SUMMARY

1. The colorimetric method for estimation of acetoacetate by reaction with diazotized *p*-nitro-aniline has been adapted for measurements of oxaloacetate in amounts of 0.003–0.03 μ mole.

2. The steady-state level of oxaloacetate in the livers of normal and fasted rats was found to be about 4 μ moles/100 g. dry wt. of tissue. The level was essentially unchanged in starvation when the level of acetoacetate increased tenfold.

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Oak-bark Tannins

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The barks of the two common oaks, the pedunculate oak (*Quercus pedunculata* Ehrh.) and the sessile oak (*Quercus sessiliflora* Salisb.) have been the traditional tanning material of Britain and northern Germany since medieval times. The principal botanical difference between the species is that the pedunculate oak has acorns on a long peduncle and stalkless or nearly stalkless leaves, whereas the sessile oak has sessile or stalkless acorns and petiolated leaves, but intermediate or hybrid forms are common. For commercial purposes, the timber and bark of these trees are not differentiated. Our experience of this tanning material shows that best-quality oak bark contains

12–16% of tannin [cf. Howes (1953) who gives 12–14%].

Little information on oak-bark phlobatannins is available, but (+)-catechin and (+)-gallo catechin have recently been isolated from this source (Mayer, 1956; cf. Casparis & Reber, 1929). The nature of the partial-type formula for oak-bark phlobatannins has now been investigated. Phlobatannins have recently been isolated in high yield from the extractives of *Acacia catechu* heartwood and harvested *Uncaria gambir* leaves (Hathway & Seakins, 1957*a*), which have the same analytical properties and similar elementary analyses and absorption spectra to those of the polymers of

catechin oxidation (Hathway & Seakins, 1955; 1957b; Hathway, 1958). There is therefore a strong supposition that these phlobatannins are formed by aerobic oxidation of catechin epimers by polyphenoloxidases in the heartwood and detached leaves respectively (Hathway & Seakins, 1957a). The isolation of the free plant phenolics and the purification of a phlobatannin fraction from oak bark, and an investigation of the polyphenoloxidase activity of the cambium, have been undertaken. The phenolics of mature leaves and the distribution of tannin extractives in stem bark have also been explored.

EXPERIMENTAL

General. Evaporations were carried out in N_2 under reduced pressure at a temperature below 35° . Paper chromatography was carried out in all-glass apparatus in a constant-temperature enclosure at 25° . Chromatograms were dried at room temperature, unless otherwise stated. Melting points were determined on a Kofler block. Specific rotations were determined for the D line of sodium, with a 1 dm. microtube. Five pairs of readings with the solutions and five pairs with a solvent blank were taken in each case, at 20 – 25° .

Systematic separation of an 80% methanolic extract of oak bark into fractions of chemically similar substances

Slivers of stem bark were stripped from a 20-year-old oak sapling immediately after felling. A portion was used for the analysis of moisture, and of tannin and non-tannin extractives (*Official Methods of Analysis*, 1957). Acids and salts were estimated by titration with $0.02N$ -NaOH on portions withdrawn from such an aqueous extract as that required by the *Official Methods*, before and after its passage through a Zeo-Karb 225 cation-exchange resin (2 mg.equiv./ml.) in the H^+ cycle. The stem bark, which had a 60% moisture content, contained 12.6% of tannin and 6.9% of non-tannin extractives on the moisture-free bark. Each 100 g. of extractives contained 60 mg.equiv. of acid and 70 mg.equiv. of salt.

A portion (500 g.) of fresh bark was percolated with methanol (1.5 l.) for 5 days, after which the cell-wall fraction was removed by filtration through a sintered-glass funnel, and pressed and washed (5×100 ml.) with 80% methanol. Partial evaporation of the combined filtrate and washings left a soln. (400 ml.) which was continuously extracted for 4 days with peroxide-free ether in a Schacherl apparatus, ice-water being pumped through the condenser. Evaporation of the solvent left a solid residue which was dried over P_2O_5 at 20° *in vacuo* for 2 days. The ether-soluble fraction (4.3 g.) constituted 2.2% by wt. of the moisture-free bark.

Residual ether was removed from the aqueous phase, which was continuously extracted for 200 hr. with four changes of ethyl acetate in a Schacherl apparatus. Evaporation of the solvent left solid residues (5 g.) which constituted 2.5% by wt. of the moisture-free bark.

Residual ethyl acetate was removed from the aqueous phase, which was then percolated (40 ml./hr.) through a

column (bed-volume, 20 ml.) of Zeo-Karb 225 cation-exchange resin (1.95 mg.equiv./ml.) in the H^+ cycle, which was washed free from acid. The total effluent (800 ml.) was evaporated to 500 ml., when a trace of insoluble material was removed by filtration through a few centimetres of Celite no. 535 (Johns-Manville Inc.) on a sintered-glass funnel. The filtrate contained 10% of tannin (hide-powder test).

Paper chromatography of the ether-, ethyl acetate- and water-soluble phenolics

A 2% methanolic solution of the fraction under investigation was spotted (5 μ l.) at a distance of 2 cm. from both edges of the lower left-hand corners of Whatman no. 2 filter papers, 25.5 cm. square, and chromatographed by the ascending method with 3% (v/v) acetic acid containing 2% (v/v) formic acid as first-way solvent system. The solvent was irrigated to within 1 cm. of the upper edge of the paper, which was subsequently dried, and developed in the other dimension with butan-2-ol-acetic acid-water (14:1:5, by vol.) for 20 hr. With the water-soluble phenolics, 50% (v/v) acetic acid was used as the second-way solvent system. Phenolics were detected by dipping the papers in a 1:1 (v/v) mixture of 0.3% $FeCl_3$ and 0.3% $K_3Fe(CN)_6$ solutions, and by means of a spraying reagent consisting of a mixture of 1 vol. of 12N-HCl with 2 vol. of a 10% (w/v) solution of vanillin in ethanol.

Separation of the ether- and ethyl acetate-soluble phenolics by partition chromatography on silica gel

Chromatographic grade silica gel (100 g., mesh size 100–200; L. Light and Co. Ltd.) was stirred with two 1 l. portions of 2N-HCl at 100° for 2 hr.; the supernatant was then decanted, and the sediment washed free from acid to Congo red. The wet slurry was washed with 2 l. of methanol, air-dried and reactivated at 110° for 8 hr.

Suitable tubes were packed with the silica gel (80 g.) which had been triturated with water (52 ml.) and slurried with peroxide-free ether by Bradfield, Penney & Wright's (1947) method. The eluent was prepared by equilibration of peroxide-free ether with excess of water, followed by retention of the upper phase. The orange powder from the ether extraction (4.3 g.) was triturated with portions of wet ether (total, 100 ml.), and the solution decanted from insoluble material (500 mg.) was applied to the top of the column, which was subsequently percolated (2 ml./min.) with wet ether.

With the ethyl acetate-soluble phenolics, the column was packed with silica gel which had been triturated with the same proportion of water and slurried with ethyl acetate. The cream powder (3.5 g.), corresponding to the first two fractions in Table 2, was applied to the top of the column, which was eluted with wet ethyl acetate.

Isolation of leucodelphinidin

The first 100 ml. of eluate obtained from the column on which the ethyl acetate-soluble phenols were adsorbed contained an unresolved mixture of phenolics (2 g.). Evaporation of the next 200 ml. of eluate left 1 g. of *leucodelphinidin*, which separated in crystals, m.p. 155 – 160° , R_f 0.4 in butan-2-ol-acetic acid-water (14:1:5, by vol.) (Found: C, 53.0; H, 5.1. $C_{15}H_{14}O_8 \cdot H_2O$ requires C, 53.0; H, 4.7%) which afford delphinidin on treatment with acid. The *hepta-acetate* melted at 140 – 144° (Found: C, 56.5; H, 5.2. $C_{20}H_{18}O_{15}$ requires C, 56.0; H, 5.6%).

Investigation of the ethyl acetate-soluble fraction for leucoanthocyanins

A portion (20 mg.) of each ethyl acetate-soluble fraction and one broad-bean (*Vicia faba*) testa were treated with 3 ml. of 2*N*-HCl at 100° for 20 min., and the supernatant was decanted into a narrow test tube and extracted with enough *n*-amyl alcohol to give an upper phase of sufficient depth for spotting on starting lines, 3 cm. from the lower edges of Whatman filter paper no. 1, 57 cm. in length. The applications were repeated with intermittent drying, until the colour of each spot was intense enough to ensure visibility of the anthocyanidins on the developed chromatograms. Single-way ascending chromatography was effected with water-acetic acid-12*N*-HCl (10:30:3, by vol.), and *m*-cresol-5.5*N*-HCl-acetic acid (1:1:1, by vol.) solvent systems (Bate-Smith, 1954).

Purification of the phlobatannin fraction by cellulose-column chromatography

Solka Floc cellulose (Grade, BW 200) (Johnsen, Jørgensen, and Wettre Ltd., Wood Pulp Agents, 26, Farringdon St., London, E.C. 4) was stirred twice with large portions of 2*N*-HCl and washed free from acid to Congo red on a sintered-glass filter. The iron-free pulp was washed with methanol, air-dried and homogenized in acetone by a top-drive macerator, and the slurry was transferred to a chromatography tube made out of Pyrex pipe-line components. The column (60 cm. × 5 cm.) was developed with acetone, and stored for 48 hr. and developed under pressure with aq. 10% (v/v) formic acid until free from acetone. A volume (125 ml.) of the aqueous solution containing the water-soluble phenolics was applied to the top of the column, from which the sugars and mobile phenolics were eluted with 5 l. of 10% (v/v) formic acid. Oak-bark phlobatannin occupied a zone stretching from 2.5 to 7.5 cm. from the top of the column.

Fractionation of an 80% methanolic extract of oak leaves

Mature sun-leaves (80 g.), harvested in August, were plunged into ice-cold 80% methanol (500 ml.) and homogenized in a top-drive macerator for 5 min. The homogenate was filtered through sintered glass, and the filtrate was extracted with successive portions of light petroleum (b.p. 40–60°) until free from chlorophyll and related compounds. Partial evaporation of the lower phase afforded an aqueous solution which was successively extracted with ether and ethyl acetate. The ether-soluble fraction accounted for 0.25% (by wt.) of the leaves, and the ethyl acetate-soluble fraction for a further 0.5%. The extracts were examined for phenolics by conventional two-dimensional paper chromatography.

Isolation of (–)-epicatechin from Acacia catechu heartwood. Chips (100 g.) of *Acacia catechu* heartwood, extracted with acetone by the method of Rao & Seshadri (1948), afforded (–)-epicatechin (1 g.) which crystallized from water, forming needles, m.p. 243–244°, $[\alpha]_D - 69^\circ$ (c, 1; ethanol).

Epigallocatechin. A specimen was made available for the purpose of chromatographic identification through the courtesy of Dr E. A. H. Roberts, of the Indian Tea Association, London, S.E. 1.

Acetone-dried powder of oak cambium. A 20-year-old sapling was felled, and the stem bark was immediately peeled. Cambium detached from the freshly exposed surfaces of the bark and sapwood was plunged into 2 l. of acetone at below 0°, and sliced, and homogenized in a top-drive macerator. The homogenate obtained was filtered through sintered glass, and the solid (20 g.) was immediately washed five times with 500 ml. portions of ice-cold acetone and dried over paraffin wax at 0° *in vacuo*. The crude preparation had a polyphenoloxidase activity of purpurogallin number (P.N.) of 0.003. P.N. represents milligrams of purpurogallin formed from pyrogallol in 5 min. at 20°/mg. dry wt. of enzyme preparation. A Hilger Uvispek spectrophotometer was used for the P.N. determination by Keilin & Mann's (1938) method. This preparation was ground in a chilled Wiley mill, and homogenized five times with 500 ml. portions of ice-cold acetone, and filtered and dried over paraffin wax at 0° *in vacuo*. Wood fibres were removed. This powder (0.09 enzyme unit) had a P.N. of 0.005. One enzyme unit (E.U.) corresponds to the quantity of enzyme which produces 1 g. of purpurogallin in 5 min. at 20°.

Manometry. Use was made of Haldane's (1921) constant-pressure respirometer (Hathway & Seakins, 1957a). Both the reaction and the compensation vessels were shaken at 30 ± 0.05° at a speed sufficient to maintain the reaction mixture as a foam.

5-Methoxy-4-methylresorcinol. A specimen, m.p. 120° (Found: C, 62.1; H, 6.7. Calc. for C₉H₁₀O₃: C, 62.4; H, 6.5%), was prepared by the method of Robertson & Whalley (1951).

4-Nitrocatechol. A specimen, m.p. 174–175° (decomp.), was prepared from *o*-dibenzoyloxybenzene by the method of Burton & Prall (1951).

Synthesis of 5-methylpyrogallol

Orcyl aldehyde (I), m.p. 180° (Adams & Levine, 1923), was converted into the dicarbomethoxy derivative (II), m.p. 84–85°, and oxidized by permanganate to dicarbomethoxyorsellinic acid (III), m.p. 132–134°, by the method of Hoesch (1913). Purdie methylation of III, followed by partial hydrolysis by the method of Robertson & Stephenson (1932), gave methyl orsellinate (IV), which crystallized from dilute methanol, forming prisms, m.p. 142°.

Preparation of methyl haematommate (V). Powdered, anhydrous ZnCl₂ was suspended in an ether soln. (300 ml.) of methyl orsellinate (7.1 g.); Zn(CN)₂ (11 g.) was added and HCl gas was conducted through the reaction mixture at 0° for 4 hr. The mother liquor was decanted from the crystalline aldimine hydrochlorides, which were washed four times with 100 ml. portions of absolute ether. The aldimine hydrochlorides were dissolved in 100 ml. of water and the soln. was heated at 100° for 40 min. After cooling for 16 hr., the product was collected and steam-distilled. Almost pure methyl haematommate (V) (3.1 g., 38% yield) separated from 5 l. of distillate at 0°, and crystallized from 100 ml. of methanol, forming glistening needles, m.p. 147° (Found: C, 57.1; H, 4.8. Calc. for C₁₀H₁₀O₅: C, 57.1; H, 4.8%), which were dried over P₂O₅ at 65° *in vacuo*. The non-volatile solid which separated from the residual liquor after steam-distillation, crystallized from water (charcoal), forming parallelepipeds, m.p. 130° (Found: C, 57.0; H, 4.6), of methyl isohaematommate, in 4% yield. Repetition of this preparation, with BF₃-ether complex

Fig. 1. Two-dimensional chromatogram of the ether-soluble phenolics. The material was applied in the position marked $\odot$ and run first in 6% (v/v) acetic acid containing 2% (v/v) of formic acid, followed by butan-2-ol-acetic acid-water (14:1:5, by vol.). Spots located by $\text{FeCl}_3\text{-K}_3\text{Fe}(\text{CN})_6$: 1, (+)-gallocatechin; 2, (+)-catechin; 3, unknown; 4, epigallocatechin; 5, epicatechin; 6, methyl gallate.

(1 ml.) (Eastman Kodak Co., Rochester, U.S.A.) gave a 45% yield of methyl haematommate. Both preparations are improved variations of Curd, Robertson & Stephenson's (1933) preparation.

Hydrolysis of methyl haematommate gave atranol (VI), which crystallized from benzene in the form of pale-yellow needles, m.p. 124°, which were converted by the Dakin reaction into 5-methylpyrogallol (VII) by the method of Pfau (1926). 5-Methylpyrogallol crystallized from benzene, and sublimed at 105°/0.01 mm. Hg, forming long needles, m.p. 119° (Found: C, 60.1; H, 5.5. Calc. for $\text{C}_7\text{H}_6\text{O}_3$: C, 60.0; H, 5.8%), which were dried over P_2O_5 at 65° *in vacuo*.

In the aerobic oxidation experiments, 0.1 m-mole of 5-methylpyrogallol, and mixtures of 0.1 m-mole of this substance and 0.1 m-mole of phloroglucinol or 5-methoxy-4-methylresorcinol, underwent auto-oxidation at $30 \pm 0.05^\circ$, in 20 ml. of phosphate buffer, pH 7, for 2 days. Similar enzymic experiments were carried out in 3 hr.

RESULTS

The present work consists of a study *in vitro* of the aerobic oxidation of the (+)-gallocatechin and leucodelphinidin metabolites by oak-cambium polyphenoloxidase to a polymer comparable with the phlobatannin, which has now been isolated in 2% yield from the bark.

Young oak bark contains 50–60% of moisture and extractable 'tannins' account for about 12% of the dry bark. The bark was extracted with 80% methanol, the solvent removed and the phenolics and tannins in the resulting aqueous solution were fractionated by solvent extraction.

A two-dimensional chromatogram of the ether-soluble phenolics showed the presence of three constituents (Fig. 1). Two of these metabolites were chromatographically indistinguishable from catechin and gallocatechin respectively, and both compounds gave the vanillin reaction. The absence of epicatechin and epigallocatechin was shown by the use of the authentic substances as markers. The third component did not react with vanillin, and was indistinguishable from methyl gallate on the chromatogram.

The ether-soluble phenolics were separated by partition chromatography on silica gel, with wet ether as eluent (Table 1). (+)-Catechin and (+)-gallocatechin were isolated from well-separated bands, and the physical properties and elementary analyses of these substances were in excellent agreement with previous findings (Freudenberg & Purrmann, 1924; Mayer, 1956). No crystalline material was obtained from the first band, however, which showed that the most mobile constituent was not methyl gallate.

The ethyl acetate-soluble phenolics were further fractionated on a time basis (Table 2). Two-dimensional chromatograms (Fig. 2) showed that the first two fractions contained three vanillin-positive constituents which were of lower R_f values in the second-way solvent system than those of the ether-soluble phenolics. All four fractions on treatment with warm acid generated mixtures of anthocyanidins, which were identified by single-

Table 1. *Separation of the ether-soluble phenolics by partition chromatography on silica gel*

Total recovery was 3.75 g. from an ether-soluble fraction weighing 3.8 g. A narrow pink band 1.0–1.5 cm. from the top of the column, and a yellow band somewhat lower were not investigated

Description of band	Eluate (ml.)	Phenolics recovered from eluates
1. Greenish brown	60	Oily residue (750 mg.) contained the phenolic, R_F 0.95 in butan-2-ol-acetic acid-water, but no crystalline material was obtained
2. Pink	500	(+)-Catechin (1.7 g.) crystallized from hot water (8 ml.) forming needles, m.p. and mixed m.p. 173–175°, $[\alpha]_D^{25} + 8^\circ$ (c, 1; acetone) (Found: C, 50.0; H, 5.8. Calc. for $C_{15}H_{14}O_6$, $4H_2O$: C, 49.8; H, 6.1%)
Absence of bands	200	—
3. Yellow	500	(+)-Gallocatechin (1.3 g.) crystallized from hot water (8 ml.), forming needles, m.p. 189–191°, $[\alpha]_D^{25} + 15^\circ$ (c, 1; 50% (v/v) acetone) (Found: C, 52.7; H, 5.6. Calc. for $C_{15}H_{14}O_7 \cdot 2H_2O$: C, 52.7; H, 5.3%)

Table 2. *Ethyl acetate extraction of the aqueous extract*

Fraction	Extraction (hr.)	Residue (g.)	Examination of the residue by paper chromatography
1	75	2.8	Phenolic, R_F 0.4 in butan-2-ol-acetic acid-water, is dominant
2	25	0.7	
3	25	0.5	Phenolic, R_F 0.28 in the same solvent system, is dominant, and the tannins begin to appear on the chromatograms
4	75	1.0	
Total 200

Fig. 2. Two-dimensional chromatogram of the ethyl acetate-soluble phenolics, the same solvent systems being used as in Fig. 1. Spots located by vanillin: 1, unknown; 2, leucodelphinidin; 3, unknown.

way paper chromatography in two different solvent systems as cyanidin and delphinidin (Bate-Smith, 1954), and the location of the absorption bands of these pigments on paper agreed with the recorded wavelengths. At least two of the constituents (Fig. 2) are therefore leucoanthocyanins, which also have the same hydroxylation pattern as the related cyanidin and delphinidin. The chromatogram in Fig. 2 shows that one constituent pre-

dominates in the first two fractions. This major constituent should therefore be capable of purification by partition chromatography. Despite the fact that the first band contained all three phenolics, crystalline leucodelphinidin was isolated from the second band, which was eluted from a silica gel column with wet ethyl acetate. This compound, which was characterized by a hepta-acetate derivative, gave delphinidin on treatment with acid. (+)-Catechin accounts for 1% of the dry weight of young oak bark, (+)-gallocatechin for up to 1% and leucodelphinidin for approximately 0.5%.

A two-dimensional chromatogram of the cation-free water-soluble extractives run first in *N*-acetic acid containing 2% (v/v) of formic acid, followed by 50% (v/v) acetic acid, revealed the presence of mobile, vanillin-positive phenolics, in addition to the phlobatannin which remained at the origin. The extract also contained free sugars. A portion of the extract was therefore adsorbed on the top of a large cellulose column, which was then eluted with 10% formic acid to remove contaminating sugars and mobile phenolics. The chemical nature of mixtures of phenolics present in fractions of effluent is described in Table 3. Since the anthocyanidins generated from these fractions by treatment with acid were separated as round spots on a paper chromatogram, it is unlikely that the corresponding leucoanthocyanins are polymeric. These leucoanthocyanins are probably glycosides.

Table 3. Purification of the phlobatannin fraction by cellulose-column chromatography

Vol. of eluate (l.)	Examination of mixed products
0.6-1.4	Sugar extractives (anthrone reagent)
1.0-1.4	Vanillin-positive phenolic, R_F 0.72 in 6% (v/v) acetic acid containing 2% (v/v) of formic acid, affords cyanidin by acid treatment
1.5-1.7	Vanillin-positive phenolic, R_F 0.64 in the same solvent system, affords cyanidin and delphinidin by acid treatment
1.8-2.9	Vanillin-positive phenolic, R_F 0.52 in the same solvent system, gives mostly delphinidin on acid treatment
3-5	Vanillin-positive phenolic, R_F 0.3 in this solvent system, gives mostly delphinidin on acid treatment

Phlobatannin (1 g.) of low R_F was eluted with aq. 70% (v/v) formamide, pH 7, from a zone stretching from 2.5 to 7.5 cm. from the top of the column. The eluate, $\lambda_{\max}$ 285 m μ , was evaporated *in vacuo*, and the residue (Found: C, 53.3; H, 5.1. $C_{15}H_{10}O_7$, $2H_2O$ requires C, 53.3; H, 4.2%), which was washed with water and dried at 65° *in vacuo*, gave a vanillin reaction

The top zone of the column was eluted with 70% formamide (Hathway, 1956a). This eluate showed intense absorption at 285 m μ (Fig. 4) and, on evaporation, the phlobatannin, which accounted for 2% of the dry weight of young bark, analysed for $C_{15}H_{10}O_7 \cdot 2H_2O$.

A methanolic extract derived from mature sun leaves was fractionated in the same way as the bark. Catechin and galliccatechin were identified in the ether-soluble fraction, and the three vanillin-positive phenolics in the ethyl acetate-soluble fraction. Besides these compounds some plant pigments, including quercetin, were present.

An acetone-dried powder of oak cambium which was substantially free from phenolics, had marked polyphenoloxidase activity. The crude enzyme preparation was tested with (+)-galliccatechin in phosphate buffer at 30° and pH 7, and the aerobic oxidation of this substance went smoothly to completion. At the level of enzyme concentration used, the rate of uptake of oxygen diminished very slowly during the course of reaction with this substrate. Such progress curves contrast with those for the aerobic oxidation of (+)-catechin by mushroom, potato and tobacco polyphenoloxidases (Hathway & Seakins, 1957a), as they show less enzyme inactivation. The enzyme concentrations in the present work were smaller than the previous ones, and these results suggest that the cambium enzyme is particularly suited to the oxidation of (+)-galliccatechin. Progress curves for the aerobic oxidation of (+)-catechin, (+)-galliccatechin and leucodelphinidin at the same enzyme concentration are recorded in Fig. 3. The rate of uptake of oxygen diminished much more rapidly with (+)-catechin

Fig. 3. Progress curves for the oxidation of galliccatechin, catechin and leucodelphinidin by oak-cambium polyphenoloxidase. In addition to 20 ml. of 0.07M-phosphate buffer, pH 7, and 5×10^{-4} E.U. of oak-cambium polyphenoloxidase (●), the reaction flask contained 0.1 m-mole of galliccatechin (○); catechin (△); leucodelphinidin (□); or 0.1 m-mole of catechin and 1 m-mole of 4-nitrocatechol (▲). A compensatory flask contained phosphate buffer.

Fig. 4. Absorption spectra of oak-bark phlobatannin (A), and the aerobic oxidation polymers of (+)-galliccatechin (B), leucodelphinidin (C) and 5-methylpyrogallol with or without the addition of phloroglucinol or 5-methoxy-4-methylresorcinol (D). The spectra were measured in aqueous solution (pH 7).

and leucodelphinidin than with (+)-gallo catechin. Thus the (+)-gallo catechin metabolite is oxidized more rapidly than the other metabolites, and units derived from the oxidative polymerization of (+)-gallo catechin would therefore be expected to contribute extensively to the structure of oak-bark phlobatannin. The aerobic oxidation of catechin is arrested by the presence of Nakabayashi's (1954) 4-nitrocatechol inhibitor, at the relative concentrations of inhibitor to substrate which he employed. This inhibitor is efficient for catechol and catechin substrates.

The absorption spectrum of oak-bark phlobatannin exhibits a phenolic absorption band at 285 m μ and diminishing general absorption in the region 300–600 m μ . The absorption spectra of (+)-gallo catechin and leucodelphinidin-oxidation polymers are also precisely similar to that of oak-bark phlobatannin (Fig. 4).

The aerobic oxidation of an equimolecular mixture of 5-methylpyrogallol and phloroglucinol, and of 5-methylpyrogallol and 5-methoxy-4-methylresorcinol, gave polymers which showed a strong absorption band at 285 m μ and a shoulder at 350 m μ , which were identical to those in the spectrum of 5-methylpyrogallol-oxidation polymer alone (Fig. 4).

The analyses on bark detached from different parts of the stem of freshly felled trees show that the concentration of tannin diminishes from ground level to the crown (Table 4).

DISCUSSION

Examination of the leaves (of *Quercus*) shows the presence of (+)-catechin, (+)-gallo catechin and several vanillin-positive spots, some of which yield delphinidin on treatment with hot acid and may therefore be concluded to be leucodelphinidins. Besides these compounds some plant pigments, including quercetin, were also present. The occurrence of the (+)-catechin, (+)-gallo catechin and leucodelphinidin metabolites in the mature leaves suggests that in the Fagaceae they originate there, and Hillis (1956*a, b*) has reached the same conclusion about the origin of the leucoanthocyanins

of certain eucalypts (cf. Jaccard, 1938). On account of Davis's (1955) microbial studies and important work involving the use of ¹⁴C-labelled cinnamic and shikimic acids and L-phenylalanine (Brown & Neish, 1955; Geissman & Swain, 1957; Underhill, Watkin & Neish, 1957; Watkin, Underhill & Neish, 1957), it is probable that the (+)-catechin, (+)-gallo catechin and leucodelphinidin metabolites of *Quercus* spp. are formed in the leaves by reaction sequences of the sort considered by Neish and his co-workers and Hathway (1956*b*).

The bark extracts contain the same two catechins (which in this case were isolated in a crystalline state by column chromatography) and several leucoanthocyanins, one of which has also been obtained in a crystalline state and shown to be leucodelphinidin. This is the first time that a crystalline leucoanthocyanin yielding a commonly occurring anthocyanidin has been isolated. In addition to these crystalline compounds, several other low molecular-weight phenols are present, together with a large amount of phlobatannin ('condensed' tannin).

Examination of the cambium showed the presence of a polyphenoloxidase, which acted more readily on the pyrogallol type [(+)-gallo catechin and leucodelphinidin] phenols than on the catechol type [(+)-catechin] to form polymers.

From earlier work it appears likely that phlobatannin is formed from the oxidation of 'flavans' by polymerization through quinone. An examination of the possible linkage has therefore been made, since for catechin this is head-to-tail (Hathway, 1958; Hathway & Seakins, 1955; 1957*b*). With (+)-gallo catechin and leucodelphinidin as substrates for the cambium oxidase, a polymer is obtained which has a similar ultraviolet spectrum to that of the phlobatannin isolated from the bark, and is in contrast with the results obtained earlier with (+)-catechin and polyphenoloxidases (Hathway & Seakins, 1957*a*). The spectrum obtained is in fact similar to the ultraviolet spectrum of catechol-oxidation products (Hathway & Seakins, 1957*b*), and suggests strongly that tail-to-tail polymerization occurs. Confirmation of this hypothesis is obtained by the oxidation of 5-methylpyrogallol

Table 4. *Distribution of tannin in stem bark*

Forest	Date of felling	Age (years)	Location on stem	Tannin percentage on moisture-free bark basis
Goytre	November 1955	20	Crown	11.5
			Butt	13.5
Chepstow	November 1955	20	Crown	11.3
			Butt	13.1
Dean	October 1955	70	Crown	7.8
			Centre	10.6
			Butt	11.0

with and without added phloroglucinol components. Aerobic oxidation of a mixture of 5-methylpyrogallol and 5-methoxy-4-methylresorcinol, and of 5-methylpyrogallol and phloroglucinol, by polyphenoloxidase, led to polymers which showed the characteristic absorption of the polymers of gallo catechin and catechol oxidation. 3-Hydroxy-5-methyl-*o*-benzoquinone and 5-methoxy-4-methylresorcinol or phloroglucinol do not therefore undergo oxidative coupling. Aerobic oxidation of (+)-gallo catechin by oak-cambium polyphenoloxidase proceeds by quinone-polymerization, and affords a product, the tail-to-tail polymer units of which account for the agreement in spectrum of the polymer with those of the catechol polymers including 5-methylpyrogallol polymer (cf. Hathway & Seakins, 1957*a*). A partial-type formula is shown in Fig. 5. The phlobatannin and the polymer obtained by the enzymic oxidation of (+)-gallo catechin have the analytical properties of a tannin (Gnam, 1949; Schmidt, 1955). These polymers are precipitated from solution by gelatin or on refluxing with formalin-hydrochloric acid mixture, and are retained by hide powder (Grassmann, Endisch & Kuntara, 1951). The reaction of the polymer (and of oak-bark phlobatannin) with vanillin (Procter & Paessler, 1901) shows that polymerization does not involve the phloroglucinol residue of the monomer.

It is concluded therefore that the pyrogallol phenols are formed in the leaves, translocated to the cambium and undergo oxidation there, and the resulting phlobatannin (which is a tail-to-tail polymer) is stored in the bark. Confirmation of this is obtained in the distribution of 'tannins' in the stem bark. The results on the distribution of 'tannins' in oak stem bark agree with similar ones relating to the stem bark of *Betula alba* (Jakimoff & Tolski, 1930), *Castanea mollissima* (Clarke, Steiner & Frey, 1942) and *Quercus laevis* (Rogers, Calderwood & Beebe, 1950) (Dicotyledonae), and *Larix*

siberica (Egorov & Gershenzon, 1939), *Pinus echinata* (Snow, 1949), *P. ponderosa* (Kurth & Hubbard, 1951) and *P. radiata* (Anderson, 1952) (Gymnospermae). Field experiments involving girdled trees are in progress to test this supposition. The presence of phlobatannin in the bark and its absence from the heartwood implies that the cambium does not participate in the formation of heartwood extractives, and there is indirect evidence for this. Anderson (1953) found that the heartwood produced in pines, by stimulation of the cambium, did not contain all the phenols of normal heartwood, and Erdtman (1949) found a lignan in wound heartwood, which was different from that of normal spruce heartwood. The presence of gallo catechin and epigallo catechin in the cacao bean (Forsyth, 1952) and tea leaf (Roberts, 1952) indicates that quinone polymerization accounts for phlobatannins which arise during subsequent fermentation. Low polymers derived from epigallo catechin and epigallo catechin gallate have recently been studied in connexion with tea fermentation (Roberts, 1957), and in agreement with the conclusions drawn from the present work, the evidence favours a tail-to-tail constitution. The isolation of mollisacacidin, 7:3':4'-trihydroxyflavan-3:4-diol, from the heartwood (Keppler, 1957), and the detection of this and other leucoanthocyanins in the stem bark of *Acacia mollissima* (Roux, 1957), are of probable significance in relation to the stem bark tannins (Hathway & Seakins, 1957*a*). Related 3:4-dihydroxystilbene glycosides have been isolated by countercurrent extraction and column chromatography on nylon from amongst the cambium extractives of spruce (*Picea excelsa*) (Grassmann, Deffner, Schuster & Pauckner, 1956; Grassmann, Endres, Brockhaus & Merkle, 1957*a*; Grassman, Endres, Pauckner & Mathes, 1957*b*). Since a polyphenoloxidase has also been found in this tissue (W. Grassmann, personal communication), it is possible that the stem bark tannins may be accounted for by the aerobic oxidation through quinone of such dihydroxystilbene aglycones by polyphenoloxidase. The phenolic and phlobatannin extractives of another important tannin material, *Eucalyptus redunca* heartwood, are now being investigated in this Laboratory. The present conclusion concerning the formation of oak-bark phlobatannins connects the formation of these compounds with the plant-browning reaction (Szent-Györgi & Vietorisz, 1931).

SUMMARY

1. (+)-Catechin and (+)-gallo catechin, and leucodelphinidin, were respectively isolated from the ether- and ethyl acetate-soluble fractions of oak-bark phlobatannins. Leucodelphinidin,

Fig. 5

$C_{15}H_{14}O_8 \cdot H_2O$, has been characterized by a hepta-acetate derivative. (+)-Catechin accounts for 1% of the dry weight of young bark, (+)-gallocatechin for up to 1% and leucodelphinidin for 0.5%.

2. Oak-bark phlobatannin, $C_{18}H_{10}O_7 \cdot 2H_2O$, was isolated in considerable yield by cellulose-column chromatography, and exhibits an absorption band at $285 m\mu$ and diminishing general absorption in the region 300–600 $m\mu$.

3. Oak cambium exhibits polyphenoloxidase activity which is inhibited by 4-nitrocatechol. (+)-Gallocatechin was oxidized by this enzyme faster than (+)-catechin and leucodelphinidin.

4. (+)-Gallocatechin and leucodelphinidin oxidation polymers exhibit the same absorption spectrum, have the same analytical properties and similar elementary analyses to those of oak-bark phlobatannin.

5. Aerobic oxidation of mixed synthetic substrates, such as 5-methylpyrogallol and 5-methoxy-4-methylresorcinol, or 5-methylpyrogallol and phloroglucinol, by polyphenoloxidase gave polymers which showed the characteristic absorption of (+)-gallocatechin-oxidation polymer, oak-bark phlobatannin, and 5-methylpyrogallol-oxidation polymer.

6. The conclusion is drawn that phlobatannin is formed by the aerobic oxidation in the cambium of principally (+)-gallocatechin through a tail-to-tail quinone-polymerization mechanism.

7. The increase in tannin concentration of oak stem bark from crown to butt suggests a downward movement of the phenolic metabolites from the leaves through the phloem.

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