LEUCOANTHOCYANINS AS THE POSSIBLE PRECURSORS OF EXTRACTIVES IN WOODY TISSUES

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Summary

Leucoanthocyanins in plant tissues have been estimated by measuring the anthocyanidins produced after heating with *iso*propanol–hydrochloric acid in a closed tube. Various tissues of *Eucalyptus sieberiana* F. Muell., *E. obliqua* L'Herit., *E. regnans* F. Muell., and *E. maculata* Hook. were studied by this method. The amount of leucoanthocyanins was observed to decrease with increasing degree of maturity of the different leaves on a stem. Most of the leucoanthocyanins appeared to be in the colourless leaf tips. The amount of leucoanthocyanin in the bark decreased during the growing season, and the amount in the cambium decreased rapidly once differentiation of the tissues began. The sapwood of *E. sieberiana* contained about half that in the heartwood. Similar results were found in transverse discs from logs of *E. marginata* Sm. which contained also an outer heartwood zone. The extraneous materials in this latter zone were much more water soluble, although the alkali solubles of both the inner and outer heartwood zones were the same. The acetone solubles in *E. marginata* sapwood were largely leucoanthocyanins.

Chromatographic examination with different solvents of the alcohol extracts from different tissues of *E. sieberiana* showed that the anthocyanin in the leaves was different from that in the bark. The amount of flavone glycosides decreased rapidly in samples taken progressively further away from the leaves. On the other hand, a mauve fluorescent component increased. Examination of the reaction products obtained after heating with *iso*propanol–hydrochloric acid showed that leucoanthocyanins which yielded delphinidin and cyanidin were present in all tissues except the cambium which yielded only cyanidin; the kino also yielded only cyanidin.

The view is expressed that the leucoanthocyanins in the xylem originate from the leaves and that they are the immediate precursors of bark and heartwood extractives. Evidence is given of the ease with which leucoanthocyanins are converted to other flavonoids.

I. Introduction

Leucoanthocyanins or leucoanthocyanidins are detected by heating the sample with aqueous or alcoholic hydrochloric acid when a red colour, largely due to anthocyanidin, is produced. They have been shown to be widespread in plant tissues (e.g. Robinson and Robinson 1933; Robinson 1937; Lawrence *et al.* 1939; Bate-Smith 1954; and Bate-Smith and Lerner 1954). Positive reactions to the above test have been reported for many species (Isenberg and Buchanan 1945; Stearns and Hartley 1952; Towers and Gibbs 1953) although leucoanthocyanins were not recognized as the responsible substances.

Bate-Smith (1953) showed that they occur much more commonly in the tissues of the woody plants than in those of the herbaceous plants. Large amounts have been shown to be present in the bark of certain eucalypts (Hillis 1954). Robinson and Robinson (1933) reported that they are found in larger amounts in the heartwood than in the sapwood and they (1935) isolated the first leucoanthocyanidin from the heartwood of *Peltogyne pubescens* Bth. Recently Pigman *et al.* (1953) isolated a leuco-

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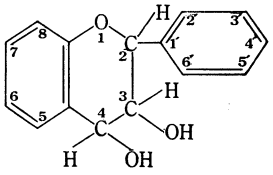
anthocyanin from the inner bark of *Picea mariana* B.S.P. and King and Bottomley (1954) isolated a leucoanthocyanidin from the heartwood of *Acacia melanoxylon* R. Br.

Bate-Smith and Lerner (1954) considered that the production of leucoanthocyanins is part of a primitive metabolic pattern associated with, but not essential to, a woody habit of growth. They also discussed the relationship of these substances to lignin and tannin.

Whilst it is agreed that under special circumstances they may be the precursors of anthocyanins (Robinson and Robinson 1939; Rutzler 1939), the existing contention is that they are derived, at least in part, from a common pigment precursor (Scott-Moncrieff 1939).

Recent work (e.g. Bauer, Birch, and Hillis 1954; Swain 1954) has shown that the leucoanthocyanidins have the basic structure:



This is the cyclic form of the compound C_6–CH(OH)–CH(OH)–CH(OH)–C_6 which Robinson (1936) postulated arose from sugars and was the precursor of the flavonoids and the anthocyanidins. Consequently leucoanthocyanidins could be one of the first forms of polyphenolic compounds synthesized in the plant and could be the precursor of flavonoids, anthocyanidins, and tannins found in various woody tissues.

Szent-Györgyi (1937) and Reichel and Burkhart (1938) have considered that certain flavones play an important part as components of the oxidation-reduction systems of the plant. Leucoanthocyanidins can be theoretically transformed to flavonoids and anthocyanidins by dehydrogenation, hydrogenation, dehydration, and pyran-ring opening. The ubiquitous leucoanthocyanins could play, then, an important role in the respiration of plant tissues, and the more stable compounds usually isolated from these tissues being the product of the respiration therein.

This present work reports the examination of tissues of certain species of eucalypts. Leucoanthocyanins were estimated to ascertain whether large amounts are found in those tissues where sugars are formed or accumulate, which would be expected if leucoanthocyanins are an early stage in the path of sugars to tannin. Also a chromatographic examination of extracts from these tissues was made to gain an idea of the nature of the accompanying components and whether there was any relationship between them.

II. Material Used

(*a*) *Leaves*

Leaves from branchlets of the following species were examined: *Eucalyptus sieberiana* F. Muell. (silvertop ash), *E. obliqua* L'Herit. (messmate), *E. capitellata* Sm. (brown stringybark), *E. regnans* F. Muell. (mountain ash), *E. elaeophora* F. Muell. (long leaved box), and *E. maculata* Hook. (spotted gum). They were obtained at different periods during the unusually wet and late spring of 1954.

A notable feature of the spring growth of *E. sieberiana* is the bright red colour of the leaves. Other eucalypts show a red or red-brown colour but not to the same intensity. At the height of spring growth a typical stem of *E. sieberiana* will carry ten or more red leaves with the third and fourth from the apex being the most intensely coloured. The number of red leaves decreases as the season progresses.

In the youngest completely unfolded leaf, the extreme tip (one-fifth of the length) is almost devoid of any colour. Adjacent to it is a pink-red area (one-third of the length) and the rest of the leaf is a very pale pink; the marginal vein is distinctly pink and the midrib greenish yellow. With the exception of the tips, which are pale green, the whole of the third and fourth unfolded leaves are red in colour, but the veins appear to be less intensely red. The sixth to eighth leaves, still with pale green tips, show signs of green on both sides of the midrib in the central portion; this area increases in older leaves until only the marginal vein is red and finally this too becomes green. When green first appears in the leaf the veins become redder than formerly.

(*b*) *Stems of Branchlets*

The external portion of the new season's growth of *E. sieberiana* is reddish yellow near the apex, becoming brilliant red further along, the colour reaching a maximum at about the position where the fifth or sixth leaf is attached. This colour is present in the next 2–3 ft or more but decreases in intensity. The stems are still red some distance from where the leaves have become completely green. The external layer of the under-portions of the branchlets takes on a green colour at a distance much closer to the apex than does the upper surfaces.

A similar pattern of colour changes was observed with other species but it was less marked.

(*c*) *Cambial Zone*

The cambial zone was gently removed with a "Perspex" scraper immediately after the phloem and bark was lifted. When it was removed from a log of *E. sieberiana* collected on November 15, 1954 the colourless material became distinctly pink in about a minute, becoming increasingly brown after 5 min. This transient pink colour did not appear when samples were collected later in the season.

(*d*) *Bark Samples*

Sections of the tree trunk were collected at the same time as the leaves and were stored at 5°C. The samples of bark were taken from different positions on the tree at a distance between 0·05–0·10 in. from the cambial zone.

(*e*) *Wood Samples*

The samples of sapwood and heartwood of *E. sieberiana* were taken from the transverse sections referred to in Section II (*d*). As is the case with many tree species, the change of sapwood to heartwood was abrupt, with a change in the colour of the wood and an increase in the amount of extraneous materials present in the cavities of the various cells particularly the rays and vertical parenchyma.

Occasionally in log cross sections of jarrah (*E. marginata* Sm.) a clear red coloured outer heartwood zone contrasts with the inner heartwood possessing the more usual brown-red colour. Zones up to 1·25 in. wide in a 12-in. diameter section have been observed, and also a lenticular shaped zone (0·5 × 3 in.) was seen in a cross section in which the normal abrupt change of sapwood to heartwood occurred elsewhere. The transverse discs of *E. marginata* were collected from mature trees (more than 100 years old) and from regrowth trees (about 50 years old) grown in Western Australia.

(*f*) *Kino*

Liquid kino was collected from the heartwood of *E. sieberiana* (log, December 7, 1954) and 1·5 in. from the boundary with the sapwood. Solid kino which was fresh in appearance was collected in a crevice in the bark from another tree of this species.

III. Experimental Procedure

(*a*) *Estimation of Leucoanthocyanin*

(i) *Method.*—A development of the method introduced by Pigman *et al.* (1953) and further applied by Hillis (1954) was used as follows: immediately after preparation, the sample was weighed and heated with 10 ml of *iso*propanol: 3·0N HCl (5: 1)

Table 1

Distribution of leucoanthocyanin in a Eucalyptus obliqua leaf

Section	1	2	3	4	5	6
Distribution (%)	1·67	2·67	5·26	3·99	3·85	3·04

in a tube (15 × 1 cm) equipped with a ground-glass stopper which was held in place with spring clips. The tube was heated in boiling water for exactly 40 min with occasional tipping to mix the contents, and then cooled to room temperature in a beaker of cold water. The filtrate from the tube was quantitatively diluted with *iso*propanol until the transmission was between 20–30 per cent., then the optical density was determined accurately between 520 and 565 mμ with a Unicam spectrophotometer model S.P. 500. Chromatographic examination (see Section III(*b*)) indicated that delphinidin was the main anthocyanidin produced and the maximum absorption (550–555 mμ) supported this (Bate-Smith 1954). The leucoanthocyanin was calculated from the anthocyanidin produced on the provisional assumptions that the conversion was complete and that delphinidin was the only anthocyanidin produced. The results were corrected for the effect of dilution, then calculated to dry weight.

The leaves were analysed within a few hours of collection, as it was found that the leucoanthocyanin decreased on storage, particularly in the pale tips of the leaves which quickly shrivelled and became discoloured. The *E. obliqua* leaf (Table 1; Fig. 1) had been stored for 2 days when the amount of leucoanthocyanin was reduced, mostly in the tip.

(ii) *Effect of Natural Red Colour.*—No attempt was made to correct the results for the natural red colour of the leaves as it was found that it did not make any real difference. This colour was reddest in the third unfolded leaves from the apex and was found to be 5·4 and 8·1 per cent. of the total red colour produced after heating with *iso*propanol-hydrochloric acid.

(iii) *Effect of Background Colour.*—In addition to the leucoanthocyanins, the *iso*propanol in the reagent removes many other substances, and of these, chlorophyll appears to be the principal source of the background colour. This colour interferes somewhat with the estimation of anthocyanidin. Although there is much less interference when aqueous hydrochloric acid is employed for the conversion to anthocyanidin, the author has found this reagent to be less sensitive and less suitable for quantitative work. Several attempts were made to estimate the background colour but, as it was not possible to do so accurately, no corrections have been made.

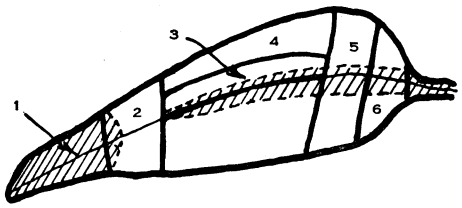


Fig. 1.—Sections taken from an *E. obliqua* leaf. Approximately natural size. Unshaded portions red.

(iv) *Sampling.*—In order to ascertain whether leucoanthocyanins are distributed uniformly, an *E. obliqua* leaf which was mainly red was sectioned as shown in Figure 1. The leucoanthocyanin content of the portions is shown in Table 1. Obviously, the analysis of a random section of a leaf would not represent that of the whole leaf. The present procedure has been to take all of the small leaves (less than 2 in. long) and one-half of the larger ones after slitting them along the midrib.

To ascertain the effect of increasing the ratio of sample to reagent beyond the optimum limits, a complete leaf was finally minced, then thoroughly mixed, and various amounts taken and analysed as in Section III(*a*)(i), the whole procedure being completed as quickly as possible. The reaction products were diluted so that the suitable transmission was obtained. It was found that when the ratio of sample to reagent was increased there was a linear decrease in the anthocyanidin produced, e.g. a 10 per cent. decrease when a 4 times dilution was needed to obtain a suitable transmission, 20 per cent. for a 7·5 times dilution, and 40 per cent. for a 15 times dilution. Almost all the dilutions needed in the routine analyses of leaves were less than a 10 times dilution, and all results have been corrected for dilution effects. Standard conditions of technique were observed in all the leucoanthocyanin estimations and the results are reproducible to within 5 per cent.

(*b*) *Paper Chromatography of Extracts of Tissues*

The solvent-ascent method was used (at 22°C) in the normal manner. The ethanol extracts and the *iso*propanol-hydrochloric acid reaction products were

TABLE 2

RATIO OF DELPHINIDIN TO CYANIDIN IN DIFFERENT TISSUES AFTER *iso*PROPANOL–HYDROCHLORIC ACID TREATMENT

Solvent	Location									
	Red Leaves	Green Leaves	Petiole of 3rd Leaf	Red Outer Layer of Stem		Green Outer Layer 12. in from Petiole of 3rd Leaf	Bark	Cambium	Heart-wood	Kino
				2 in. from Petiole of 3rd Leaf	12 in. from Petiole of 3rd Leaf					
CH_3COOH: HCl: H_2O (30: 3: 10 v/v)	4: 1	3: 1	1: 2	2: 1	2: 1	3: 1	4: 1	Delphinidin only	4: 1	Delphinidin only
CH_3COOH: HCl: H_2O (10: 2: 20 v/v)	1: 2	1: 1	1: 4	1: 1	2: 1	3: 1	4: 1	—	4: 1	8: 1
Wavelength at max. absorption (mμ)	555	555	550	550	550	555	550	550	555	555

prepared from similar amounts of tissue and kino samples using the same proportion of liquid to sample. The samples were repeatedly chromatographed and R_F values were within $\pm$ 0·02 R_F. Delphinidin, cyanidin, and quercetin were used as controls.

The resolving solvents used were (i) *n*-butanol: acetic acid: water (40: 10: 50 v/v); (ii) *n*-butanol: 2N HCl (1: 1 v/v); (iii) acetic acid: conc. HCl: water (30: 3: 10 v/v) (the "Forestal solvent" (Bate-Smith 1954)); (iv) acetic acid: conc. HCl: water (10: 2: 20 v/v) (Bottomley 1954); (v) phenol: 2N acetic acid+HCl (1: 1 v/v) (Hillis 1950).

The chromatograms were examined as soon as they were removed from the tanks in order to locate the anthocyan(id)ins, then, as soon as the sheets were air dry, were again examined under ultraviolet light before and after fuming with ammonia. The sheets were also sprayed with ammonia-silver nitrate or dipped in a freshly-mixed solution of ferric chloride and potassium ferricyanide (Barton, Evans, and Gardner 1952).

Delphinidin was usually present in the reaction products from the leucoanthocyanins in greater amounts than cyanidin but the ratio varied according to the location of the sample in the tree (see Table 2). The ratios obtained with the Forestal solvent (30: 3: 10 v/v) are probably the most reliable.

(*c*) *Wood Analysis*

In order to indicate the changes which take place when heartwood is formed in *E. marginata*, the hot water, alkali, and acetone soluble materials were determined. The first solvent removed low molecular weight carbohydrates or low molecular weight polyphenols or both, the second certain carbohydrates or polyphenolic substances or both, and the third mainly low molecular weight polyphenols. The methods used were as follows:

(i) *Hot Water Soluble Material.*—Two grams of accurately weighed sample (ground to pass a 20 mesh sieve) were heated with 300 ml of distilled water on a boiling water bath for 1 hr. The suspension was filtered hot through a coarse porosity alundum crucible (R.A. 98) and all the sawdust transferred to the crucible and washed thoroughly with 500 ml of boiling water. The extracted sawdust was dried under vacuum at 45°C to constant weight.

(ii) *Alkali Soluble Material.*—As in Section III(*c*)(i) but with 300 ml 0·1N NaOH instead of distilled water.

(iii) *Acetone Soluble Material.*—Ten grams of accurately weighed sample were extracted with acetone in a Soxhlet apparatus on a boiling water bath for 10 hr. The extracted material was dried under vacuum at 45°C to constant weight.

IV. Results and Discussion

(*a*) *The Distribution of Leucoanthocyanins*

The analyses of leaves from young stems of different trees are given in Tables 3 and 4. The amount of leucoanthocyanin present decreased as the maturity of the leaves increased.

When a whole leaf was heated with the *iso*propanol–hydrochloric acid reagent, the reddest part was the tip. The pale tips were removed from each successive leaf

TABLE 3

DESCRIPTION AND LEUCOANTHOCYANIN CONTENT OF EUCALYPTUS SIEBERIANA LEAVES

Results calculated on volatile-free leaves

Leaf No.	Leaves Collected 15.xi.54			Leaves Collected 24.xi.54						Leaves Collected 7.xii.54			Leaves Collected 14.xii.54		
				25-Ft Tree			4-Ft Tree								
	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)
1	Yellow-red	0·75	0·63	—	—	—	—	—	—	Yellow-red	0·75	2·26	Red	0·75	0·84
2	Red	1·0	1·16	Red	0·75	1·31	—	—	—	Red	0·75	2·06	Red	0·75	
3	Max. red	1·25	1·08	—		—	—	—	—	Red	1·125	1·91	Max. red	1·25	0·78
4	Green along midrib	2·0	0·82	Max. red	1·25	1·22	Max. red	1·0	1·04	Max. red	1·25	1·88	Red	1·75	0·68
5	Greener	2·75	0·64	—	—	—	—	—	—	Red	1·5	1·58	Red	2·0	0·69
6	Greener	3·5	0·49	Green along midrib	2·5	1·18	—	—	—	Red	1·75	1·70	Red	2·5	0·57
7	Greener	3·5	0·52	—	—	—	Red	2·5	0·68	Red	2·0	1·31	Green	2·75	0·48
8	—	—	—	Greener	3·0	0·60	—	—	—	Green along midrib	2·875	1·28	—	—	—
9	—	—	—	—	—	—	—	—	—	Damaged	—	—	—	—	—
10	—	—	—	All green	3·0	0·60	—	—	—	Weak red	3·5	0·14	—	—	—
12	—	—	—	—	—	—	All green	1·75	0·77	—	—	—	—	—	—
14	—	—	—	—	—	—	Green	—	0·22	—	—	—	—	—	—
16	—	—	—	—	—	—	Green	—	0·15	—	—	—	—	—	—

on a freshly-collected stem and heated in each case with the same proportion of the reagent; the intensity of colour showed that the amount of leucoanthocyanin in the leaf tip steadily decreased as the leaf became more mature.

The outer layers removed from different points of a young stem of *E. sieberiana* were analysed. The anthocyanidins produced by both leucoanthocyanins and anthocyanins showed that these substances were most highly concentrated at a point 7–8 in. from the third red leaf and they decreased gradually on either side of this point.

The two samples of bark collected from a *E. sieberiana* tree showing vigorous leaf growth (November 15, 1954; Table 5) contained more leucoanthocyanin than those collected from different trees later in the season (Table 5).

The leucoanthocyanin present in the cambial zone (Table 5) decreased as the season progressed and, at the same time, the nature of the samples of this zone changed from a gel to an increasingly "stringy" condition. The latter physical condition indicates that more mature cells were present. This association is similar to the decrease in leucoanthocyanin in leaves of increasing maturity. The production of pink colour in the first samples (November 15, 1954) of cambial zone may have been due to enzymatic oxidation of the leucoanthocyanins to anthocyanins under the slightly acid (pH 5·4) and aerobic conditions that existed. The occurrence of pink coloured wood in some freshly-cut billets of *E. regnans* and other species may be due to such a mechanism. Both "pale" and "pink" wood contain the same amount of leucoanthocyanin, but under the influence of light and air there is a greater tendency for those with the lowest pH (as low as 3·0) to form the bright pink colour.

The above evidence suggests that there is association between active cell division and leucoanthocyanin content. Further support was found in the examination of kino which Jacobs (1938) showed was produced in the cambial region as a result of injury and the healing of the wound involved initially, the abundant proliferation of the cambium cells, with the production of parenchyma cells. The leucoanthocyanin in *E. sieberiana* kino was high—8·36 per cent. in the solid sample and 6·45 per cent. in the liquid.

The leucoanthocyanin content of *E. sieberiana* heartwood was double that of the sapwood of the same tree. According to one theory of heartwood formation, the zone of origin of the extractives is at the sapwood-heartwood boundary where there is intense cell activity in the rays and vertical parenchyma just before heartwood formation. The sapwood and heartwood in various logs of *E. marginata* were selected for intensive investigation because in this species not only is there a very great increase in the amount of extractives when heartwood is formed but two distinct heartwood zones are often apparent. Therefore to test whether leucoanthocyanins could be the precursor of the extractives in this species, transverse discs were examined by estimating the leucoanthocyanin in 30-μ tangential sections taken every centimetre or less, and the results are shown in Table 6 and Figure 2. There was an increase at the sapwood-heartwood boundary, a fairly constant amount across the outer heartwood zone, and then a decrease (compare with decrease of pinosylvin in pine heartwood (Erdtman, Frank, and Linstedt 1951)). The amount in included sapwood (Section 7, sample B (Table 6)) was much less than elsewhere. However, no localized increase in leucoanthocyanin content was found at the sapwood-heartwood boundary, which,

TABLE 4

DESCRIPTION AND LEUCOANTHOCYANIN CONTENT OF EUCALYPT LEAVES

Results calculated on volatile-free basis

Leaf No.	*E. obliqua*												*E. regnans*			*E. maculata*		
	Leaves Collected 24.xi.54			Leaves Collected 2.xii.54			Leaves Collected 7.xii.54			Leaves Collected 14.xii.54			Leaves Collected 1.xii.54			Leaves Collected 14.xii.54		
	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)
1	Pink leaf	0·875	4·24	Yellow-brown	1·0	1·84	Yellow-brown	1·25	11·3	Pale green-red	1·25	0·67	Pink	0·375	} 3·49	Pink-green	1·0	} 0·078
2	Greener	1·5	2·82	Pink	1·5	1·90	Pink-red	1·0	>15	Greener	2·0	6·75	Red	0·625		Pink-green	1·0	
3	Greener	3·0	1·62	Pink-red	1·75	1·39	Pink	2·75	8·2	Green	3·75	4·5	Max. red	1·25	2·27	Red	2·0	0·096
4	—	—	—	Pink	2·5	1·63	Greener	3·25	7·0	—	—	—	Greener	1·625	2·24	Red	2·0	—
5	—	—	—	Greener	3·5	1·35	Green	—	8·0	—	—	—	Greener	3·0	1·02	Greener	3·0	0·012
6	—	—	—	Green	3·75	0·66	—	—	—	—	—	—	Greener	4·0	0·86	Greener	3·25	—
7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Greener	3·75	0·008
8	—	—	—	—	—	—	—	—	—	—	—	—	All green	3·0	0·76	—	—	—

if it had occurred, would have given a result similar to that obtained from the section including a kino vein (Section 11, sample A (Table 6)). If leucoanthocyanins are the precursors they are not formed in this region.

Table 7 gives typical analyses of different zones of *E. marginata*. The variation between the alkali soluble materials in the two heartwood zones are insignificant but the dissimilarity in the hot water soluble materials indicates a difference in constitution of the phenolic extractives. One explanation of the difference in these solubilities may be that the heartwood extractives are formed from low molecular weight substances and their conversion to the usual insoluble substances has in these

TABLE 5

LEUCOANTHOCYANIN CONTENT OF DIFFERENT TISSUES OF EUCALYPTUS SIEBERIANA AND E. OBLIQUA

The different values refer to samples from different parts of the tree

Date Collected	Leucoanthocyanin Content (%)				
	E. sieberiana				*E. obliqua*
	Bark	Cambium	Sapwood	Heartwood	Bark
15.xi.54	1·80, 1·72	1·24, 1·28, 1·36, 1·40	0·043, 0·044, 0·050	0·10, 0·10	—
24.xi.54					
25-ft tree	0·25, 0·37	—	—	—	0·057
4-ft tree	0·15, 0·20	0·32, 0·31	—	—	—
7.xii.54	0·53, 0·45	0·37 (base of trunk) 0·36 (top of trunk)	0·027	0·060	—

abnormal cases been delayed. If this is true then the analyses of the 50-yr old jarrah samples indicate that the conversion has not commenced. In view of their water solubility the outer heartwood extractives could have been transported in the sap to the sapwood-heartwood boundary.

Alkali was necessary to remove all the heartwood extractives, but the hot water soluble and alkali soluble materials of the sapwood contained large amounts of non-phenolic substances not removed from the heartwood. If the alkali soluble materials present in the heartwood zones were considered to be polyphenolic substances, then a low percentage of leucoanthocyanins would be found in the materials occurring in the outer heartwood zone and a still lower one in the inner heartwood. On the other hand, the percentage of leucoanthocyanins in the acetone soluble material was high in the sapwood but decreased in the outer heartwood (Table 7).

Chromatographic examination showed that the anthocyanidins formed from the leucoanthocyanins did not decrease in any tissues to the same extent as the anthocyanins and flavone glycosides when the sample was taken at increasing distances from the leaves.

Both types of leucoanthocyanins were present in all differentiated tissues and they may well be formed initially in the leaves and transported along with carbohydrates and other materials to the rest of the tree. In support of this view, Lindner, Fitzpatrick, and Weeks (1950) found that any type of phloem blockage may produce

TABLE 6

ANALYSIS OF SECTIONS TAKEN FROM TRANSVERSE DISCS OF EUCALYPTUS MARGINATA

Section	Leucoanthocyanin Content (%)		Section	Leucoanthocyanin Content (%)	
	Sample A	Sample B		Sample A	Sample B
1	1·50	1·51	9	3·35	3·00
2	1·58	1·30	10	2·82	1·65
3	3·43	3·01	11	4·35	2·50
4	4·13	2·83	12	2·24	1·60
5	4·62	2·42	13	4·10	2·12
6	4·56	2·79	14	1·44	1·86
7	3·19	0·20	15	2·34	1·15
8	4·00	2·10	16	4·61	—

polyphenol accumulation; from their description these polyphenols could have been leucoanthocyanins. Using staining and other techniques Wood (1933) found that the younger leaves of eucalypts and acacia contained the most tannin. At first it was distributed in all cells of the leaf but later was found only in the palisade and conducting

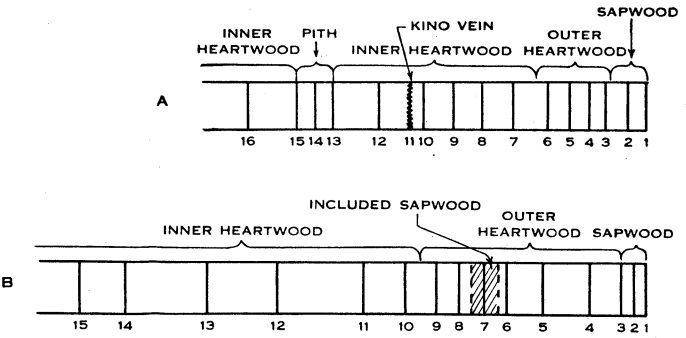


Fig. 2.—Locations of analytical sections in *E. marginata* log cross sections.

tissues. The amount of tannin was found to decrease in the leaf as the quantity of lignin increased so that it was suggested that the phenolic nucleus in the tannin provided a starting point for the lignone complex of lignin. Wood also pointed out that considerable quantities of tannin in the leaf were transported to the bark. All of the polyphenolic substances mentioned in this present work would react similarly with the histological stains used by Wood.

Leucoanthocyanins appear to be one of the products accumulated by unhealthy tissue as Stearns and Hartley (1952) found that virus-affected peach root

gave a violet colour with methanolic hydrochloric acid. This colour is given by materials containing leucoanthocyanins in the presence of coniferyl aldehyde or wood lignin (Adler 1951). Lindner *et al.* (1950) found that whereas virus-infected woody

TABLE 7

ANALYSIS OF EUCALYPTUS MARGINATA (JARRAH)

Results calculated on volatile-free wood. S = Sapwood, O = outer hardwood, I = inner hardwood

Sample	Zone	(1) Hot Water Solubles (%)	(2) O·1N NaOH Solubles (%)	(3) Acetone Solubles (%)	(4) Leuco-anthocyanins (%)	$\frac{(4)}{(3)} \times \frac{100}{1}$ (%)
A	S	17·8	31·9	4·81	2·44	50·7
	O	18·4	29·0	12·2	2·45	20·1
	I	8·0	30·8	4·14	1·64	—
B	S	15·3	29·2	2·58	0·85	32·9
	O	15·9	30·9	16·1	2·90	18·0
	I	12·1	28·7	9·17	2·37	—
C	S	14·4	26·6	2·21	1·49	67·4
	O	9·1	26·9	10·5	0·93	8·80
	I	1·8	28·4	0·71	0·40	—
D	S	18·1	28·3	4·80	1·40	29·2
	O	15·4	30·8	11·8	3·30	28·0
	I	9·75	30·8	5·86	2·60	—
E	S	15·8	31·7	3·60	2·74	76·2
	O	8·9	31·0	19·4	2·30	11·8
	I	1·6	29·7	4·63	0·56	—
Regrowth 1	S	10·8	25·9	1·15	—	—
	I	31·2	39·7	—	—	—
Regrowth 2	S	14·6	28·0	2·25	0·35	15·6
	O	26·7	37·4	7·94	2·69	33·9
	I	26·2	36·3	5·36	1·95	—
Regrowth 3	S	14·0	24·8	0·83	0·54	65·1
	O	26·4	38·5	8·05	1·78	22·1
	I	25·5	34·7	4·69	1·78	—

plants gave good reactions with their polyphenolic stain, the virus-infected herbaceous plants gave no reaction; a similar observation has been made regarding the distribution of leucoanthocyanins in healthy tissues (Bate-Smith 1953).

(*b*) *Biogenesis of Flavonoid Materials and Tannins*

Different ratios of flavonoid materials were found after leucoanthocyanins were heated at 100°C under different conditions with *iso*propanolic and aqueous hydrochloric acid. For example, the conversion yield to anthocyanidin was reduced

by the use of aqueous acid, or heating in an open tube, or heating for prolonged periods.

The leucoanthocyanins from *Acacia melanoxylon* readily change into other flavonoids, even when stored in an acetone solution in the dark (Hillis, unpublished data). When heated with *iso*propanol–hydrochloric acid for 40 min several substances were formed, amongst which 3, 3′, 4′, 7, 8-pentahydroxyflavylium chloride and 3, 3′, 4′, 7, 8-pentahydroxyflavone have been identified. A yellow fluorescent substance with $R_F = 0$ and leucoanthocyanin properties was also present. Swain (1954) also showed that the same classes of compounds are formed from leucocyanidin with dilute acid. Earlier, Forsyth (1953) found that, after 5 min heating at 100°C with 0·1N hydrochloric acid, leucocyanidin was changed to 31 per cent. (−) *epi*catechin, 6 per cent. (−) catechin, and other substances.

When 3, 3′, 4′, 7, 8-pentahydroxyflavylium chloride was heated with *iso*propanol–hydrochloric acid other flavonoids were produced. But with this substance heating for 3 hr was necessary before an 80 per cent. change occurred so that it does not affect the above observations. The only changes noticed with other classes of flavonoids were that quercetin yielded a dull yellow fluorescent compound ($R_F = 0$) on prolonged heating in an open tube with *iso*propanol–hydrochloric acid. Also kaempferol was formed when dihydrokaempferol was heated with 0·6N hydrochloric acid for a prolonged period.

Although the above *in vitro* conditions to which leucoanthocyanins have been subjected are severe, it is considered that enzymatic processes could accomplish the (and possibly other) transformations in the plant. Examples of leucoanthocyanins and other corresponding flavonoids occurring in nature are known. Chromatographic examination showed that the leucoanthocyanin and the corresponding 3, 3′, 4′, 7, 8-pentahydroxyflavone exist in *A. melanoxylon* heartwood. Forsyth (1952) isolated leucocyanidin, cyanidin glycosides, and catechins from fresh cacao bean extracts but the basic structure of these materials is ubiquitous in nature. Alston and Hagen (1955) have produced evidence that leucoanthocyanins were the precursors of the anthocyanins in the flowers of *Impatiens balsamina*.

The leucoanthocyanins are much more widespread in the plant kingdom than any other single class of flavonoid and this suggests that they may have some special function in the processes of the cell. They may play a part in the respiration of the cell as by appropriate dehydrogenation and dehydration of the C_3 portion of the molecule it is theoretically possible to transform leucoanthocyanin to most other classes of flavonoid materials. Apart from the *iso*flavones the two exceptions are the catechins and the benzalcoumaranones, the former could be formed by dehydration followed by hydrogenation, and the latter would have to be formed following a pyrone-ring opening in, say, a flavanol.

Szent-Györgyi (1937) and Reichel and Burkhart (1938) have considered that certain flavones play an important part as components of the oxidation-reduction systems of the plant. Hibbert (1941) proposed a scheme to indicate the course of the reductive and oxidative changes of C_6–C_3 units in the plant. Apparently the plant may selectively produce a given state of oxidation of the C_3 portion from some precursor which is characteristic of certain groups of plants. This implies that the

hydroxylation pattern of the benzene rings is established prior to the oxidation (or reduction) of the C_3 portion to its final state and support for this is found in evidence collected by Geissman and Hinreiner (1952).

If the different classes of flavonoids are formed from leucoanthocyanins by an oxidation-reduction-dehydration process, then the same type of tissue in different plants would be expected to have similar sets of conditions favouring a certain class of compounds. However, this holds only for the class of flavanonols which are most often found in heartwood. Theoretically, flavanonols and flavonols can be formed sequentially from leucoanthocyanins, and they have been found together in nature with the following hydroxyl pattern: 3, 3′, 4′, 7-tetrahydroxy (Oyamada 1939); 3, 3′, 4′, 5, 5′, 7-hexahydroxy (Kotake and Kubota 1940); 3, 3′, 4′, 5, 7-pentahydroxy (Pew 1948); 3, 4′, 5, 7-tetrahydroxy (Hillis 1952); 3, 3′, 4′, 5′, 7-pentahydroxy (Freudenberg and Hartmann 1954).

The author has found that usually more than one anthocyanidin is formed when plant tissues are heated with the more efficient *iso*propanol–hydrochloric acid reagent (Hillis 1954 and unpublished work). The parent leucoanthocyanins could account for the occasional occurrence in the sample of representatives of the different classes of flavonoids with different hydroxyl pattern. The conversion to the different classes of flavonoids could depend on the nature of the leucoanthocyanin. For example, the phloem of *E. sieberiana* yielded delphinidin and cyanidin more slowly on treatment with *iso*propanol–hydrochloric acid than cambium gave cyanidin.

Eberhardt (1954) found that anthocyanin formation depended on intensity of respiration as shown by the rapidity of metabolism of sugar. The maximum amount of anthocyanin was formed after the maximum respiration occurred and he considered that colour formation took place during the last phase of a two-stage colouring synthesis, the second phase being independent of respiration metabolism. In this present work, the greatest amount of leucoanthocyanin was found in the youngest eucalypt leaves. Therefore, it is noteworthy that Arney (1947) found that there was a rapidly rising rate of respiration on young strawberry leaves as they unfolded and expanded. Similar observations have been made by Marsh and Goddard (1939) and James (1953).

(*c*) *Distribution of Materials Possibly Formed from Leucoanthocyanins*

By means of semi-quantitative comparisons in the chromatographic studies, changes in amounts of the resolved components have been observed.

Work in progress indicates that one of the main resolved components is quercetin glycoside. The amount contained in the petioles of the third red leaves was less than half the amount found in the leaves, and it was considerably less in the red external portion of the stem 2 in. away, and still less 12 in. away; the green portion of the stem at this latter position contained just detectable amounts of this substance which was absent from other tissues. Another component appears to be a myricetin glycoside and it was present in the leaves in about the same amount as the quercetin glycoside but it decreased more rapidly in the above tissues. On the other hand a mauve fluorescent substance with the properties of ellagic acid increased as the above two decreased.

The amount of anthocyanins in the red external portion of the stem 7–8 in. from the third leaf appeared to be greater than that in the leaf or elsewhere on the stem. The quantity of the two main flavone derivatives appeared to decrease more than the anthocyanins when the sample was taken in increasing distances down the stem.

The main red pigment in the external portion of the stem was different from those in the leaves. This observation has been confirmed with three different samples of *E. sieberiana*, and also on *E. obliqua* (3 samples), *E. maculata* (2 samples), *E. regnans*, *E. capitellata*, and *E. elaeophora*.

The author has shown (unpublished data) that the anthocyanins in the leaf are glycosides of delphinidin whereas the main one in the petiole and the external layer of the stem is a glycoside of cyanidin. It is possible that the delphinidin-based anthocyanins are retained by the leaf and the cyanidin-based anthocyanin is formed in the external layer of the stem. This view is supported by the observations that (i) frequently young stems had large areas of a red external layer although carrying few red leaves, and (ii) the injuries made by gall insects on pale pink leaves were surrounded by localized circles of intense red without any trace of movement into the veins.

The bark anthocyanins could be synthesized *in situ*, but it is also possible that a leucoanthocyanin originating in the leaf could be a precursor and be transformed to the anthocyanin under the influence of direct light and other agencies. The minor red colouring material in the outer layer of the stem appeared to be a delphinidin glycoside but there is no evidence that it originates in the leaves.

(*d*) *Origin of Heartwood Extractives*

The origin of the heartwood extractives is at present unknown. Some workers consider that they are formed from the starch in the sapwood cells during their sudden burst of activity prior to their death. Others consider that the extractives are transported centripetally from the cambium via the medullary rays (Jaccard 1938), but not transported centrifugally from the cambium into the bark as no trace of the heartwood phenols were found there (see Linstedt 1951). However, it has been shown in a number of eucalypts and other samples that the different transverse zones of the trunk and roots contained the same general pattern of resolvable materials (Hillis 1952).

In view of the evidence presented in this paper the author suggests that the leucoanthocyanins are the immediate precursors of the different classes of heartwood extractives based on the C_6–C_3–C_6 pattern and that these leucoanthocyanins originate in the leaves and principally the tips of the young leaves.

The leucoanthocyanins could give rise to different classes of compounds in response to different physiological conditions, diseases, temperature, or to light absorption. For example, conditions in the young leaves, the red external layer of the upper stem of certain eucalypts, and the callus tissues induced in the stems favour the formation of anthocyanins which are not found elsewhere in the tree. The transformation products are apparently left behind in these tissues which could be due to insolubility in the sap stream or to involvement in certain functions in that location. The leuco-

anthocyanins would need to join the stream of other materials in their passage from the leaves through the phloem to the sapwood. The saprophytic fungi considered to be associated with heartwood formation (Chattaway 1952) would be expected to establish a different oxidation-reduction potential to that existing in the phloem, and the leucoanthocyanins could be changed to different ratios of flavonoids and tannins in these two tissues.

This mechanism implies that the cambium does not play a part in the formation of heartwood extractives and there is support for this. Anderson (1953) found that the heartwood produced in pines as a result of stimulation of the cambium with sulphuric acid did not contain all the phenolic constituents of normal heartwood; also a different lignan has been found in wound resin (from the cambial region) and wound heartwood than has been found in the normal heartwood of certain spruce species (Erdtman 1949). Certain eucalypt kinos contain dihydrokaempferol (Hillis 1952, 1953) which has not been found in the heartwood of the same species. The argument against these cases is that the abnormal conditions induced may have been responsible for the differences. However, samples obtained during the normal growth of the tree show that leucoanthocyanins which yield cyanidin and delphinidin are present in all tissues other than the proliferating cambium and the kino formed in the cambium, whereas these latter contain only leucoanthocyanins which yield delphinidin. There does not appear to be any enrichment in the delphinidin: cyanidin ratio in the heartwood when compared with the phloem so the cambium does not supplement the supply of leucoanthocyanins (Table 2).

Numerous gaps in this thesis are being examined in order to test the validity of the postulations. The information should help in an understanding of the transformation of sapwood to heartwood, with the ultimate aim of control of heartwood formation, and, where required, an improvement in its durability.

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