Condensed Tannins

3. ISOLATION AND ESTIMATION OF (-)-7:3':4':5'-TETRAHYDROXYFLAVAN-3-OL, (+)-CATECHIN AND (+)-GALLOCATECHIN FROM BLACK-WATTLE-BARK EXTRACT*

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The bark and heartwood extractives of the black-wattle tree (*Acacia mollissima*) both contain mixtures of complex polyphenolic compounds consisting of a molecular-weight gradation within the range 300–3000 (Roux & Evelyn, 1958*a*, *c*). Keppler (1957) showed that wattle-heartwood extractives contain a (+)-7:3':4'-trihydroxyflavan-3:4-diol, which appears to be the prototype and possible precursor of some of the condensed tannins of the wood (Roux, 1958*b*), and which was found to be enantiomorphous (Clark-Lewis & Roux, 1958, 1959) with a similar monomeric (–)-leuco-fisetinidin prototype of quebracho (*Schinopsis balansae* and *S. quebracho-colorado*) heartwood tannins (Roux, 1958*a*; Freudenberg & Weinges, 1958; Roux & Evelyn, 1958*b*). Roux & Maihs (1958) have briefly reported the isolation from the fresh bark extract of (–)-7:3':4':5'-tetrahydroxyflavan-3-ol, in addition to (+)-catechin and (+)-gallocatechin. The present study provides details of the isolation, characterization and estimation of these catechins.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Mixed melting points were made on the crystalline residue remaining after dissolving equal weights of both compounds in methanol and removing the methanol under vacuum. Analyses of C, H, methoxyl and acetyl are by Weiler and Strauss, Oxford. Infrared comparisons were made by Dr J. R. Nunn, National Chemical Research Laboratory, C.S.I.R., Pretoria. Tannin analyses are by the ultraviolet photometric method for black-wattle tannins (Roux, 1951, 1957*a*, *b*), standardized against the hide-powder (shake) method.

Isolation and identification of (–)-7:3':4':5'-tetrahydroxyflavan-3-ol [(–)-robinetinidol], (+)-catechin and (+)-gallocatechin

Preliminary fractionation of bark extracts. The damp, mature (5–10 mm. thick) and freshly stripped bark from *A. mollissima* was sliced as soon as possible into slivers of 1 mm. thickness by cutting across the grain with a stainless-steel knife, and the slivers were immediately covered with commercial ethyl acetate. Extraction at room tem-

perature proceeded over 5 days with daily renewal of the ethyl acetate. The combined extracts were evaporated to dryness in a rotary evaporator under vacuum, yielding a pale cream-coloured product. This ethyl acetate-soluble extract contained 92.5% of tannins, and the tannin content of the whole extract represented 62% of the total content of the bark. A methanol-soluble extract obtained by the same method contained 70% of tannins, representing 96.5% of the total bark tannins. The ethyl acetate-soluble fraction is of lower (921) average molecular weight (Evelyn, 1958) compared with that of the whole fresh-bark extract (1275) but still contains much high-molecular-weight tannins. The latter are removed by one of two alternative procedures: *Procedure 1.* The ethyl acetate-soluble tannins (10 g.) are dissolved in ethyl acetate (100 ml.), and CHCl_3 (100 ml.) is added in small quantities with vigorous shaking. The white precipitate formed was filtered from the straw-coloured liquid under anhydrous conditions, and the latter evaporated to dryness under vacuum (0.9 g.). *Procedure 2.* A similar product was obtained by chromatography on a Solka Floc cellulose (Brown Co., Berlin, New Hampshire, U.S.A.) column (4 cm. $\times$ 55 cm.), prepared by introducing the cellulose in a slurry with water and washing with 2% acetic acid (8 hr.) and water (8 hr.). The ethyl acetate-soluble tannins (25 g.) in water (125 ml.) were introduced and the column was developed with water. After the emergence of the first polyphenols in the eluent (FeCl_3 test), 625 ml. was collected in one fraction over 8 hr. From this fraction 16 g. of extract was recovered by evaporation under vacuum.

Separation of catechins by paper chromatography. The products from separation procedures 1 and 2 were streaked (400 mg. per sheet) on to a Whatman no. 3 (18½ in. $\times$ 22½ in.) paper sheet and the chromatogram was developed in 2% acetic acid by upward migration. The air-dried sheets were examined under ultraviolet light. Fluorescent bands were observed at R_f values 0.43 (dark-blue A band), 0.48 (dark-blue B band), 0.56 (dark-blue C band), 0.64 (light-blue E band) and 0.73 (dark-mauve H band). Bands B and C were separated by a very narrow bright-blue fluorescent band which serves as an excellent marker and enabled these bands to be demarcated accurately.

Strips were cut corresponding to bands B and C, and these were eluted with 70% ethanol (Nordström & Swain, 1953*a*). Band C eluate was evaporated under vacuum until the ethanol was removed. Microcrystals separated on standing. Yield: 2.4 g. from 720 g. of ethyl acetate extract enriched by procedure 1, and 0.25 g. from 102 g. of ethyl acetate extract enriched by procedure 2. Band B eluates were evaporated to dryness under vacuum (yield 13–15%

* Part 2. Roux & Evelyn (1958*b*).

of enriched fraction), and streaked on to washed (Nordström & Swain, 1953a) Whatman no. 3 chromatographic sheets (200 mg. per sheet). The chromatograms were developed in butan-1-ol-acetic acid-water (6:1:2) and examined under ultraviolet light. Two dark-mauve bands B_1 (R_f 0.70) and B_2 (R_f 0.53) were visible and separated by a light-blue band B_3 (R_f 0.64). Bands B_1 and B_2 were cut from the sheets and eluted as before. After removal of ethanol and concentration under vacuum, crystals separated from each of the aqueous concentrates.

(-)-7:3':4':5'-Tetrahydroxyflavan-3-ol. The crystals from band C consisted only of component H (see Roux, 1953; Roux & Evelyn, 1958a) having R_f values 0.76 (butan-1-ol-acetic acid-water, 4:1:5), 0.67 (water-saturated butan-2-ol) and 0.47 in 2% acetic acid. Component H crystallized from water as a white hydrate, m.p. 207°, $\lambda_{\max}$ 282 m μ (Found: C, 56.2; H, 5.8; loss at 100°, 8.8. Calc. for $C_{15}H_{14}O_6 \cdot 1.5H_2O$: C, 56.8; H, 5.4; H_2O , 8.5%). After drying over P_2O_5 at room temperature or at 80° under vacuum, one molecule of water was retained (Found: C, 58.6; H, 5.4. Calc. for $C_{15}H_{14}O_6 \cdot H_2O$: C, 58.4; H, 5.2%), but this was lost by drying at 100° for 2 hr. (Found: C, 61.7; H, 5.1. Calc. for $C_{15}H_{14}O_6$: C, 62.1; H, 4.9%). A blue with $FeCl_3$ indicated the presence of a pyrogallol unit; a very weak reaction with vanillin-toluene-*p*-sulphonic acid reagent (Roberts, Cartwright & Wood, 1956) indicated the absence of phloroglucinol nucleus, and a yellow with bis-diazotized benzidine (Lindstedt, 1950) indicated the probable presence of a resorcinol nucleus.

Fusion with KOH under anhydrous conditions resulted in degradation to β -resorecylic acid (also some resorcinol) and gallic acid. In the partitioning mixtures water-saturated butan-2-ol and butan-1-ol-acetic acid-water it migrated at the same rate as (+)-catechin and (+)- and (-)-7:3':4':5'-trihydroxyflavan-3:4-diol, suggesting (1) the presence of a total of five hydroxy groups on the C_{15} skeleton, and (2) that the compound was a catechin containing pyrogallol and resorcinol nuclei, e.g. 7:3':4':5'-tetrahydroxyflavan-3-ol. Its melting point and specific rotation, $[\alpha]_D^{20} - 10.7 \pm 0.3^\circ$ in acetone-water (1:1) (c, 1.42), suggested its identity with (-)-robinetinidol, m.p. 207–209°, $[\alpha]_D^{25} - 11.5^\circ$, obtained shortly before by Weinges (personal communication and 1958) from the catalytic reduction of (+)-dihydorobinetin by the method of Freudenberg & Roux (1954) (see also Roux & Freudenberg, 1958) followed by the catalytic hydrogenolysis of the resulting (+)-7:3':4':5'-tetrahydroxyflavan-3:4-diol at position 4.

Component H from wattle does not assume a pink on drastic drying as does synthetic (-)-robinetinidol (Weinges, 1958), but a mixed m.p. of the natural and synthetic compounds showed almost no depression (m.p. 204°), and they also show superimposable infrared-absorption curves over the range 2.5–15 μ . On two-dimensional chromatograms with water-saturated butan-2-ol for direction 1 and 2% acetic acid for direction 2, the natural and synthetic compounds run to identical positions.

7:3':4':5'-Tetramethoxyflavan-3-ol. (-)-7:3':4':5'-Tetrahydroxyflavan-3-ol (500 mg.) from wattle-bark extract was methylated with diazomethane by the method described for (-)-leuco-fisetinidin (Roux & Evelyn, 1958b), the reaction mixture being kept at -5° for 2 days in the presence of excess of diazomethane. After concentration of solvents the product was poured into water and the white

flocculent precipitate recrystallized from ethanol (0.41 g.). The melting point, 141–142° increasing to 143–144° after two further recrystallizations, was similar to that of synthetic 7:3':4':5'-tetramethoxy-(-)-robinetinidol (Weinges, 1958) and a mixed m.p. of the natural and synthetic derivatives showed no depression (Found: C, 65.9; H, 6.7; OMe, 35.4. Calc. for $C_{19}H_{22}O_8$: C, 65.9; H, 6.4; OMe, 35.8%). The specific rotation, $[\alpha]_D^{20} - 21.6 \pm 0.4^\circ$ in tetrachloroethane (c, 2.3), was also similar to that of the synthetic derivative (-22.3°) and their infrared-absorption curves were identical over the range 2.5–15 μ .

3:7:3':4':5'-Penta-acetylflavan. Component H (490 mg.) was acetylated with acetic anhydride (5 ml.) and sodium acetate (1 g.) as described (Roux & Evelyn, 1958b) for 3:4-diacetoxy-7:3':4'-trimethoxyflavan. The solid obtained (0.6 g.) was repeatedly recrystallized from ethanol to m.p. 132–133°, $[\alpha]_D^{20} + 4.6 \pm 0.3^\circ$ in tetrachloroethane (c, 2.15) (Found: C, 60.0; H, 4.9; CO.CH₃, 42.0. $C_{23}H_{24}O_{11}$ requires C, 60.0; H, 4.8; CO.CH₃, 43.0%). This derivative was not reported by Weinges (1958) but (personal communication) was prepared by him, m.p. 131–133°, $[\alpha]_D^{25} + 4.9^\circ$ in tetrachloroethane (c, 2). Mixed melting points of the synthetic and natural derivatives showed no depression, and their infrared-absorption curves were superimposable over the range 2.5–15 μ .

(+)-Catechin. The crystals from band B_1 correspond to extract component G (0.83 g. from 720 g. of ethyl acetate-soluble tannins with separation procedure 1), and were recrystallized from water, m.p. 172–174°, $[\alpha]_D^{21} + 17.0^\circ$ in acetone-water (1:1) (c, 1.1), $\lambda_{\max}$ 280 m μ (Found: C, 56.8; H, 5.7. Calc. for $C_{15}H_{14}O_6 \cdot 1.5H_2O$: C, 56.8; H, 5.4%). A mixed melting point with (+)-catechin from gambier extract from the leaves of *Uncaria gambir* showed no depression, and the infrared-absorption curves of (+)-catechin and component G were superimposable over the range 2.5–15 μ . With $FeCl_3$ (green), bisdiazotized benzidine (ochre) and vanillin-toluene-*p*-sulphonic acid (violet) reagents, constituent G gave the same colour reactions as (+)-catechin and migrated to identical positions in two-way solvent systems which separate optical antipodes (Roberts & Wood, 1953). R_f values in water (0.35), water-saturated butan-2-ol (0.67) and butan-1-ol-acetic acid-water (4:1:5) (0.75) are in agreement with those of (+)-catechin (Bradfield & Bate-Smith, 1950; Roux & Evelyn, 1958c). The penta-acetyl derivative of component G, m.p. 131–132°, $[\alpha]_D^{20} + 40.1^\circ$ in tetrachloroethane (c, 1.3), was formed as above for 3:7:3':4':5'-penta-acetylflavan. Mixed m.p. with an authentic specimen of penta-acetyl-(+)-catechin showed no depression.

(+)-Gallocatechin. The microcrystals from band B_3 , composed mainly of component A_2 , were recrystallized from water (30 mg. from 720 g. of ethyl acetate-soluble tannins with procedure 1), m.p. and mixed m.p. with (+)-gallocatechin from *Casuarina equisetifolia* (Roux, 1957c) 182–184°, $[\alpha]_D^{20} + 10.7^\circ$ in acetone-water (1:1) (c, 0.5), $\lambda_{\max}$ 271 m μ . The crystals tended to darken rapidly on drying (Found: C, 55.8; H, 5.4. Calc. for $C_{15}H_{14}O_7 \cdot H_2O$: C, 55.6; H, 5.0%). Infrared-absorption curves of component A_2 and (+)-gallocatechin were identical over the range 2.5–15 μ .

Component A_2 developed the same coloration with $FeCl_3$ (blue), bisdiazotized benzidine (ochre) and vanillin-toluene-*p*-sulphonic acid (violet) reagents as with (+)-gallocatechin, and migrated to identical positions on two-way

chromatograms under conditions which allow for the separation of optical antipodes (Roberts & Wood, 1953). R_F values of A_2 in water (0.32), butan-1-ol-acetic acid-water (4 : 1 : 5) (0.57) and water-saturated butan-2-ol (0.54) are in agreement with those of (+)-gallo catechin (Bradfield & Bate-Smith, 1950; Roux & Evelyn, 1958c).

Examination of wattle extract for the presence of (-)-epicatechin. The catechin-enriched fractions of wattle-bark extracts, bands B and C, and also band A of lower R_F in 2% acetic acid, were examined by two-way chromatography and spray reagents as described above for the presence of (+)- or (-)-epicatechin, with (-)-epicatechin from cocoa beans (Forsyth, 1954) as reference compound. With reference to the work of Roberts & Wood (1953), the apparent absence of epicatechins in fresh bark extracts was established, and no significant quantities of (-)-epicatechin could be detected in the commercial extract.

Estimation of catechins in black-wattle extracts

The percentages of (-)-7:3':4':5'-tetrahydroxyflavan-3-ol, (+)-catechin and (+)-gallo catechin in methanol and ethyl acetate extractives of fresh bark were determined by the measurement of the colour density of their spots on two-way chromatograms with a simple densitometer (Block, 1950; Block, Durrum & Zweig, 1955) procedure previously used for amino acids. All chromatograms were run by the ascending method, at 21° in water-saturated butan-2-ol in the short direction (11½ in.; 15 hr.) and, after drying, in the long direction (18½ in.; 5-6 hr.).

The catechins dissolved in methanol (1% solution) were spotted on the sheets in 10 µg. increments over the range 10-100 µg., and the extracts in 0.5 mg. increments (10% solution) over the range 1-8 mg. The dried chromatograms were sprayed with one of the following: (a) ammoniacal $AgNO_3$ reagent containing 14 g. of $AgNO_3$ /100 ml. Chromatograms were sprayed with this freshly made reagent until damp, left 2-3 min. for full development of brown colour and finally washed in a dish, first with water (three thorough washings) followed by treatment with 0.5% sodium thiosulphate solution for 2 min. and washing in running water for 30 min. (b) Benzidine reagent (Lindstedt, 1950), consisting of a solution of 5 g. of benzidine, 14 ml. of conc. HCl and 980 ml. of water freshly mixed with two-thirds of its volume of aqueous 10% sodium nitrite solution. After spraying until the maximum colour density had developed,

the chromatograms were washed in running water for 30 min. to remove excess of benzidine reagent.

Densitometer and method of measurement. The maximum colour density of the spots, after spraying, was measured by using a densitometer and method similar to that described by Block *et al.* (1955). The colour density of each spot was determined by using the blank paper of the particular chromatogram as 100% transmission. A straight-line relationship between log concentration and galvanometer readings was obtained for each catechin. From these standard curves an approximate estimation of catechins in the methanol and ethyl acetate-soluble extracts of the fresh bark was made (Table 1). (+)-Catechin and (-)-robinetinidol run well in advance of other components on two-way paper chromatograms (components G and H respectively, see Roux & Evelyn, 1958b), but gallo catechin (component A_2) is closely associated with others in the A complex, and also with B and F, and its estimation is complicated by the close proximity of these reducing bodies when the $AgNO_3$ spray reagent is used. With the benzidine reagent, however, the ochre coloration of (+)-gallo catechin permits the more accurate estimation, both visual and photometric, of this compound in the presence of other bodies which furnish yellow colorations. The value obtained for (+)-catechin with the benzidine reagent (1.2%) was very similar to that obtained with the $AgNO_3$ spray (1.4%), whereas that for (+)-gallo catechin with the benzidine reagent was half (1.8%) the value obtained with the $AgNO_3$ spray (3.6%).

DISCUSSION

Black-wattle-bark extracts contain polyphenolic and carbohydrate fractions in the approximate proportion of 3 : 1. The carbohydrate fraction consists partly of a mixture of sucrose, glucose and fructose (paper chromatography), as well as about 7% of the total extract weight of hydrocolloid gums (Roux, 1952). From the polyphenolic fraction, comprising 60% of the commercial bark extract, fisetin, gallic acid and resorcinol (Roux, 1952), and syringic acid (King, 1956), have been isolated in exceptionally low yield. Roux (1953) provisionally identified (+)-catechin and (+)-gallo catechin by paper chromatography from amongst the main polyphenolic components of the fresh-bark extract. King (1957) isolated milligram quantities of (+)-catechin (m.p. 173-174°) and an impure substance (m.p. 166°) which was surmised to be a 3:7:3':4':5'-penta-hydroxyflavan on the basis of degradative and quantitative colorimetric evidence only. *epi*Catechin (rotation not designated) was also shown to be present by paper chromatography, but King could not establish the presence of gallo catechin by this method. The above-described and previous work on wattle extract has emphasized the difficulty experienced in isolating crystalline components from the polyphenolic fraction. The presence of a high percentage of tannins varying in average molecular weight over the range 600-3000 (Roux & Evelyn, 1958a, c) appears to complicate separation procedures.

Table 1. *Estimation of catechins in black-wattle extract*

Values are expressed as percentage of dry extract and, in parentheses, as percentage of the tannin content.

Bark extract	(-)-Robine-tinidol	(+)-Cate-chin	(+)-Gallo-catechin
<i>AgNO₃ spray</i>			
Methanol	0.7 (1.0)	1.1 (1.6)	2.8* (4.0)*
Ethyl acetate	0.8 (0.9)	1.4 (1.5)	3.6* (3.9)*
<i>Benzidine spray</i>			
Ethyl acetate	—	1.2 (1.3)	1.8 (1.9)
<i>By isolation</i>			
Ethyl acetate	0.33	0.12	0.0004

* Values erroneously high due to interference or overlap of other polyphenols. See text.

In the present work (–)-7:3':4':5'-tetrahydroxy-flavan-3-ol, (+)-catechin and (+)-gallocatechin have been isolated in sufficient quantity for their accurate identification. (–)-*epi*Catechin appears to be absent from both fresh-bark and commercial extracts (compare King, 1957). The high melting point (207°) of (–)-7:3':4':5'-tetrahydroxyflavan-3-ol [(–)-robinetinidol] emphasizes the relative impurity (m.p. 166°) of that substance, which King (1957) correctly surmised to be a 3:7:3':4':5'-penta-hydroxyflavan. The shift in the specific rotation of penta-acetyl-(–)-robinetinidol relative to that of the parent compound (positive), to the tetramethyl ether (positive), pentamethyl ether (positive), tetramethyl-3-acetyl (positive) and tetramethyl-3-tosyl (negative) derivatives bears the same relationship as penta-acetyl-(+)-catechin to the corresponding (+)-catechin derivatives (see

Weinges, 1958). The shifts relative to the tetramethyl-3-mesyl derivatives constitute an anomaly. This evidence (also communicated by Dr Weinges) nevertheless confirms his deduction of the agreement in absolute configuration between (+)-catechin and (–)-robinetinidol. From the inter-relationships between the catechins, leuco-anthocyanidins and 2:3-dihydroflavonols (Weinges, 1958; Clark-Lewis & Roux, 1958) it follows that the flavans of the resorcinol series present in *A. mollissima*, namely (–)-robinetinidol from the bark and (+)-mollisacacidin from the wood, have the same (2:3-*trans*) configurations in C atoms 2 and 3.

The concentration of (+)-catechin in the methanol or ethyl acetate extracts of the fresh bark (1.1–1.4%) is higher than that found by King & White (1956) for the commercial extract (0.02%).

Table 2. Distribution of C₁₅ 'flavonoid' compounds of the resorcinol series in Nature

Family: LEGUMINOSAE		
Compound	Species	Reference
<i>Catechins or flavan-3-ols</i>		
(–)-7:3':4':5'-Tetrahydroxy- (robinetinidol)	<i>Acacia mollissima</i>	Roux & Maihs, 1958
<i>Flavan-3:4-diols</i>		
(+)-7:3':4':5'-Tetrahydroxy-	<i>Robinia pseudacacia</i>	Weinges, 1958
(+)-7:3':4'-Trihydroxy-	<i>A. mollissima</i>	Keppler, 1957
(mollisacacidin, gleditsin)	<i>Gleditsia japonica</i>	Mitsuno & Yoshizaki, 1957
7:4'-Dihydroxy-	<i>A. pycnantha</i> , <i>Guibourtia</i> spp.	Roux, 1959
<i>2:3-Dihydroflavonols</i>		
7:3':4':5'-Tetrahydroxy-	<i>R. pseudacacia</i>	Freudenberg & Hartmann, 1954
7:3':4'-Trihydroxy- (fustin)	<i>Gleditsia triacanthos</i>	Chadenson, Molho-Lacroix, Molho & Mentzer, 1955
	<i>G. japonica</i>	
<i>Flavonols</i>		
7:3':4':5'-Tetrahydroxy- (robinetin)	<i>R. pseudacacia</i>	Schmidt & Pietsch, 1931
7:3':4'-Trihydroxy- (fisetin)	<i>Gleditsia monosperma</i>	Mitsuno & Yoshizaki, 1957
	<i>G. japonica</i>	
<i>2:3-Dihydroflavones</i>		
7:3':4'-Trihydroxy- (butin)	<i>Butea frondosa</i>	Perkin & Hummel, 1904
7:4'-Dihydroxy- (liquiritigenin)	<i>Glycyrrhiza glabra</i>	Shinoda & Ueda, 1934
<i>isoFlavones</i>		
7-Hydroxy-4'-methoxy-	<i>Trifolium subterraneum</i>	Bradbury & White, 1951
	<i>T. pratense</i>	
Family: ANACARDIACEAE		
<i>Flavan-3:4-diols</i>		
(–)-7:3':4'-Trihydroxy- [(–)-leuco-fisetinidin]	<i>Schinopsis quebracho-colorado</i>	Roux, 1958a; Freudenberg & Weinges 1958
<i>2:3-Dihydroflavonols</i>		
7:3':4'-Trihydroxy- (fustin)	<i>Rhus succedanea</i>	Oyamada, 1934
	<i>R. cotinus</i> , <i>R. glabra</i> , <i>R. vernicifera</i> , <i>R. trichocarpa</i>	
<i>Flavonols</i>		
7:3':4':5'-Tetrahydroxy-	<i>Schinopsis</i> spp.	Kirby & White, 1955
7:3':4'-Trihydroxy- (fisetin)	<i>S. quebracho-colorado</i>	Perkin & Gunnell, 1896
	<i>Rhus</i> spp. (see above)	
7:3'-Dihydroxy-4'-methoxy-	<i>Schinopsis</i> spp.	Kirby & White, 1955
7:4'-Dihydroxy-	<i>Schinopsis</i> spp.	Kirby & White, 1955

The complex condensed tannins of black-wattle bark show light-absorption and degradative behaviour typical of the flavans, and the catechin, 3:7:3':4':5'-pentahydroxyflavan (robinetinidol) has for many years been regarded as a possible prototype of wattle tannins (Roux & Maihs, 1958). No direct chemical or biochemical evidence as for the leuco-fisetinidins of quebracho tannins (Roux, 1958*b*; Roux & Evelyn, 1958*b*) has, however, been obtained to support this surmise. The work of Hathway (1959) on oak-bark tannins suggests that pyrogallol catechins in addition to leuco-anthocyanidins, participate in condensed tannin formation.

The simultaneous presence of (+)-catechin and (+)-gallocatechin in wattle bark is of interest in view of their parallel occurrence in the bark tannins of *Casuarina equisetifolia* (Roux, 1957*c*), *Quercus sessifolia*, *Castanea vesca* (Mayer & Bauni, 1956, 1957) and *Quercus pendunculata* (Hathway, 1959).

Similarly, the presence of (-)-robinetinidol [and also of (+)-mollisacacidin] in *Acacia mollissima* lends further emphasis to the almost exclusive occurrence amongst the Leguminosae and two genera of the Anacardiaceae (namely *Rhus* and *Schinopsis*), of all those C₁₅ 'flavonoid' compounds of the resorcinol series which have hitherto been isolated and identified in Nature (Table 2). The only known exceptions amongst a broader group of C₁₅ compounds are the resorcinol-containing chalcones, butein and dahlia chalcone, and benzilidenecoumaranones (aurones), for example sulphuretin, from the fluorescence of certain Compositae (see Nordström & Swain, 1953*b*; Bate-Smith, Swain & Pope, 1953). This rather remarkable distribution, if also confirmed by future investigation, may be of taxonomic significance, and provides confirmation of plant classifications proposed on the basis of morphological and anatomical characteristics similar to that found by Erdtman (1955) for the Coniferae.

SUMMARY

1. (-)-7:3':4':5'-Tetrahydroxyflavan-3-ol [(-)-robinetinidol] has been isolated from black-wattle-bark (*Acacia mollissima*) extracts in 0.3% yield.

2. (+)-Catechin and (+)-gallocatechin were isolated in lower yields from the extracts, and (-)-epicatechin has been shown to be absent from fresh-bark and commercial extracts.

3. The concentration of the catechins in the fresh-bark extracts and in their tannin fractions is estimated by means of paper chromatography in conjunction with the measurement of maximum colour density of the spots with a densitometer.

4. The presence of (-)-robinetinidol in *Acacia mollissima* places further emphasis on the almost exclusive occurrence amongst the Leguminosae and two genera of the Anacardiaceae of those C₁₅ 'flavonoid' compounds of the resorcinol series which have hitherto been isolated.

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The Enzymic Synthesis of Aryl Sulphamates

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Boyland, Manson & Orr (1957) have shown that the administration of certain arylamines to rats or to rabbits is followed by the urinary excretion of the corresponding aryl sulphamates, compounds whose natural occurrence had not previously been recognized. This was followed by the demonstration of the synthesis *in vitro* of 2-naphthyl sulphamate by rat-liver preparations (Roy, 1958) and of phenyl sulphamate by sheep intestinal-mucosal enzymes (Kent & Pasternak, 1958). These results were of interest, as the reactions involved might be expected to be related to those involved in the synthesis of the sulphamate groups of heparin (Foster & Huggard, 1955). In the light of the present ideas on the synthesis of sulphuric acid esters (Lipmann, 1958) it seemed likely that the synthesis of aryl sulphamates would involve sulphate transfer from 'active sulphate', adenosine 3'-phosphate 5'-sulphatophosphate, this reaction being catalysed by a specific sulphokinase, arylamine sulphokinase. The present paper describes an investigation into the enzymic synthesis of aryl sulphamates and, in particular, into the influence of certain steroids on this reaction. A preliminary account of the work has already appeared (Roy, 1959).

EXPERIMENTAL

Preparation of the enzyme system. This was carried out essentially as previously described (Roy, 1956). With 5 g. samples of liver the tissue was treated with 3 vol. of 0.15 M-KCl containing mM-ethylenediaminetetra-acetate (EDTA), pH 7, in a glass homogenizer, for two 60 sec. periods.

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Larger amounts of tissue were treated with 3 vol. of the KCl solution in an Atomix blender (M.S.E. Ltd.) for two 30 sec. periods. The resulting tissue suspension was centrifuged for 2 hr. at 20 000 g and the supernatant precipitated between 1.5 and 2.3 M-(NH₄)₂SO₄, to give the enzymically active fraction. This was dissolved in water so that 1 ml. of the resulting solution corresponded to 500 mg. of liver; the protein concentration was approximately 18 mg./ml.

The sulphate-activating system, a mixture of adenosine triphosphate (ATP) sulphurylase and adenosine 5'-sulphatophosphate phosphokinase (Lipmann, 1958), was prepared by heating the above enzyme solution at 52° (DeMeio, Wizerkaniuk & Schreiberman, 1955) for 30 min. and removing the denatured protein by centrifuging.

The sulphate-activating system from baker's yeast was prepared exactly as described by Nose & Lipmann (1958).

The sulphokinase fraction of the enzyme was prepared from the complete enzyme system by dialysing the latter for 48 hr. against repeated changes of 100 vol. of water at 0°, a procedure which destroyed the sulphate-activating system because of the latter's instability in the absence of SO₄²⁻ ions. The resulting suspension was made 0.03 M in glycylglycine and adjusted to pH 8 with N-Na₂CO₃. After the suspension had stood for a few hours any remaining insoluble material was removed by centrifuging. The enzyme suspension obtained after dialysis could be used without further treatment, but a considerable proportion of the sulphokinase was present in the insoluble fraction and was rather unstable in this state, the activity being destroyed on freezing. The soluble sulphokinase preparations obtained after glycylglycine treatment were stable and could be stored frozen for long periods.

Preparation of adenosine 3'-phosphate 5'-sulphatophosphate. This was prepared enzymically by incubating 100 ml. of a mixture containing 50 ml. of the above unfractionated enzyme preparation, 10 ml. of 0.2 M-phosphate buffer (pH 7.2), 10 ml. of 0.04 M-MgSO₄-0.06 M-K₂SO₄ and 400 μmoles of ATP (crystalline sodium salt, Sigma Chemical Co., adjusted to pH 7.2 with N-NaOH) for 1 hr. at 37°.