The Formation of Polyphenols in Trees

2. THE POLYPHENOLS OF *EUCALYPTUS SIEBERIANA* KINO*

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The polyphenolic components in the cambial tissues of several eucalypt species are mainly C_6 compounds in the form of ellagitannins; shikimic acid is also present in appreciable quantities (Hillis & Carle, 1958, 1962; W. E. Hillis, unpublished work). When the cambial tissues of eucalypts are injured in the growing season, a faint-brown liquid commonly exudes and dries to a brown or brown-red, brittle, semi-transparent solid. This astringent exudate, known as kino, contains very largely C_{15} compounds, usually in the form of leuco-anthocyanins (Hillis & Carle, 1960, 1963; W. E. Hillis, unpublished work). Consequently, it appears that acetate units become available in relatively larger amounts in damaged cambium to enable the formation of the A ring of the C_{15} flavonoid components of kino. Attempts to indicate the processes governing this increased availability have been made with the use of enzyme inhibitors and are described in the present paper.

Several types of linkages between the leuco-anthocyanin units of condensed tannins have been proposed (Hathway, 1962; Hergert, 1962; Swain, 1962). Some of these linkages have resulted during the study of the behaviour of model compounds under conditions *in vitro*. Similar linkages may also form *in vivo* when polyphenols remain in plant tissues for long periods and during their isolation and purification. However, little attention has been given to the possibility that these linkages may supplement or modify those originally formed. An examination of polymerized polyphenols within a short period of their formation should yield information on the nature of 'primary' linkages. The definition of such linkages would assist the determination of the extent of linkages formed subsequently. Kinols can be collected within 5 days of formation (Hillis & Hasegawa, 1963) and are suitable material for study. This present work reports an investigation of the kino of *Eucalyptus sieberiana* F. Muell. (family Myrtaceae), which is very largely composed of polymerized leuco-delphinidin. The polymers formed by monomeric leuco-delphinidin (3',4',5,5',7'-pentahydroxyflavan-3,4-diol) and the related gallo catechin under conditions *in vitro* and those isolated from plant tissue have been studied previously (Hathway, 1958; Roberts, 1957).

* Part I: Hillis & Hasegawa (1963).

EXPERIMENTS AND RESULTS

Melting points are uncorrected. Analyses are by Dr K. W. Zimmermann and associates, C.S.I.R.O., Melbourne. Infrared-absorption spectra were prepared by Mr K. H. Harrington of these Laboratories. Two-dimensional chromatograms were developed in butan-1-ol-acetic acid-water (6:1:2, by vol.) followed by 6% (v/v) acetic acid, and one-dimensional chromatograms in water, water-saturated phenol or water-saturated butan-2-ol. The anthocyanidins were examined with acetic acid-conc. HCl-water (30:3:10, by vol.), formic acid-conc. HCl-water (5:2:3, by vol.), 2N-HCl-acetic acid (7:3, v/v) and butan-1-ol-acetic acid-water (6:1:2, by vol.) by using paper washed with HCl. The chromatograms were examined by methods described by Hillis & Carle (1960).

Collection and examination of kino samples

The kino was collected every 12 hr. from an incision made into the cambial region of an area that exuded kino. The kino exudation had commenced naturally in a 6-year-old *Eucalyptus sieberiana* tree. About 200 mg. was collected daily for 10 days. The colourless viscous liquid had pH 3.5 (by indicator paper). The samples were examined within 48 hr. of collection or dried *in vacuo* over P_2O_5 ; λ_{max} 221 and 278 m μ , λ_{min} 258 m μ in ethanol (Found: C, 55.7; H, 3.9; ash, 0.4. $C_{15}H_{13}O_8$ requires C, 56.0; H, 4.0%). The i.r.-absorption curve (KCl disk) showed sharp peaks at 5.90, 6.20, 6.50, 6.92 and broad peaks at 7.5, 8.3, 8.7, 9.1, 9.7 μ . The curve was similar to that of leuco-cyanidin (Freudenberg & Weinges, 1958b).

Freshly dried kino was an almost colourless glassy solid, which when stored in the absence of light became increasingly brown after 7 days, or when placed under u.v. light became distinctly red in 3 hr. Aqueous solutions of the kino gave an immediate precipitate with a gelatin-salt reagent (Atkin & Thompson, 1937), indicating strong tanning properties. Except for faint traces of gallic acid, the kino formed a streak in all chromatographic solvents and reacted strongly with phenolic reagents and particularly with $FeCl_3$ -potassium ferricyanide (dark blue) and vanillin-HCl (brilliant red) reagents.

Kino (264 mg.) was dissolved in water (20 ml.) and dialysed through Visking Cellulose Dialysis Tubing (av. pore size 2.4 m μ ; Visking Co., Chicago, U.S.A.) into stirred water (400 ml.) for 30 hr., when the water was changed and the dialysis continued for a further 96 hr. The dialysis was conducted in diffuse light. The diffusate and the non-diffusible material were evaporated under vacuum at 40°. The former fraction (17% of the kino) contained polymeric material and a small amount of gallic acid. The chromatographic properties of the diffusate indicated that it was less

highly polymerized than the undialysed fraction and the original kino. The i.r.-absorption curve of the non-diffusible material was very similar to that of the original kino, except that the intensity of the carbonyl band at 5.9μ was considerably reduced.

Kino (5 mg.) was fused with KOH at 220° under N_2 for 4 min., the acidified products were extracted with ether, and gallic acid, phloroglucinol and traces of protocathechuic acid in the extract were identified co-chromatographically by using two-dimensional chromatograms. A weak reaction was obtained with quinonemonochloroimide so that a small proportion of *p*-hydroxybenzyl alcohol groups (Gierer, 1954) was possibly present.

A solution of the kino in 0.1 N-HCl (5 ml.) or 0.1 N-acetic acid (5 ml.) was heated at 99° in a sealed tube in the absence of light for 5 min. and 1.5 hr. respectively. After cooling, the liquids were extracted with amyl alcohol (0.4 ml.) and the extracts examined by two-dimensional chromatography. Faint traces of catechin, ellagic acid, gallic acid, myricetin and three substances with the colour reactions and expected chromatographic properties of monomeric leuco-delphinidins were detected (Hillis & Carle, 1960); no evidence of galocatechin was found.

Chromatographic examination (Harborne, 1958a) showed that delphinidin and traces of cyanidin were the only anthocyanidins produced when kino (100 mg.) was heated with butan-1-ol-conc. HCl (19:1, v/v) at 99° for 40 min. The solvent was removed at 50° under vacuum and the delphinidin isolated with the aid of a polyamide (type TPU 2; Knapsack-Griesheim A.-G., Frankfurt, Germany) column (Chandler & Swain, 1959) and purified with Whatman no. 3 paper and acetic acid-conc. HCl-water (30:3:10, by vol.). The u.v.-absorption spectra before and after the addition of aluminium chloride were found to be identical with those of authentic delphinidin (Harborne, 1958b). The cyanidin was identified chromatographically (Harborne, 1958a) by direct comparison with authentic material.

Kino (12–16 mg.) was dissolved in ethanol (5 ml.), diluted with water to 25 ml. and portions (1 ml.) were heated with butan-1-ol-conc. HCl (19:1, v/v; 10 ml.) at 99° under standard conditions (Swain & Hillis, 1959). The max. extinction (at $555m\mu$) was determined and the $E_{1\%}^{1cm}$ values of eight samples of kino were found to be between 0.80×10^2 and 1.07×10^2 (average 0.92×10^2). The ϵ_{max} value (at $555m\mu$) of delphinidin was calculated from the extinction of solutions (0.010%, w/v) of delphinidin chloride (Fluka A.-G., Buchs S.G., Switzerland) in the same solvent and found to be 39 400 [ϵ_{max} calculated from the data of Hayashi (1962) 43 700 in ethanol-0.1% HCl]. On this basis the percentage conversions of the kino samples into delphinidin were between 6.15 and 8.23% (average 7.08%, equivalent to 7.90% of delphinidin chloride). When a dilute solution of kino was heated at 80° for 5 hr. there was a 35% reduction in the yield of anthocyanidin. An aqueous solution of kino became brown when stored for 1 week with an accompanying 43% reduction in the yield of anthocyanidin.

When kino was heated with 6N-HCl or butan-1-ol-conc. HCl (19:1, v/v), phloroglucinol was formed (Hillis, 1960). The phloroglucinol formed by catechin under similar conditions was identified co-chromatographically with two-dimensional chromatograms. For quantitative estimations of the formation of phloroglucinol, kino or catechin (20–

30 mg.) was heated with 5 ml. of butan-1-ol-conc. HCl (19:1, v/v) in a sealed tube at 99° for 40 min. Measured amounts were applied to a Whatman no. 1 paper, developed in one dimension with 6% (v/v) acetic acid (for catechin) or in two dimensions with butan-1-ol-acetic acid-water (6:1:2, by vol.) then 6% (v/v) acetic acid (for kino). After drying, the chromatograms were dipped in diazotized *p*-nitroaniline in sodium acetate solution (Swain, 1952), washed and dried. Within 3 hr., the size and intensity of the spot were estimated with a Chromoscan J 64 Densitometer (Joyce, Loeb and Co., England) and the amount of phloroglucinol present was estimated from a standard curve for phloroglucinol over the range 0.2–0.6 μ g. Catechin formed 1.28% and kino 2.31% of phloroglucinol.

The strong-blue-white-fluorescent components formed when catechin was heated with butan-1-ol-conc. HCl (19:1, v/v) for 30 min. possessed R_F values 0.60 and 0.0 in butan-1-ol-acetic acid-water (6:1:2, by vol.) and 6% (v/v) acetic acid respectively. The two blue-white-fluorescent components formed by kino possessed R_F values 0.77 and 0.60 in butan-1-ol-acetic acid-water (6:1:2, by vol.) and 0.20 and 0.18 in 6% (v/v) acetic acid respectively. The fluorescent colours under u.v. light and R_F values were similar to those of 3',4,5'-trihydroxy- and 2,3',4,5'-tetrahydroxystilbene, and, like the stilbenes, the intensity of the fluorescence of the components increased when exposed to NH_3 vapour and turned a reddish colour when sprayed with diazotized *p*-nitroaniline. Small amounts of gallic acid and traces of four other components were present in the acid-degradation products, but sugars could not be detected.

Finely powdered kino was exposed to u.v. light for 40 hr. The absorption spectra of the bright-red powder were very similar to those of the original kino; there was no increase in intensity in the regions of 5.9μ and $400m\mu$, showing that quinone groupings (Teuber & Staiger, 1955; Ley & Mueller, 1956; Moriconi, Rakoczy & O'Connor, 1962) were absent.

Acetylated kino. Kino (250 mg.) was acetylated with acetic anhydride (2.0 ml.) and pyridine (2.0 ml.) for 18 hr. at room temperature and poured on to crushed ice. The white amorphous product was dissolved in acetone, filtered and poured into water; the product was dried at 40° for 48 hr. *in vacuo* (Found: C, 56.1; H, 4.3; $CO \cdot CH_3$, 45.1. $C_{15}H_7O_5(CO \cdot CH_3)_6$ requires C, 56.5; H, 4.4; $CO \cdot CH_3$, 45.0%).

Methylated kino. Kino (440 mg.) was dissolved in methanol (10 ml.) and methylated with excess of ethereal diazomethane at 5° for 24 hr. The insoluble material was dissolved in methanol, filtered and poured into ether and the insoluble material dried at 40° for 48 hr. *in vacuo*. (Found: C, 60.9; H, 5.8; $O \cdot CH_3$, 38.0; ash, 0.5. $C_{15}H_8O_3 \cdot (O \cdot CH_3)_5$ requires C, 61.4; H, 5.9; $O \cdot CH_3$, 39.6%). The i.r.-absorption curve showed peaks at 3.40, 5.82, 6.31, 6.69, 6.89, 7.10, 7.52, 8.23, 8.92, 10.0 μ . [Ganguly, Seshadri & Subramanian (1958) report for methyl-leuco-delphinidin 6.26, 6.86, 7.20, 8.26, 8.79 μ .]

Acetylation of methylated kino. Methylated kino (100 mg.) was dissolved in pyridine (1 ml.), acetic anhydride (1 ml.) was added and the mixture allowed to stand for 18 hr. at room temperature and then poured on to crushed ice. The precipitate was washed well with water and dried at 40° for 48 hr. *in vacuo* (Found: C, 62.4; H, 5.8; $O \cdot CH_3$, 36.3; $CO \cdot CH_3$, 5.56. $C_{15}H_7O_5(O \cdot CH_3)_5(CO \cdot CH_3)$ requires C, 63.2; H, 6.0; $O \cdot CH_3$, 37.1; $CO \cdot CH_3$, 10.3%).

Examination of methylated kino

When methylated kino was heated at 99° with butan-1-ol-conc. HCl (3:1, v/v) for 40 min. anthocyanidins A, B and C were formed in the approximate proportions 1:10:10. Appreciable amounts of an orange-coloured compound and traces of a yellow-fluorescent component were also formed. Penta-*O*-methyl-leuco-delphinidin formed a red colour slightly more quickly when treated in the same way, but the proportions of the anthocyanidins formed were about 2:5:2. Whereas similar amounts of the yellow-fluorescent and the orange components were present in about the same amounts the chromatographically immobile material was considerably less. Anthocyanidins A, B and C were strongly salmon-pink-fluorescent under u.v. light and had the properties shown in Table 1.

The above reaction mixture obtained after heating methylated kino with strong acid was partly neutralized with aqueous Na₂CO₃ solution, streaked on to Whatman no. 3 papers, and developed with formic acid-conc. HCl-water (5:2:3, by vol.). The bands containing anthocyanidins B and C were extracted at room temperature with methanol containing 0.1% of HCl and the absorption maxima determined (see Table 1). An impurity (with $\lambda_{\max}$ 363 m μ and presumably a chalcone; cf. Jurd, 1963) masked the true extinction at 440 m μ so that the $E_{440}/E_{\max}$ ratio (Harborne, 1958*b*) could not be determined.

Anthocyanidin A had the expected R_F values of a tetra-*O*-methyl-delphinidin and anthocyanidin B the same R_F values as penta-*O*-methyl-delphinidin prepared by methylating an ethereal solution of delphinidin with diazomethane for 4 min. When the R_M values (calculated from published R_F values; Harborne, 1958*a*) of known anthocyanidins were plotted against functional groups, extrapolation of the curve to the R_M values of anthocyanidin C indicated that such a value could be possessed by hexa-*O*-methyl-delphinidin or by 3',4',5,5',7,-pentamethoxyanthocyanidin. The absorption maximum of C (Table 1) is appreciably higher than those of the known anthocyanidins lacking a C-3 substituent (Harborne, 1958*b*; Jurd, 1963). Hexa-*O*-methyl-delphinidin was prepared in low yield from hexa-*O*-methylmyricetin (14 mg.) by the reductive acetylation method of King & White (1957). It possessed the same chromatographic properties as anthocyanidin C (Table 1) and these two compounds are provisionally considered to be identical.

An acetone solution of methylated kino (120 mg.) was refluxed for 4 hr. with powdered KMnO₄ (300 mg.), the acetone was evaporated, water added, the solution was acidified, SO₂ passed in and the clear liquid was then extract-

ed repeatedly with ether. The ether extract yielded a homogeneous cluster of needles (15 mg.) which after sublimation had m.p. 166–168° unchanged by admixture with 3,4,5-trimethoxybenzoic acid. The ether extract was examined by thin-layer chromatography with silica gel GF 254 (E. Merck, Darmstadt) and the solvents chloroform-ethyl acetate-formic acid (5:4:1, by vol.) and toluene-ethyl formate-formic acid (5:4:1, by vol.). Markers of several methoxybenzoic acids were added to the chromatograms, which after development were examined under u.v. (254 m μ) light. The only compounds visible were 3,4,5-trimethoxybenzoic acid and a trace of a compound which was not 3,4-dimethoxybenzoic acid.

Administration of enzyme inhibitors

Young trees of *E. sieberiana* and *E. regnans* were treated in the early and late spring of three consecutive years. Solutions of inhibitors (and other liquids) were administered to the cambial region by means of hypodermic syringes, or by making small incisions through the bark to the cambium and then adding the inhibitor solution with a syringe. Each solution was added to three incisions which were at the same level around the tree. With both methods of administration about 0.5 ml. was added to each area.

The aqueous solutions used were: (i) 0.1-M-sodium arsenite, (ii) 0.1-M-sodium arsenate, (iii) 0.1-M-NaF, (iv) 0.37 mM-methylene blue, (v) 1 mM-2,4-dinitrophenol, (vi) 16 μ M-sodium salt of *p*-chloromercuribenzoate, (vii) 1 mM-indolylacetic acid, (viii) 0.1-M-iodoacetic acid, (ix) 0.1-M-KCN, (x) 0.1-M-FeSO₄, (xi) 1 mM-disodium salt of EDTA, (xii) 0.1-M-sodium borate, (xiii) 0.1-M-potassium hydrogen phthalate and (xiv) water. Ethanol was also added. In addition, mixtures of sodium arsenite with KCN or 0.1-M-hydroxylamine sulphate were added. Also, air was forced under the bark and a portion of the bark cut out.

The injection areas were examined daily (with *E. sieberiana*) or 6–8 weeks later (with *E. regnans*). During the following year, the bark was removed and the injection area examined. The response of the trees to the injections made in late spring were much less marked than to those made in early spring and the latter gave in the majority of cases very similar results with both species in different years.

Some solutions resulted in the formation of callus tissue around the injection area. Other solutions formed callus tissue and also killed the bark for several inches above and below the point of injection. Neither of these changes was invariably associated with the exudation of kino from the affected region. With one exception, administration of arsenite resulted in the vigorous exudation of kino, and in addition kino veins extended beyond the points of injection with overall lengths of 10–20 in. With this exception, only a small amount of kino had exuded (from *E. regnans*) but the size of kino veins (and the amount of kino in them) was much greater than those formed as a result of other injections done at the same time. One injection of arsenite into *E. sieberiana* produced 0.3 g./day for 40 days. The period between injection and appearance of exuded kino appeared to depend on climatic conditions and ranged between 21 and 30 days; the nature of the inhibitor had no apparent effect on the period. Kino exudation also resulted from the injection of solutions of KCN, sodium arsenate, FeSO₄ and iodoacetic acid, but the response to them was less marked than to arsenite. Their effectiveness was in the order listed but the degree of response to FeSO₄ and iodoacetic acid was

Table 1. Properties of anthocyanidins obtained from methylated kino

	Anthocyanidins		
	A	B	C
$\lambda_{\max}$ in methanol-1% HCl (m μ)	—	527	521
R_F in			
Formic acid-conc. HCl-water (5:2:3, by vol.)	0.48	0.69	0.83
Acetic acid-conc. HCl-water (30:3:10, by vol.)	0.90	0.94	0.98
Butan-1-ol-acetic acid-water (6:1:2, by vol.)	—	0.93	0.93
2N-HCl-acetic acid (7:3, v/v)	0.50	0.63	0.79

erratic. The other solutions, as well as ethanol and air, usually resulted in the formation of kino veins, the size of which was variable but much smaller than those resulting from the above inhibitors.

The kino samples which exuded in response to the injection of inhibitors were examined chromatographically. Those produced when cyanide or hydroxylamine was present appeared to be slightly less polymerized than naturally formed kino but in other cases no differences were evident.

DISCUSSION

Damage of the cambium of eucalypts usually results in the formation of kino veins. To minimize the difficulties that this behaviour could cause in the interpretation of the effect of the administration of enzyme inhibitors, a wide range of solutions of inhibitors and other substances were administered during a 3-year period to *E. sieberiana* and *E. regnans* trees growing in different localities. An inhibitor was considered to have an influence only if it resulted in the exudation of kino from the injection area.

The formation and exudation of kino was most vigorous when arsenite was injected into active cambial tissues; the response to arsenate was less vigorous. Administration of arsenite to different tissues can result in the accumulation of pyruvate (Dawes & Holms, 1958) by inhibiting its utilization in the major pathway of the tricarboxylic acid cycle (ap Rees & Beevers, 1960b; Beevers, 1961). Arsenite can have other effects (ap Rees & Beevers, 1960a), but the increased availability of pyruvate (and acetyl-CoA) for the existing enzyme systems can explain the formation of the acetate-engendered portions of the substances in kino. In most cases, the administration of iodoacetate resulted in the exudation of kino. This inhibitor also lacks specificity, but like arsenite can inhibit the oxidation of pyruvate (Webb, Saunders & Thienes, 1949). Kino exudation also resulted from the administration of potassium cyanide and ferrous sulphate, but the lack of response to the other enzyme inhibitors which affect different areas of metabolism (Beevers, 1961; Ordin & Skoe, 1963) is consistent with the above view. Ellagitannins (lacking acetate-engendered moieties) are present in greater quantity in the polyphenols of undamaged active eucalypt cambium than are catechin and leuco-anthocyanins (Hillis & Carle, 1958). Presumably the utilization of pyruvate by the mitochondrion is so great that little is available for other pathways.

Entry of air to active cambium of many eucalypts frequently results in the formation of kino veins. Consequently, it is notable that Goldstein, Swain & Tjhi (1962) found increased aeration resulted in increased leuco-anthocyanin formation by tissue cultures derived from *Acer pseudo-platanus*.

In most cases less than 10 % of the extractives from wood and bark are monomeric flavonoid components. Freudenberg & Weinges (1958a, b, 1959, 1962) suggested that, during long storage under slightly acid conditions in plant tissues, C-C linkages form between leuco-anthocyanin or catechin monomers to produce polymers; but Hathway (1958) has suggested that C-C linkages and quinone groups, resulting from aerobic oxidation at pH 7 or from the action of polyphenol oxidase, are involved in the polymerization of gallo catechin and leuco-delphinidin. Chromatographic examination and dialysis of freshly formed *E. sieberiana* kino show that monomeric substances are almost completely absent. Consequently, in this case at least, the polymers of polyphenols are formed quickly and presumably during cellular metabolism. Prolonged storage in plant tissues is therefore not essential for the formation of polyphenol polymers, although such storage may result in the formation of secondary linkages in the polymers. Administration of peroxidase and catalase inhibitors (cyanide and hydroxylamine) together with arsenite did not significantly alter the chromatographic properties of the resultant kino, so that apparently these enzymes are not responsible for polymerization.

To avoid any changes in the polymers, purification of the kino was not attempted. Consequently the trace amounts of gallic acid and ellagic acid in the acid-degradation products probably arose from ellagitannin copolymers. These polymers are present in uninjured cambium (Hillis & Carle, 1960) and they could account for the peaks in the regions of 5.90 and 5.82 μ of the i.r.-absorption curves of kino and methylated kino respectively. Comparison of the u.v.- and i.r.-absorption curves of kino and methylated kino with those of ellagitannins suggests that these copolymers are present in very small amounts. On the other hand, the traces of myricetin may have arisen from the leuco-delphinidin units in the main polymer (cf. Swain, 1954). The stilbene-like substances and phloroglucinol which formed when the kino was heated with butanol-hydrochloric acid probably arose from the flavan-3,4-diol units in the polymers, as under the same conditions catechin behaves similarly; the former substances are apparently formed from the isoflavene resulting from aryl migration (Freudenberg, Carrara & Cohn, 1925; Baker, 1929). However, the very high ratio of delphinidin to other products formed on acid degradation indicates that the kino is very largely composed of polymerized leuco-delphinidin.

The average yield of delphinidin chloride (7.90 %) obtained when *E. sieberiana* kino is heated with butanol-hydrochloric acid is higher than those of fisetinidin chloride from the 'trimeric' (6.75 %), 'pentameric' (4.78 %) and 'decameric' (4.95 %)

leuco-fisetinidins isolated from *Acacia mearnsii* heartwood (Roux & Paulus, 1962). Pure monomeric leuco-delphinidin could not be prepared, but the yields of anthocyanidin from kino are about one-third of the reported yields from monomeric 3',4',7-trihydroxy- and 3',4',5',7-tetrahydroxy-flavan-3,4-diol (Roux & Evelyn, 1958; Roux & Paulus, 1962). One possible cause of the lower yields from kino could be the existence of C-C linkages between some of the units of the polymers. The colourless appearance of fresh kino, the absence of benzoquinone bands in the 400 m μ region (Teuber & Staiger, 1955), the absence of a strong C=O band at 5.9 μ and the almost sole formation of 3,4,5-trimethoxybenzoic acid on permanganate oxidation of methylated kino indicate that orthoquinone residues and C-C linkages involving the B (2-phenyl) nuclei are absent. The intense reaction of the kino with vanillin-hydrochloric acid also indicates that few C-8 positions are involved in linkages.

There is little evidence for the existence of the above C-C linkages in kino. However, under mild conditions, aqueous solutions of *E. sieberiana* kino slowly turn brown and the subsequent yield of delphinidin is appreciably lower. Accordingly, these or other additional linkages may form in the polyphenol polymers stored in bark and wood tissues.

The formation of 3-hydroxy-3',4',5,5',7-pentamethoxy- and 3,3',4',5,5',7-hexamethoxy-flavylium chloride when methylated kino is heated with butanol-hydrochloric acid shows that ether linkages involving phenolic hydroxyl groups are absent. However, this formation is consistent with the view (Hillis & Urbach, 1959; Roux, 1959) that the linkages in leuco-anthocyanin polymers involve the C-3 or C-4 (or both) hydroxyl groups. The analyses of kino and its derivatives show a higher degree of correspondence to those calculated for formula (I) than for other postulated formulae. There was, however, a significant difference between the calculated and observed acetyl content of the acetyl derivative of methylated kino when about half the calculated value was found. A similar observation has been made with the same derivative of leuco-fisetinidin tannin (Roux & Paulus, 1961). Roux & Paulus (1961, 1962) favour the C-4 hydroxyl group

as the one most likely to be involved in the ether linkages between the units of the 'trimeric', 'pentameric' and 'decameric' leuco-fisetinidins isolated from *Acacia mearnsii* heartwood.

The 4-hydroxyl group is largely responsible for the redness induced in leuco-anthocyanins by sunlight (Roux, 1963; Roux & de Bruyn, 1963); consequently it is probable that some of these hydroxyl groups exist free in kino as it becomes red in these conditions. In addition, when heated with butanol-hydrochloric acid, catechin yields 1.3 % but kino 2.3 % of phloroglucinol. The higher yield from kino indicates that, before the formation of anthocyanidin, the *sec.-p*-hydroxybenzyl alcohol moiety is present in the polymers, as Adler, Pepper & Ericksoo (1957) showed that under similar conditions this moiety readily liberates the phenolic residue. Methylated kino does not become red on exposure to u.v. light but this could be due to the methylation of a phenolic hydroxyl group which acts in conjunction with the 4-hydroxyl group.

The available evidence is consistent with the view that a large proportion of the linkages between the units of the leuco-delphinidin polymers of *E. sieberiana* kino are ether linkages involving the C-3 or C-4 hydroxyl groups, or both. However, an adequate explanation for the low yield of anthocyanidin from these polymers on acid treatment, relative to that from the monomers, has not been found.

SUMMARY

1. *Eucalyptus sieberiana* kino collected within a few days of formation is composed almost entirely of polymeric polyphenols.

2. The polymers appear to be largely made up of leuco-delphinidin units with ether linkages involving either or both of the C-3 or C-4 hydroxyl groups.

3. The formation of leuco-anthocyanins by injured cambial tissues appears to be due to a blockage in the utilization of pyruvate in the tri-carboxylic acid cycle.

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Where R = —OH when R' = —O—
 or R = —O— when R' = —OH

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Studies on Metabolism of Vitamin A

7. LOWERED BIOSYNTHESIS OF ASCORBIC ACID IN VITAMIN A-DEFICIENT RATS*

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Existing information on the effect of vitamin A deficiency on the biosynthesis of ascorbate in animals is contradictory. Thus some workers have produced evidence that vitamin A deficiency decreases the tissue and urinary concentrations (Sure, Theis & Harrelson, 1939; Sangiorgi, 1942; Sutton, Kaeser & Hansard, 1942; Jonsson, Obel & Sjöberg, 1942, 1945) and synthesis *in vitro* (Chatterjee, Kar, Ghosh & Guha, 1961) of ascorbate in rats, and others have claimed that the administration of ascorbate leads to a general improvement in the health and longevity of the vitamin A-deficient animals (Mayer & Krehl, 1948). In contrast, several other investigators claimed that no such relationship exists (Sharman, 1949; Guerrant, 1948; Mapson & Walker, 1948). The most signifi-

cant of these reports seems to be that of Mapson & Walker (1948), who concluded that the decreased tissue and urinary concentrations of ascorbate in vitamin A-deficient rats are caused by inanition and not by the deficiency.

We have previously shown that the tissue concentrations of free phenylalanine and tyrosine are markedly increased in vitamin A-deficient rats (Malathi, Seshadri Sastry & Ganguly, 1961). Ascorbate has been implicated in the metabolism of these two amino acids (La Du & Zannoni, 1961; Knox & Goswami, 1961). It therefore appeared that the increase in these two amino acids might be due to blocks in their metabolic pathway caused by inadequate availability of ascorbate during vitamin A deficiency. In a preliminary communication we have shown that the synthesis of ascorbate is considerably lowered in vitamin A-deficient rats

* Part 6: Subba Rao & Ganguly (1964).