann.* **571**, 41.
 Schmidt, O. T. & Lademann, R. (1951b). *Liebigs Ann.* **571**, 232.
 Schmidt, O. T. & Schmidt, D. M. (1952). *Liebigs Ann.* **578**, 25.
 Schmidt, O. T., Schmidt, D. M. & Herok, J. (1954). *Liebigs Ann.* **587**, 67.
 Schou, S. A. (1927). *Helv. chim. acta*, **10**, 907.
 Stewart, C. M., Amos, G. L. & Harvey, L. J. (1953). *Aust. J. biol. Sci.* **6**, 21.
 Swain, T. (1952). *Biochem. J.* **53**, 200.
 Swain, T. & Hillis, W. E. (1959). *J. Sci. Fd Agric.* **10**, 63.
 White, T. (1956). *Symp. Soc. Leath. Tr. Chem. Vegetable Tannins*, p. 7.
 White, T., Kirby, K. S. & Knowles, E. (1952). *J. Soc. Leath. Tr. Chem.* **36**, 148.
 Wiesner, J. (1878). Quoted by Brauns, F. E. in *The Chemistry of Lignin*, (1952), pp. 29, 35 et seq. New York: Academic Press Inc.
 Yoshida, S. & Hasegawa, M. (1957). *Arch. Biochem. Biophys.* **70**, 377.
 Young, S. (1883). *Chem. News*, **48**, 31.
 Zeisel, S. (1885). In *Technique of Organic Chemistry*, (1954), vol. 6, p. 454. Ed. by Cheronis, N. D. New York: Interscience Publications Inc.
 Zimmermann, M. H. (1957a). *Plant Physiol.* **32**, 288.
 Zimmermann, M. H. (1957b). *Plant Physiol.* **32**, 399.