evidence against the suggestion that selenium is involved in electron transport.

The associated defects of failure to reaccumulate potassium and of decline in oxygen uptake, after cooling, are part of the inability of vitamin E-deficient tissues to stand up to certain conditions *in vitro*. However, it is possible that the defects shown up in vitamin E-deficient tissues by the stress of cooling *in vitro* can also be brought about *in vivo* by some other stress. Analyses of the dystrophic muscles of vitamin E-deficient animals have shown high sodium and low potassium contents. These were attributed to expansion of the extracellular space. But when the values given are recalculated it becomes clear that these changes are at least in part due to loss of potassium and gain of sodium in the intracellular phase (Blaxter & Wood, 1952; Morgulis & Osheroff, 1938). We can postulate that vitamin E-deficient tissues are unable to recover from the situations that stress the mechanisms responsible for ion movements.

The way in which selenium prevents dietary liver necrosis is as yet unknown, but perhaps it acts by preventing the situation which stresses the ion-transport mechanism. If this were found to be so, then we would have an explanation for the need for simultaneous deficiency of vitamin E and selenium for dietary liver necrosis to occur.

SUMMARY

1. Rats were made deficient in vitamin E by feeding them on casein or yeast diets.

2. Liver slices from these animals were unable to reaccumulate potassium removed by leaching in the cold.

3. Cooled slices show a falling off in oxygen uptake.

4. These defects were not prevented by feeding with selenium but were promptly reversed by giving α -tocopherol or by the addition of anti-oxidants to the incubation solution.

5. It is concluded that the defect *in vitro* in oxygen uptake by vitamin E-deficient tissues follows disturbances of ionic composition due to cooling.

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Condensed Tannins

15. INTERRELATIONSHIPS OF FLAVONOID COMPONENTS IN WATTLE-BARK EXTRACT*

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Wattle-bark extracts (*Acacia mearnsii*, formerly *A. mollissima*), known commercially as 'mimosa' extract, is mainly composed of polymeric leuco-

robinetinidin and leuco-fisetinidin tannins (Roux, 1957a, b, 1958; Roux & Evelyn, 1958a) accompanied by closely related flavonoids, (–)-robinetinidinol (Roux & Maihs, 1960), robinetin (Kirby & White, 1955) and fisetin (Roux, 1952). Isolation of (+)-catechin and (+)-gallocatechin (Roux &

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Maihs, 1960) from the bark extract also established the presence of two minor groups corresponding to cyanidin and delphinidin in their patterns of phenolic hydroxylation.

Subsequent to studies of interrelationships of components of the robinetinidin and fisetinidin analogues in the heartwoods of black-wattle and locust trees (*Robinia pseudacacia*) (Roux & Paulus, 1962a; Roux, 1962), a more detailed study of wattle-bark extract has now been made by using countercurrent-partition and paper-chromatographic methods for the separation of new components.

EXPERIMENTAL AND RESULTS

Two-dimensional chromatograms were run first in butan-1-ol-acetic acid-water (6:1:2, by vol.) and then in 2% (v/v) acetic acid on Whatman no. 1 paper. Comparisons of infrared spectra were made by the KBr-disk method by Dr P. R. Enslin, National Chemical Research Laboratory, C.S.I.R., Pretoria, South Africa. Analyses of C and H were by Mr K. Jones, Microanalytical Laboratory, C.S.I.R., Pretoria, South Africa.

Flavonoids based on the 3',4',5',7 and 3',4',7 patterns of phenolic hydroxylation

Preparation of extract and preliminary enrichment of flavonoid components. Bark (3.5 kg.) was collected from mature (40 ft.) trees, cut into slivers with a stainless-steel knife and immersed in commercial ethyl acetate within 1-2 hr. of being stripped from the tree. After 24 hr. the ethyl acetate extract was concentrated to a dry powder in a rotary evaporator. Wax was removed from the pale-buff solid (700 g.) by extraction with light petroleum (b.p. 80-110°) in a large Soxhlet extractor.

The wax-free extract (100 g.) was subjected to a preliminary enrichment by using the countercurrent-partition principle with ethyl acetate as upper and water as lower phases in ten 2 l. separating funnels. After completion of the separation procedure the contents from both upper and lower phases of each funnel were concentrated under vacuum. The enrichment was repeated seven times and the contents of the corresponding tubes were united. Most of the flavonoid components were present in tubes 9 and 10.

Separation of components. The solids from tubes 9 and 10 were combined (126 g.) and dissolved in 400 ml. of the lower phase of a water-butan-2-ol-light petroleum (b.p. 40-150°) (5:4:1, by vol.) mixture, and the solution was introduced into the first eight tubes of a 160-tube 50 ml.-underphase automatic Craig machine (Glasapparatebau Göttingen, Helmut Rettberg). The upper and lower phases of the above mixture were used for countercurrent separation, and after 160 transfers the upper layer of every fifth tube was examined by two-dimensional chromatography on Whatman no. 1 paper with water-saturated butan-2-ol and then 2% (v/v) acetic acid.

The contents (7.23 g.) of tubes 110-130 contained relatively high concentrations of (-)-robinetinidin and (+)-catechin, and in addition (-)-fisetinidin, fustin and dihydrorobinetin. These were separated as follows: Solids from tubes 110-130 were applied to 22½ in. × 18½ in. Whatman

no. 3 paper (250 mg. per sheet) as described previously (Roux & Evelyn, 1958b; and subsequent papers in the present series) and developed in 2% (v/v) acetic acid. Bands at R_F 0.38 (catechin band) and 0.48 (robinetinidin band) were cut out and eluted in the usual manner. On concentration of the eluates from the R_F 0.48 band to a small volume, crystals of robinetinidin (350 mg.) separated, m.p. and mixed m.p. with authentic (-)-robinetinidin [(+)-3',4',5',7-tetrahydroxyflavan-3-ol] (Roux & Maihs, 1960), 204-206°.

(-)-*Fisetinidin* [(+)-3',4',7-trihydroxyflavan-3-ol]. The solution remaining after the removal of (-)-robinetinidin by crystallization was streaked on six sheets of Whatman no. 3 (prewashed with water and dried) and the sheets were developed by downward chromatography in butan-1-ol-acetic acid-water (6:1:2, by vol.). A band with R_F 0.70 was located, cut out, eluted with 70% (v/v) ethanol and concentrated to a small volume. The fraction proved to be impure and chromatographic purity was achieved by repeating the preparative chromatography on cellulose sheets with 2% (v/v) acetic acid. From the final eluates (-)-fisetinidin (13 mg.) crystallized, m.p. 210-214°. The compound was compared with authentic (+)-fisetinidin and (-)-fisetinidin (Roux & Paulus, 1961a) on two-dimensional chromatograms and was inseparable from the latter. In 2% (v/v) acetic acid with reference substance (+)-fustin (R_F 0.37 on Whatman no. 1 paper) the R_F values were: synthetic (+)-fisetinidin, 0.43; synthetic (-)-fisetinidin, 0.48; (-)-fisetinidin from black-wattle bark, 0.48. The infrared spectrum over the range 2-15 μ was identical with that of (-)-fisetinidin.

Fustin [3,3',4',7-tetrahydroxyflavan-4-one]. The eluate from the catechin band (R_F 0.38) was streaked on six sheets of Whatman no. 3 chromatographic paper and developed by downward migration in butan-1-ol-acetic acid-water (6:1:2, by vol.) as above. A band with R_F 0.70 was located with ammoniacal silver nitrate spray reagent, cut out and eluted with 70% (v/v) ethanol. Fustin (7 mg.), m.p. and mixed m.p. with (+)-fustin (Roux & Paulus, 1960) (soda-glass) 212°, crystallized from the eluates on concentration. It could not be separated from authentic (+)-fustin on two-dimensional chromatograms [R_F in 2% (v/v) acetic acid, 0.37]. By comparison (-)-fustin has R_F 0.35 (Roux, Maihs & Paulus, 1961). The infrared spectrum over the range 2-15 μ was identical with that of (+)-fustin.

Dihydrorobinetin [3,3',4',5',7-pentahydroxyflavan-4-one]. After the above partition separation of the catechin band (R_F 0.38) for the isolation of fustin, the strong catechin band (R_F 0.60) in butan-1-ol-acetic acid-water (6:1:2, by vol.) on Whatman no. 3 was cut out and eluted. (+)-Catechin (Roux & Maihs, 1960) (47 mg.) crystallized, and the solution remaining was examined in more detail. Both catechin and dihydrorobinetin occupy identical positions on two-way chromatograms with the usual solvent systems including water-saturated butan-1-ol for the first direction. The presence of dihydrorobinetin was suspected as the solution remaining after the removal of (-)-robinetinidin gave a deep red on treatment with Mg-HCl. Separation of catechin and dihydrorobinetin was obtained by streaking the solution remaining after the removal of (-)-robinetinidin on a single sheet of Whatman no. 3 and developing with aq. 75% (w/v) phenol (Koeppen, 1961). The dihydrorobinetin moved as a discrete band with R_F 0.65 whereas

catechin had R_F 0.57. From this separation 2.5 mg. of dihydrorobinetin was obtained. Two-way chromatograms showed the compound to be pure and inseparable from optically pure (+)-dihydrorobinetin (Roux & Paulus, 1962*b*) [R_F 0.35 in 2% (v/v) acetic acid, 0.69 in water-saturated butan-2-ol]. The pure dihydrorobinetin gave a deep red-blue with Mg-HCl. The infrared spectrum over the range 2.5–15 μ was identical with that of (+)-dihydrorobinetin.

Butin (3',4',7-trihydroxyflavan-4-one). Solids (1.8 g.) from tubes 131–145 of the Craig separation were further separated by chromatography on paper sheets with 2% (v/v) acetic acid as described above. A band with R_F 0.16 was cut out and eluted to give 106 mg. of solids. Two-way chromatograms showed one prominent spot and numerous minor ones. The main spot was inseparable from that of ($\pm$)-butin (Roux & Paulus, 1961*b*) on two-way chromatograms [R_F 0.20 in 2% (v/v) acetic acid, 0.83 in water-saturated butan-2-ol on Whatman no. 1 paper], and gave characteristic colour reactions on paper chromatograms and also red with Zn-HCl in solution. The isolation of the butin was not attempted.

Butein (2',3,4,4'-tetrahydroxychalcone). Solids (1.6 g.) from tubes 145–160 were streaked on six sheets of Whatman no. 3 paper and the chromatograms were developed in 2% (v/v) acetic acid. Compounds remaining at the origin were eluted and applied to six sheets, and the chromatograms were irrigated with butan-1-ol-acetic acid-water (6:1:2, by vol.). A yellow band, orange-yellow when fumed with ammonia, moved to R_F 0.75. The compound was stripped and purified by chromatography in a water-90% (v/v) formic acid (1:1, v/v) mixture. A yellow band with R_F 0.31 was eluted and its ultraviolet and visible-range spectra were determined: $\lambda_{\max}$ 260 and 383 μ (in ethanol); 256 and 456 μ (in M-sodium ethoxide); 275 and 436 μ (in 0.02M-AlCl₃ in ethanol). For synthetic butein Roux & Paulus (1961*b*) found: $\lambda_{\max}$ 262 and 382 μ (in ethanol); 262 and 450 μ (in M-sodium ethoxide); 272 and 437 μ (in 0.02M-AlCl₃ in ethanol). Chromatographic comparison with authentic butein in aq. 75% (w/v) phenol (R_F 0.73) and butan-1-ol-acetic acid-water (6:1:2, by vol.) (R_F 0.83) showed identity.

Robtein (2',3,4,4',5-pentahydroxychalcone). The contents (70 g.) of separating funnel 8 of the initial enrichment procedure were separated in the Craig machine as before. Tubes 119–160 contained 0.78 g. of solids. These were separated by the preparative method in 2% (v/v) acetic acid, and compounds remaining at the origin (0.91 mg.) were examined for chalcones. Separation on Whatman no. 3 sheets with 80% (v/v) formic acid-water (3:2, v/v) gave a yellow band with R_F 0.59. This fraction had the same R_F (0.66) as that of synthetic robtein (Roux & Paulus, 1962*b*) in butan-1-ol-acetic acid-water (6:1:2, by vol.) and gave the same characteristic brick-red when fumed with ammonia. The band from the chromatographic separation with formic acid was eluted with 70% (v/v) ethanol and its ultraviolet and visible spectra were examined: $\lambda_{\max}$ 258 and 370 μ (in ethanol); 275 and 425 μ (in 0.02M-AlCl₃ in ethanol). Synthetic robtein had: $\lambda_{\max}$ 260 and 385 μ (in ethanol); 275 and 445 μ (in 0.02M-AlCl₃ in ethanol).

(+)-*Leuco-robinetinidin* [(+)-3',4',5',7-tetrahydroxyflavan-3,4-diol] and (+)-*leuco-fisetinidin* [(+)-3',4',7-trihydroxyflavan-3,4-diol]. Previous chromatographic evidence of the presence of traces of monomeric leuco-robinetinidin and

leuco-fisetinidin in black-wattle-bark extract (Roux & Evelyn, 1958*a*) was confirmed as follows. Mature black-wattle bark (500 g.) was cut into thin slivers with a stainless-steel knife and covered with commercial ethyl acetate (2 l.) at 0°, 10–30 min. after being stripped from the tree. After extraction for 48 hr. at -15°, the solution was streaked on 15 preparative sheets (5 ml. per sheet) and the chromatograms were developed with 2% (v/v) acetic acid without delay. Bands were cut at R_F 0.50 and 0.60 and the eluates examined by one- and two-way paper chromatography (Whatman no. 1), with ($\pm$)- and (+)-3',4',5',7-tetrahydroxyflavan-3,4-diol, and (+)- and (-)-3',4',7-trihydroxyflavan-3,4-diol, as reference compounds. The eluates of both bands contained compounds corresponding to (+)-leuco-robinetinidin and (+)-leuco-fisetinidin in R_F [0.46 and 0.52 respectively in 2% (v/v) acetic acid] and in colour reactions. By comparison their enantiomers, (-)-leuco-robinetinidin and (-)-leuco-fisetinidin, had lower R_F values (0.40 and 0.47 respectively) in the same solvent (cf. Roux, Maihs & Paulus, 1961).

These leuco-anthocyanidins appear to be absent from black-wattle extracts that were either heated or handled for prolonged periods at room temperature, presumably owing to condensation.

Flavonol glycosides

Bark (0.9 kg.) stripped from immature trees (average diam. 0.25 in.) was finely cut, across the grain, to give small chips, which were extracted with commercial ethyl acetate. Chlorophyll and waxes (6.5%) were removed with light petroleum (b.p. 80–110°) from the solid extractives (76.5 g.). The pale extract (72 g.) was enriched in flavonoid components by solution in a mixture of 220 ml. of ethanol and 720 ml. of ethyl acetate, the addition of chloroform (720 ml.), and the removal of the precipitate (15 g.) by filtration.

The soluble compounds from the filtrate (57 g.) were separated by preparative chromatography on Whatman no. 3 paper (300 mg. per sheet), with 2% (v/v) acetic acid as irrigant. A broad band, R_F 0.27, dark-brown under ultraviolet light, was cut out and eluted. The combined eluates were concentrated under vacuum to a small volume at a temperature not exceeding 70°. The solution was applied to prewashed sheets (about 100 mg. of solids per sheet) and developed in water-saturated butan-1-ol. Two bands (R_F 0.51 and 0.66), yellow in visible and dark-brown under ultraviolet light, were cut out and eluted.

The combined eluates were concentrated to a small volume under vacuum at 70°, treated with charcoal and filtered. Crystals slowly separated over 10–14 days. Several crops of crystals could usually be filtered off, the first usually being very impure. In a typical isolation 15 g. of enriched extract yielded 240 mg. (1.5%) of crude product of lower R_F (myricitrin) and 63 mg. (0.4%) of crude product of higher R_F (quercitrin).

Myricitrin (3',3',4',5',5',7-hexahydroxyflavone 3-rhamnoside). The compound was recrystallized from water, m.p. 201–206° with decomposition after strong sintering at 194–197°. Authentic myricitrin from *Myrica rubra* (Shimizu, Ohta, Yoshikawa & Kasahara, 1952) melts at 197°, and a mixed m.p. showed strong sintering at 194–197° with decomposition at 202°. The compound gave the following colour reactions: yellow in visible and brown under ultra-

violet light, orange-yellow with benzidine, brown with ammoniacal silver nitrate, olive-green with ferric alum, bright yellow with ammonia and dark purple with Mg-HCl in solution. The compound was inseparable from myricitrin in the following solvents: 2% (v/v) acetic acid (R_F 0.25), water-saturated butan-1-ol (R_F 0.56) and butan-1-ol-acetic acid-water (6:1:2, by vol.) (R_F 0.66). Alkali microfusion gave phloroglucinol and gallic acid. Ultraviolet-absorption curves of the compound gave: λ_{max} in ethanol, 256 and 355 m μ (log ϵ , 3.94 and 3.83 respectively); in 0.2M-AlCl₃ in ethanol, 268 and 422 m μ (log ϵ , 3.98 and 3.85). Found for authentic myricitrin: λ_{max} in ethanol, 258 and 355 m μ (log ϵ , 4.01 and 3.94); in 0.2M-AlCl₃ in ethanol, 265 and 423 m μ (log ϵ , 4.08 and 3.96) (Found for sample dried at 110°: C, 52.5; H, 5.2. C₂₁H₂₀O₁₂·H₂O requires C, 52.3; H, 4.6%. Found for sample dried at 160°: C, 54.5; H, 5.2. C₂₁H₂₀O₁₂ requires C, 54.3; H, 4.3%). The infrared-absorption curves of the compound and of myricitrin were identical over the range 2.5–15 μ .

The pure dry compound (71.5 mg.) was hydrolysed in 20 ml. of 2N-HCl for 2 hr. at 100°. The yellow precipitate was filtered off after being kept at 0° overnight. The yield (46.5 mg.) represents 94.8% of the calculated value for myricitrin rhamnoside. The aglycone (21 mg.) after crystallization from aqueous ethanol was acetylated, and the product was recrystallized three times from aqueous ethanol to give 7 mg. of white needles, m.p. and mixed m.p. with authentic hexa-acetylmuricetin similarly prepared from myricetin (L. Light and Co. Ltd.) showed no depression, 213°. Ultraviolet-absorption curves gave: λ_{max} in ethanol, 255 (shoulder) and 375 m μ ; in 0.02M-AlCl₃ in ethanol, 275 and 438 m μ . Found for authentic myricetin: λ_{max} in ethanol, 255 and 375 m μ ; in 0.02M-AlCl₃ in ethanol, 272 and 440 m μ . The filtrate from hydrolysis was neutralized by treatment with Amberlite IRA-400 resin. Chromatography on Whatman no. 1 paper with benzene-butan-1-ol-pyridine-water (1:5:3:3, by vol.) [due to W. Matthias; cf. Herrmann (1958)] showed the sugar to be rhamnose, R_F 0.45. Aniline hydrogen oxalate (Horrocks & Manning, 1949) was used as spray reagent, the sugars appearing as yellow spots after heat treatment at 105° for 10 min.

Quercitrin. The compound was recrystallized from water, sintered at 178° and melted at 180–185°. Mixed m.p. with authentic quercitrin (L. Light and Co. Ltd.) showed no depression. On chromatograms the compound was yellow in visible and dark brown under ultraviolet light, and gave the following colours with the spray reagents: orange-red with benzidine, black with ammoniacal silver nitrate, lime-green with ferric alum, intense yellow with ammonia and deep red with Mg-HCl in solution. The compound and quercitrin were inseparable on paper chromatograms in the

following solvent systems: 2% (v/v) acetic acid (R_F 0.31), water-saturated butan-1-ol (R_F 0.66) and butan-1-ol-acetic acid-water (6:1:2, by vol.) (R_F 0.76). Alkaline microfusion gave phloroglucinol and protocatechuic acid. Ultraviolet-absorption curves of the compound gave: λ_{max} in ethanol, 256 and 352 m μ (log ϵ , 4.24 and 4.10 respectively); in 0.02M-AlCl₃ in ethanol 273 and 420 m μ (log ϵ , 4.32 and 4.12); in M-sodium ethoxide, 272, 330 and 403 m μ (log ϵ , 4.36, 4.03 and 4.15). Found for authentic quercitrin: λ_{max} in ethanol, 256 and 352 m μ (log ϵ , 4.29 and 4.17); in 0.02M-AlCl₃ in ethanol, 274 and 418 m μ (log ϵ , 4.37 and 4.18); in M-sodium ethoxide 273, 332 and 403 m μ (log ϵ , 4.43, 4.09 and 4.32) (Found: C, 56.3; H, 5.3. C₂₁H₂₀O₁₁ requires C, 56.3; H, 4.5%). The infrared spectra of the compound and of quercitrin were identical over the range 2.5–15 μ .

The compound was hydrolysed with HCl as described for myricitrin, 67.04 mg. giving 43.5 mg. of precipitate. This represents a 96.3% yield calculated for quercetin monorhamnoside. The aglycone formed had the same R_F (0.80) as quercetin on Whatman no. 1 paper in butan-1-ol-acetic acid-water (6:1:2, by vol.). The ultraviolet spectrum gave: λ_{max} in ethanol 258 and 373 m μ ; in 0.2M-AlCl₃ in ethanol, 270 and 435 m μ . Found for quercetin: λ_{max} in ethanol 257 and 373 m μ ; in 0.02M-AlCl₃ in ethanol, 270 and 435 m μ . The sugar was shown to be rhamnose by paper chromatography, as described for myricitrin.

DISCUSSION

The presence of resorcinol-pyrogallol and resorcinol-catechol flavonoid analogues in black-wattle bark (Table 1) parallels their co-existence in the heartwoods of *R. pseudacacia* (Roux & Paulus, 1962a) and of black wattle (Roux, 1962). The ratio of flavonoid compounds to polymeric tannins in wattle bark, is, however, much lower than in these heartwoods, and the isolation of bark flavonoids is further complicated by the extreme complexity of the mixture.

Although robin (3',4',5',7-tetrahydroxyflavan-4-one), the flavanone analogue of the robinetinidin group, was not isolated (Table 1), its probable occurrence in wattle bark in low concentration is implied by the known presence of robinetin, and the ease of the flavanone $\rightleftharpoons$ chalcone isomerization of 5-deoxyflavanones and corresponding chalcones. Those flavonoids that have been isolated in sufficiently high yield for more detailed characteriza-

Table 1. Analogues of the main groups of flavonoids isolated and identified in wattle-bark extract

Flavonoid type	3',4',5',7-Tetrahydroxy compound	3',4',7-Trihydroxy compound
Chalcone	Robtein	Butein
Flavanone	—	Butin
Flavonol	Robinetin	Fisetin
Flavanonol	Dihydorobinetin	Fustin
Flavan-3-ol	(-)-Robinetinidol	(-)-Fisetinidol
Flavan-3,4-diol	(+)-Leuco-robinetinidin	(+)-Leuco-fisetinidin
Tannins	Polymeric leuco-robinetinidins	Polymeric leuco-fisetinidins
(mol.wt. 600–3000)		

Table 2. Analogues of minor groups of flavonoids isolated and identified from black-wattle-bark extract

Flavonoid type	3',4',5,5',7-Pentahydroxy compound	3',4',5,7-Tetrahydroxy compound
Flavan-3-ol	(+)-Gallocatechin	(+)-Catechin
Flavonol 3-rhamnoside	Myricitrin	Quercitrin
Tannin	Leuco-delphinidin*	—

* The presence of polymeric leuco-delphinidins in black-wattle bark was established by Dr E. A. Maihs (personal communication). Polymeric leuco-cyanidins are absent, but occur in green-wattle (*A. decurrens*) bark and in black-wattle $\times$ green-wattle hybrids.

tion or that are amenable to examination by suitable chromatographic methods [(−)-robinetinidol (by polarimetry), and (+)-leuco-robinetinidin, (+)-leuco-fisetinidin and (−)-fisetinidol (by paper chromatography)] belong to the same stereoisomeric series as (+)-catechin and their counterparts in wattle and *Robinia* heartwoods. However, the direction of the rotations of fustin, dihydrorobinetin and butin from wattle bark could not be determined by either method, and their stereochemical relationship remains unknown.

The presence of an exceptionally high concentration of polymeric leuco-robinetinidin and leuco-fisetinidin tannins in wattle bark, compared with the low concentration of related flavan-3,4-diols and flavonoids, suggests that enzymic polymerization of flavan-3,4-diols to tannins occurs more rapidly and completely in barks than in heartwoods where the ratio of flavonoids to tannins is often reversed. Almost complete polymerization of flavan-3,4-diols in wattle bark thus reduces the emphasis on their interconversions into flavonoid analogues. Such interconversions, on the other hand, predominate in wattle heartwood, where monomeric flavan-3,4-diols are prominent components (Roux & Paulus, 1961a, 1962a; Roux, 1962).

The presence of myricitrin and quercitrin [myricetin 3-rhamnoside and quercetin 3-rhamnosides; cf. Hattori & Hayashi (1931) and Herzig & Schönbach (1912)] establishes further links with two minor flavonoid groups in wattle-bark extract (Table 2). Myricitrin and quercitrin represent the only glycosides isolated hitherto from wattle-bark or heartwood extract, and the sugar residue (rhamnose) of both substances is also a constituent sugar of the natural polysaccharide exudate of the bark (Stephen, 1951). These flavonol glycosides are present in relatively high concentration in immature bark, selected as starting material for their isolation. Although their concentrations appear to be almost unaltered in mature bark, the predominance of the other components complicates their isolation from this source.

The recognition of four different patterns of phenolic hydroxylation, subdivided into two major and two minor but related groups, as well as the interrelationships of components within these

groups, simplifies the study of the exceptionally complex flavonoid mixture present in black-wattle bark. The predominant groups of flavonoid compounds found in the barks and heartwoods of black wattle and related *Acacia* spp. [also in certain *Robinia*, *Gleditschia*, *Guibourtia*, *Schinopsis* and *Rhus* spp. (Roux & Maihs, 1960)] are based on a resorcinol A nucleus. They therefore differ in this respect from those found in many fruit and forest trees, e.g. *Prunus*, *Eucalyptus* and *Pinus* spp. (cf. Hergert, 1962), which are predominantly patterned on the more commonly occurring phloroglucinol A nucleus. The proliferation of the phenolic hydroxylation pattern within a single genus is mainly confined to the B nucleus of flavonoid compounds, but occurs in both A and B nuclei in many *Acacia* spp., and also in certain *Schinopsis* spp. Such a general tendency towards proliferation of heartwood and bark flavonoids within a single genus is commonly found amongst the Coniferae and appears to be a primitive rather than a modern characteristic.

SUMMARY

1. Analogues of the two predominant groups of flavonoid components, based on robinetinidin and fisetinidin, have been isolated or identified from fresh mature black-wattle bark.

2. Myricitrin and quercitrin, related to the two minor groups based on delphinidin and cyanidin, have been isolated from fresh immature black-wattle bark.

3. All flavonoid components hitherto isolated from black-wattle bark fall into one of these four patterns of phenolic hydroxylation.

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Studies on Alkaline Phosphatases

4. INFLUENCE OF DIETARY MINERALS ON pH OPTIMA AND REACTION VELOCITIES OF PHOSPHATASES OF FOWL*

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The optimum pH for alkaline phosphatase depends on such factors as the source of enzyme and the type and concentration of substrate, buffers and activators (for references see Motzok & Branion, 1961). Also, the optimum pH of fowl-intestinal phosphatase changes to more alkaline values with increasing age of the birds (Motzok & Branion, 1961). Although the pH optima for the various concentrations of glycerol 2-phosphate were quite constant for the intestinal enzyme of several breeds of chicks at 1 day of age, some differences have been observed among groups of older fowls of approximately the same age but given different experimental and commercial diets. The suggestion that the concentration of specific minerals in the diet caused some of these differences was based on the observation that the optimum pH of intestinal phosphatase was lower in hens given insoluble granite grit than in hens given oyster shell *ad libitum* with a diet containing about 1.2% of calcium (Motzok & Branion, 1961).

The present paper is mainly concerned with the effect of dietary calcium on the pH optima and reaction velocities of the alkaline phosphatase of the intestinal mucosa of fowl. Some observations on the influence of dietary magnesium, manganese and molybdenum on phosphatases of avian tissues are also recorded.

METHODS AND MATERIALS

Methods for the preparation of tissue phosphatases and for the determination of enzyme activity were as described

by Motzok (1950). Except for the studies on pH optima without added Mg^{2+} ions in the reaction mixtures (Figs. 3 and 5), phosphatase activities were determined in the presence of 5 mM- $MgCl_2$ for the enzymes of bone, liver and kidney, and 10 mM- $MgCl_2$ for the enzyme of intestinal mucosa. Columbian Rock, White Rock, Barred Plymouth Rock and Australorp fowls were used. In preliminary work (Expt. 1) hens and 2-month-old cockerels were fed on a commercial diet, with oyster shell and insoluble granite grit being provided to separate groups of birds. In the remainder of the work the experiments were started on 1-day-old chicks, which were randomized into groups of 20 and kept in metal batteries with wire floors in an air-conditioned room maintained at approx. 27°. The experimental diets and water were given *ad libitum*. In studies on the effect of calcium and magnesium a practical type of chick diet was used (Motzok, Arthur & Branion, 1956). This basal diet contained 0.2% of calcium, 0.28% (calc.) of magnesium, 80 p.p.m. of manganese and 0.48% of total phosphorus, of which 0.17% was calculated to be inorganic. Sufficient disodium hydrogen phosphate (commercial grade) was added to the basal diet to raise the concentration of total inorganic phosphorus in the diets to 0.45%. Two dietary concentrations of calcium were employed: calcium carbonate (U.S.P. grade) was added to provide a total calcium content of 0.6% in the low-calcium diet and 1.5% in the high-calcium diet. In experiments with magnesium, 2% of magnesium carbonate (U.S.P. grade) was added. In experiments with supplemental calcium the basal diet constituted 96.6% of the rations and the remaining 3.4% was made up with various amounts of calcium carbonate and finely ground cellulose (Alphacel, obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) (Expt. 2) or with wheat (Expts. 7 and 8). In experiments with added calcium and magnesium the basal diet made up 94.6% of the ration with the remainder provided by the mineral supplements and additional wheat as the other variable component. In studies with manganese

* Part 3: Motzok & Branion (1961).