Plant Proanthocyanidins. Part 4.[1] Biosynthesis of Procyanidins and Observations on the Metabolism of Cyanidin in Plants

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Evidence based on the results of feeding experiments with labelled (^{3}H,^{14}C) cinnamic acids is presented to show that the various procyanidin dimers are biosynthesised from two metabolically distinct units. One is the flavan-3-ol [(+)-catechin (10) or (−)-epicatechin (9)] and the other is the C-4 carbocation (7) or (8). It is postulated that the carbocations are derived by protonation of the flav-3-en-3-ol (6) and are intermediates in the reduction to the flavan-3-ols (9) and (10). The relationship of procyanidin biosynthesis to anthocyanidin formation in certain plants is briefly discussed.

SEVERAL observations support the view that most higher plants possess the potential to form anthocyanidins under appropriate metabolic conditions.[2] Phytochemical surveys for example show the majority of red to blue colours present in the tissues of some plants are based upon pigments such as pelargonidin, cyanidin (1), and delphinidin. In other plants anthocyanidin pigmentation may only become visible at a particular stage of growth, as for example the development of skin pigmentation in ripening fruit, the transient ' spring flush ' and the autumnal colouration of leaves of deciduous plants in the northern hemisphere. The biosynthesis of the C_6–C_3–C_6 carbon skeleton of the anthocyanidins[3] is related to that of other flavonoids and involves the condensation of a C_6–C_3 [probably cinnamate (2)] unit and three acetate units. The sequence of chemical changes in the C_3 unit which results in the formation of the anthocyanidin structure (or indeed that of the other flavonoids) nevertheless remains poorly defined. Assuming the chalcone–dihydroflavone pair (3) to be key intermediates on the biosynthetic pathway, several mechanistically plausible schemes have been proposed, and Scheme 1, route A, shows one such route based on an original suggestion of Birch.[4] However the continuing uncertainty concerning the steps which lead to the

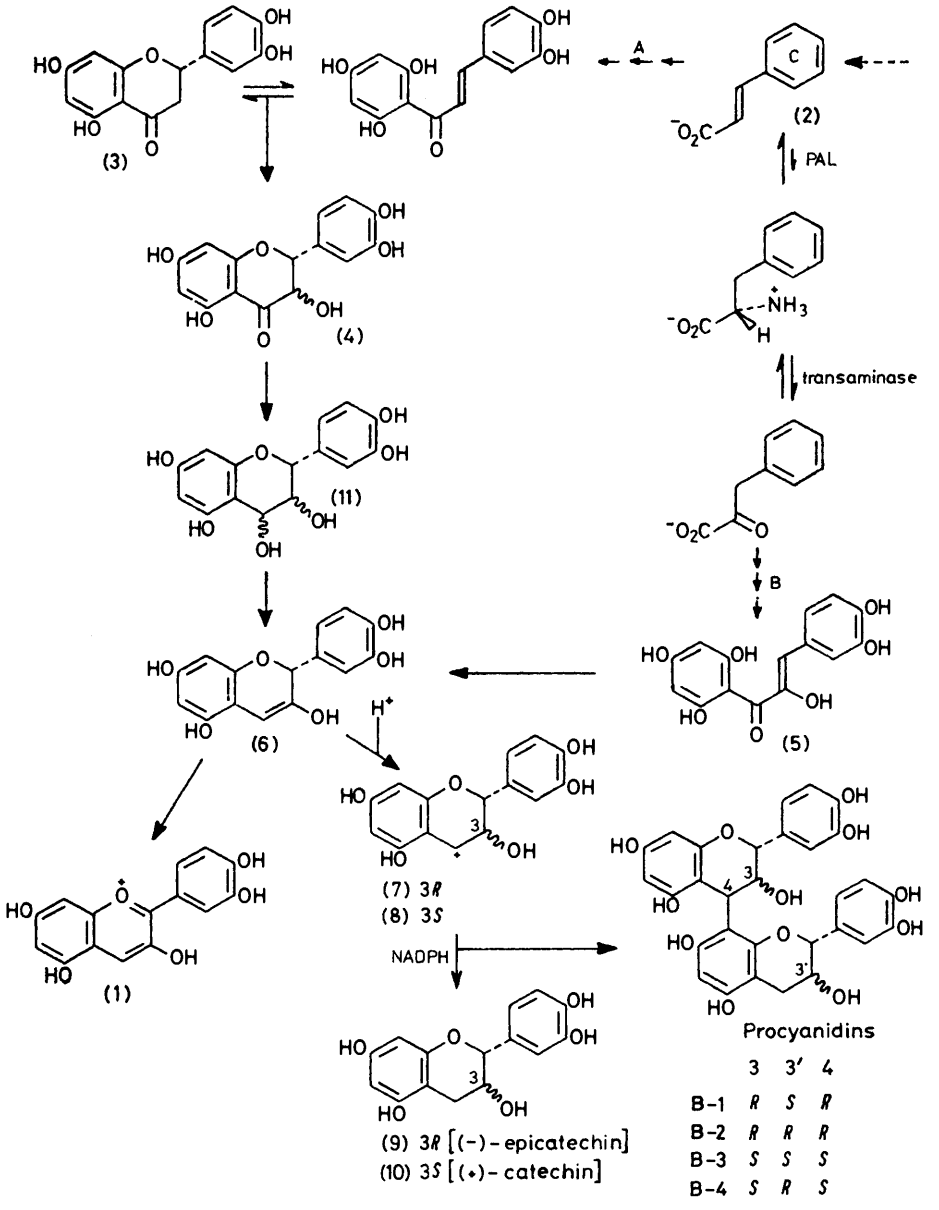
[1] Part 3, A. C. Fletcher, L. J. Porter, E. Haslam, and R. K. Gupta, preceding paper.

[2] J. B. Harborne, ' Comparative Biochemistry of the Flavonoids,' Academic Press, London and New York, 1967, p. 104.

[3] K. Hahlbrock and H. Grisebach, ' The Flavonoids,' eds. J. B. Harborne, T. J. Mabry, and H. Mabry, Chapman and Hall, London, 1975, p. 866.

[4] A. J. Birch, ' Chemical Plant Taxonomy,' ed. T. Swain, Academic Press, London and New York, 1963, p. 148.

3-hydroxy-dihydroflavone (4) has encouraged the belief that this intermediate may be biosynthesised by more than one route and also to the important suggestion that key intermediates may have been overlooked. The discovery by Roux and his colleagues [5] of the previously unrecognised α-hydroxychalcones (5) is one such observation. It is combined in an alternative scheme of biogenesis (Scheme 1, route B) which is based on an idea of Clark-Lewis [6] that a direct pathway to anthocyanidins from α-hydroxychalcones may exist in nature. Steps are embodied in Scheme 1 to lead to the flavan-3-ols (9) and (10), the other principal class of flavonoids which, like the anthocyanidins, lack oxygen at position 4 on the heterocycle. Although a final solution to this problem requires work at the enzyme level, firm circumstantial evidence is presented here which points to the intermediacy in flavan-3-ol, procyanidin, and by implication, cyanidin biosynthesis of the flav-3-en-3-ol (6). Preliminary reports of some of this work have been published.[7,8]

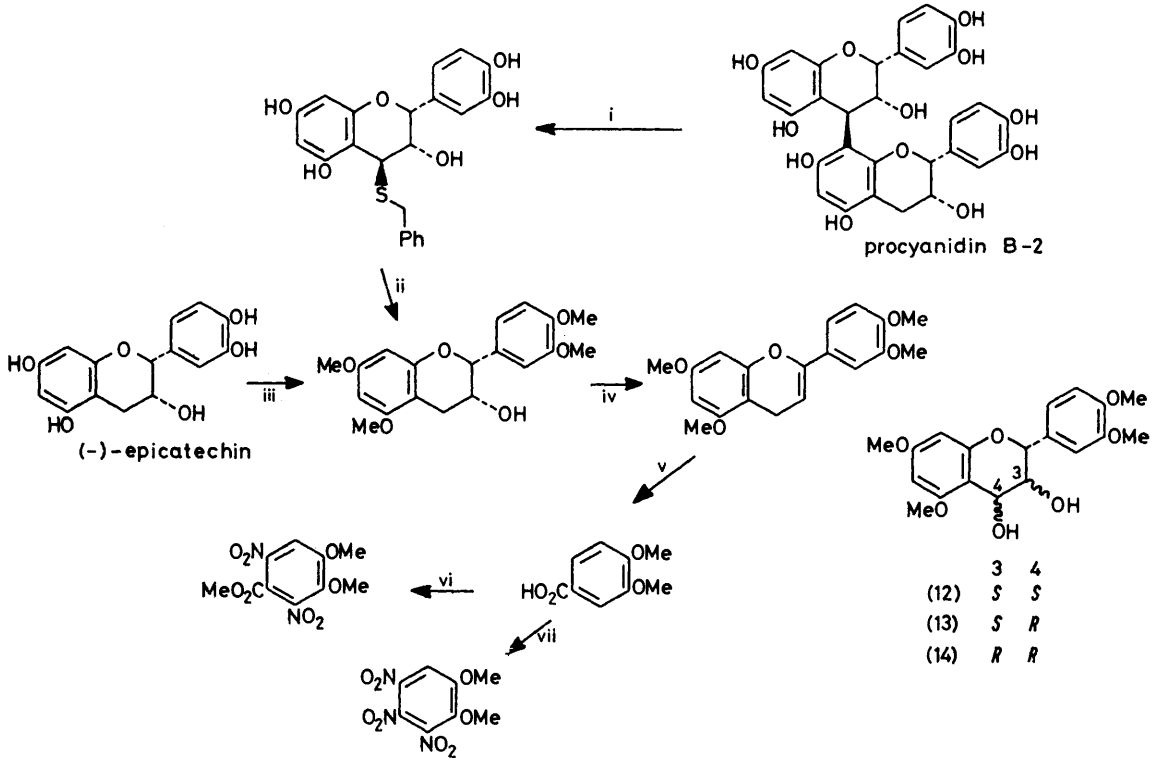


SCHEME 1 Biosynthetic pathways to cyanidin, flavan-3-ols, and procyanidins; (i) route A based on a suggestion by Birch,[4] (ii) route B based on proposals by Clark-Lewis and Skingle [6] and by Roux [5]

Procyanidin dimers of the B type are located in most vegetative tissues of plants with a woody habit of growth

[5] D. G. Roux and D. Ferreira, *Phytochemistry*, 1974, **13**, 2039.
[6] J. W. Clark-Lewis and D. C. Skingle, *Austral. J. Chem.*, 1967, **20**, 2169.
[7] D. Jacques and E. Haslam, *J.C.S. Chem. Comm.*, 1974, 231.
[8] E. Haslam, C. T. Opie, and L. J. Porter, *Phytochemistry*, 1977, **16**, 99.

and they are often concentrated in the skin and seed shells of fruit.[9,10] Invariably one or both of the monomeric flavan-3-ols (+)-catechin (10) and (−)-epicatechin (9) co-occur with the procyanidins. Both the flavan-3-mic acids (labelled separately at C-2 and -3 with ^{14}C and at H-2, -3, and -4′ with ^{3}H) were administered to the plants and harvested 3—7 days later. In experiments with *A* × *carnea* and *A. hippocastanum*, (−)-epicatechin



SCHEME 2 Degradation of (−)-epicatechin and procyanidin B-2; reagents: i, $PhCH_2{\cdot}SH{-}H^+$; ii, CH_2N_2 Raney Ni; iii, CH_2N_2; iv, TsCl–pyridine, piperidine; v, $NaIO_4$–$KMnO_4$; vi, CH_2N_2, HNO_3; vii, HNO_3

ols and procyanidins are unglycosylated. The developing fruit of *Aesculus* × *carnea* and *A. hippocastanum* [procyanidin B-2, proanthocyanidin A-2,[11] and (−)-epicatechin (9)], the young male catkin of *Salix caprea* (9), procyanidin B-2, and proanthocyanidin A-2 were isolated, and both (−)-epicatechin and procyanidin B-2 were degraded to determine the position of radioactive labelling (Scheme 2). A satisfactory mode of chemical

TABLE 1

(E)-[3-^{3}H,^{14}C]Cinnamic acid incorporation into (−)-epicatechin, (+)-catechin, and procyanidins (^{3}H : ^{14}C ratios) *

(*E*)-[3-^{3}H,^{14}C]Cinnamic acid	5.0	12.0	14.0	22.0	10.2	8.8
(−)-Epicatechin (9) [a,b]	4.9 (3.3)	9.8	13.5 (1.0)	18.6 (0.3)	9.2 (0.5)	7.2 (0.5)
Procyanidin-B-2	5.5 (0.3)	11.2	13.9 (0.23)	17.7 (0.04)		6.8 (0.04)
(−)-Epicatechin	4.6 (0.06)		12.4 (0.06)	17.2 (0.01)		
(2*R*,3*S*,4*S*)-4-Benzylthioflavan-3,3′,4′,5,7-pentaol			12.8 (0.17)	17.5 (0.03)		
Proanthocyanidin A-2 [a]	0.97 (0.5)	2.5	3.50 (0.3)	2.9 (0.05)		2.6 (0.06)
Procyanidin B-4 [b]					9.8 (0.3)	
(−)-Epicatechin					8.6 (0.06)	
(2*R*,3*S*,4*RS*)-4-Benzylthioflavan-3,3′,4′,5,7-pentaol					9.2 (0.24)	
(*E*)-[3-^{3}H,^{14}C]Cinnamic acid	12.9	13.1	13.1			
(+)-Catechin (10) [c,d,e]	11.5	10.4	10.6			
Procyanidin B-3 [c,d,e]	10.3	10.2	10.7			

[a] *Aesculus* × *carnea*. [b] *Rubus fructicosus*. [c] *Salix caprea*. [d] *Salix inorata*. [e] *Chamaecyparis lawsoniana*.

* Figures in parentheses show percentage incorporations of radioactivity into the metabolite.

and *S. inorata* [procyanidin B-3 and (+)-catechin (10)] and young shoots of blackberry and raspberry, *Rubus fructicosus* and *R. idaeus* [procyanidin B-4 and (−)-epicatechin (9)], were selected for this work. Cinna-

degradation of proanthocyanidin A-2 has not yet been achieved,[11] and the positions of labelled atoms have been inferred from the structural and the presumed biogenetic relationship of the metabolite to both (−)-epicatechin (9) and procyanidin B-2. Similar arguments were also applied in experiments to determine the bio-

[9] R. S. Thompson, D. Jacques, E. Haslam, and R. J. N. Tanner, *J.C.S. Perkin I*, 1972, 1387.

[10] K. Weinges, W. Kaltenhauser, H.-D. Marx, E. Nader, J. Perner, and D. Seiler, *Annalen*, 1968, **711**, 184.

[11] D. Jacques, E. Haslam, G. R. Bedford, and D. Greatbanks, *J.C.S. Perkin I*, 1974, 2663.

synthesis of procyanidins B-3 and -4 and the associated co-metabolites (+)-catechin (10) and (−)-epicatechin (9), respectively.

Apart from the information obtained from feeding experiments with [3-^{14}C; 2-^{3}H] cinnamic acid (Table 3), the results of various tracer experiments (summarised in Scheme 3 and in Tables 1—3) accord with the accepted patterns of flavanoid and phenol metabolism determined by earlier workers,[3] namely that a C_6–C_3 fragment is combined intact with a C_6 unit to produce the flavan ring system. The incorporation of the cinnamic acid precursor into the particular C_6–C_3 unit of the flavan nucleus necessitates, at some intermediate stage, the hydroxylation of the aryl ring (c) to produce the catechol orientation of phenolic groups. It was anticipated that such a biological transformation would result in the displacement of the 4′-proton to the 3′-position (NIH shift [12]) when the first hydroxy-group is introduced, but loss of a proton at the 3′- or 5′- position when the second hydroxy-group is inserted.[13] The results (Scheme 3, Table 2) fully substantiate this view.

Particularly significant however were the various results which demonstrated (Tables 1 and 2 and Scheme 3) the differential extent of labelling of the two ' halves ' of the procyanidin dimers B-2, -3, and -4 and proanthocyanidin A-2. They are similar in some respects to those obtained in the biosynthesis of other natural products, *e.g.* the monoterpenes [14] and the betalains,[15] and indicate that although the two halves of these flavan dimers are composed of structurally identical monomer units they are nevertheless derived from metabolically distinct intermediates. This evidence thus appears to rule out suggestions made earlier that the procyanidins are derived by dehydrogenation of monomer flavan-3-ols.[9,10] On the basis of chemical analogy and model reactions the idea that the ' upper ' flavan-3-ol units of procyanidin dimers are derived from intermediates such as the carbocations (7) and (8) and that the ' lower ' flavan-3-ol unit is derived from (−)-epicatechin (9) or (+)-catechin (10) now appears a more acceptable hypothesis.

Various workers have suggested [16-18] that the flavan-3,4-diols (11) might give rise to the appropriate carbocation (7) or (8), and attempts have been made to provide evidence in support of this idea. Although in principle the flavan-3,4-diols (11) may be envisaged to be in equilibrium with the carbocations (7) and (8), to date no flavan-3,4-diol corresponding to the 3′,4′,5,7-phenolic hydroxylation pattern has been isolated from the vegetative tissues of a plant.[19,20] ' Cold trap ' experiments with *Salix caprea* have similarly failed to reveal their presence in the tissues of that plant. Thus [3-^{3}H;^{14}C]cinnamic acid was administered to *Salix caprea* catkins and the phenolic metabolites were isolated and methylated (diazomethane). Dilution of the extract in one case with the diol (12) [21] and in the other with the diol (13) [21] gave, after re-extraction and isolation, (12) or

TABLE 2

(*E*)-[2-^{14}C; 3,4′-$^{3}H_2$]cinnamic acid incorporation into (−)-epicatechin and procyanidins [a] *

(*E*)-[2-^{14}C; -4′-^{3}H]Cinnamic acid	6.6	6.6	6.6
(*E*)-[2-^{14}C; -3-^{3}H]Cinnamic acid		7.1	9.0
(−)-Epicatechin (9)	3.20 (0.27)	9.2 (0.25)	10.5 (0.1)
3′,4′,5,7-Tetramethoxyflav-2-ene	3.00	3.00	2.8
Veratric acid	[1.00]	[1.00]	[1.00]
Methyl 2,6-dinitroveratrate	[0.85]	[0.88]	[0.89]
3,4,5-Trinitroveratrole	[0.62]	[0.63]	[0.64]
Procyanidin B-2	3.18 (0.17)	8.5 (0.04)	10.9 (0.03)
Proanthocyanidin A-2	2.59 (0.18)	4.6 (0.13)	5.7 (0.07)

[a] *Aesculus* × *carnea*.

* Figures in parentheses show percentage incorporation of radioactivity; figures in square brackets show tritium content expressed as a fraction.

TABLE 3

(*E*)-[2-^{3}H, 3-^{14}C]cinnamic acid incorporation into (−)-epicatechin, (+)-catechin, and procyanidins

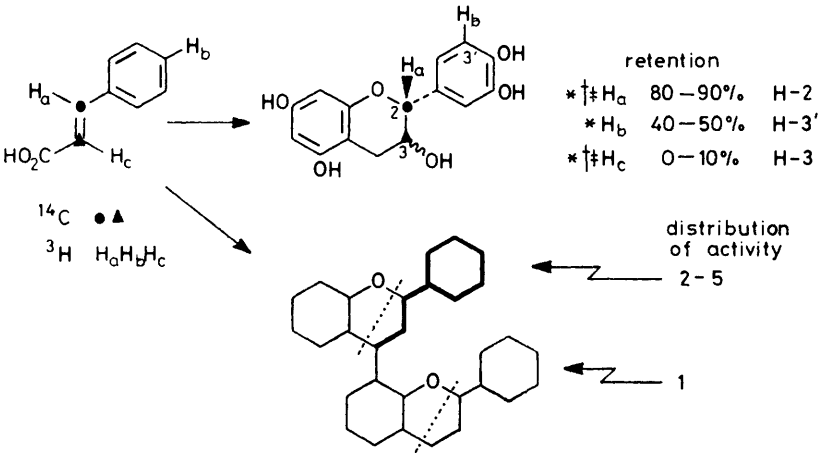
(*E*)-[2-^{3}H; 3-^{14}C]Cinnamic acid	7.1 [a]	7.6 [b]	7.5 [c]	11.8 [d]	12.7 [d]
(−)-Epicatechin (9)				0.3	0.15
(+)-Catechin (10)	0.1	0.2	0.5		
Procyanidin B-2				0.6	0.2
Proanthocyanidin A-2				0.7	0.7
Procyanidin B-3	0.2	0.6	0.1		

[a] *Salix caprea*. [b] *Salix inovata*. [c] *Chamaecyparis lawsoniana*. [d] *Aesculus* × *carnea*.

[12] J. W. Daly, D. M. Jerina, and B. Witkop, *Experientia*, 1972, **28**, 1129.
[13] T. Nagatsu, M. Levitt, and S. Udenfriend, *J. Biol. Chem.*, 1964, **239**, 2910.
[14] D. V. Banthorpe, B. V. Charlwood, and M. J. O. Francis, *Chem. Rev.*, 1972, 115.
[15] T. J. Mabry, A. S. Dreiding, and H. Wyler, *Helv. Chim. Acta*, 1965, **48**, 361.
[16] T. A. Geissman and N. N. Yoshimura, *Tetrahedron Letters*, 1966, 2669.
[17] L. L. Creasey and T. Swain, *Nature*, 1965, **208**, 151.
[18] I. C. du Preez and D. G. Roux, *J. Chem. Soc.* (*C*), 1970, 1800.
[19] K. Weinges, W. Bahr, W. Ebert, K. Göritz, and H.-D. Marx, *Fortschr. chem. org. Naturstoffe*, 1969, **27**, 158.
[20] E. Haslam, ' The Flavonoids,' eds. J. B. Harborne, T. J. Mabry, and H. Mabry, Chapman and Hall, London, 1975, p. 510.
[21] L. Jurd and R. Lundin, *Tetrahedron*, 1968, **24**, 2653.

(13) which showed no incorporation of the radioactively labelled substrates. Analogously in *Aesculus* × *carnea* the results of a ' cold trap ' experiment with the diol (14) [22] were negative after administration of [^{3}H;3-^{14}C]-cinnamic acid and methylation of the extract.

When [2-^{3}H; 3-^{14}C]cinnamic acid was utilised as the labelled precursor with procyanidin-metabolising plants, the various flavan-3-ols [(9) and (10)] and procyanidins (B-2 and -3) which are biosynthesised all showed greater (5—20%) from H-2 of the various flavan units. This observation implies that at some stage the cinnamic acid unit is incorporated into a fragment which permits random exchange of the hydrogen atoms at C-3 on the C_6–C_3 unit. Such an intermediate might be the α-hydroxychalcone (5).[5] This idea merits serious consideration, since (5) could arise if in these experiments the cinnamic acid is in equilibrium with L-phenylalanine (catalysed by L-phenylalanine ammonia lyase [23]) and



SCHEME 3 Radiochemical tracer experiments in flavan-3-ol and procyanidin biosynthesis; summary of results

* *Aesculus* × *carnea*, *A. hippocastanum* {epicatechin, procyanidin B-2, proanthocyanidin A-2
† *Rubus idaeus*, *R. fructicosus* {epicatechin, procyanidin B-4
‡ *Salix caprea*, *S. inorata*, *Chamaecyparis lawsoniana* {catechin, procyanidin B-3

than 90% loss of the tritium label (Table 3). These observations suggest that the biosynthetic pathways to both the flavan-3-ols (9) and (10) and the carbocations (7) and (8) pass through an intermediate which lacks a 3-hydrogen atom on the heterocycle. It is postulated that this intermediate is the flav-3-en-3-ol (6), and that this may partition by stereospecific reduction to either (+)-catechin (10) or (−)-epicatechin (9) and by stereospecific protonation to the carbocation (7) or (8). The formation of the various procyanidins can then be visualised as occurring by reactions between the appropriate flavan-3-ol (9) or (10) and carbocation (7) or (8). Each of these reactions can be reproduced exactly in the laboratory, and they have been discussed in detail elsewhere.[1] If it is assumed that reduction of the flav-3-en-3-ol (6) is a two-step process in which proton addition to C-3 precedes delivery of a hydride ion (or its biological equivalent from, say, NADPH) then the carbocations (7) and (8) may result from a metabolic situation in which the supply of biological reductant is rate-limiting, and they may then ' leak ' from the active site of the enzyme.

A notable feature of the experimental data obtained by feeding [3-^{3}H]cinnamic acid (Scheme 3, Tables 1 and 2) is the small, variable but consistent loss of tritium label hence, by transamination, phenylpyruvic acid and, contrary to the generally accepted view, the latter compound is the true biochemical C_6–C_3 substrate incorporated into the flavan-3-ol pathway (Scheme 1B). Similar results were obtained from the administration of [2-^{14}C; ^{3}H; 3-$^3H_2$]-L-phenylalanine (^{3}H : ^{14}C 10 : 1) to *Aesculus* × *carnea*. The residual tritium label in the isolated metabolites [(−)-epicatechin (9) and procyanidin B-2] was at positions 2 and 2', but the ^{3}H : ^{14}C ratios (1.26 and 1.88, respectively) were again indicative of a non-selective exchange at the same position in the flavan precursor.

Robinson, in his earlier investigations of leucoanthocyanidin (*syn*-procyanidin) chemistry,[24] frequently commented on the possible relation which these compounds might have to anthocyanidin formation in plants. The theory of biosynthesis as outlined does now permit an explanation of the potential of procyanidin-containing plants to form anthocyanidins under appropriate metabolic conditions. The autumnal colouration of leaves, the ripening of fruit, and the transient ' spring flush ' may all occur when the supply of biological reductant is cut off and the intermediate (6) is diverted by oxidation to the distinctive pigmentation.

[22] B. R. Brown, M. J. Betts, and M. R. Shaw, *J. Chem. Soc.* 1969, 1178.

[23] E. A. Havir and K. R. Hanson, *Biochemistry*, 1968, **7**, 1896.

[24] R. Robinson, *Nature*, 1936, **137**, 172.

EXPERIMENTAL

Methods for paper chromatographic analysis and for the isolation of natural procyanidins and preparation of derivatives were as previously described.[9] Radioactive counting was carried out with a Packard Tri-Carb Scintillation counter. Phenols were dissolved in methanol (1 ml) and Unisolve (10 ml), and phenol derivatives were dissolved in Unisolve (10 ml). Standardisation was carried out both externally (instrumentally) and internally. Internal standardisation was achieved by addition of 20 μl of [1-^{14}C]- or [1,2-$^{3}H_2$]-n-hexadecane of known activity. (2*R*,3*S*,4*S*)-3′,4′,5,7-Tetramethoxyflavan-3,4-diol and (2*R*,3*S*,4*R*)-3′,4′,5,7-tetramethoxyflavan-3,4-diol were prepared by reduction of tetra-*O*-methyltaxifolin by the method of Jurd and Lundin;[21] (2*R*,3*R*,4*R*)-3′,4′,5,7-tetramethoxyflavan-3,4-diol was prepared from tetra-*O*-methyl-(−)-epicatechin by the procedure of Brown, Betts, and Shaw.[22]

Benz[^{3}H]*aldehyde*.—Phenylglyoxylic acid (1.23 g), β-amino-oxindole hydrochloride (0.15 g), and tritiated water (1 ml; 1 Ci) were heated under reflux in nitrogen for 1 min. Benzaldehyde (4.13 g) was added and the mixture fractionally distilled. The fraction of b.p. 121—175 °C was distilled onto magnesium sulphate (0.08 g) and redistilled to give benz[^{3}H]aldehyde (2.05 g, 49%; *ca.* 3 μCi mg^{-1}).

(E)-[3-^{3}H; ^{14}C]*Cinnamic Acid*.—Benz[^{3}H]aldehyde (0.085 g), benz[^{14}C]aldehyde (0.014 5 g, 50 μCi), and malonic acid (0.197 4 g) were dissolved in anhydrous pyridine (0.5 ml) containing piperidine (3 drops), and the mixture was refluxed for 24 h. The solution was poured into ice–hydrochloric acid (10N; 0.5 ml). The precipitate was collected and recrystallised from water to give (*E*)-[3-^{3}H; ^{14}C]cinnamic acid (0.075 g, 1.26 μCi mg^{-1}; ^{3}H : ^{13}C 5.0 : 1).

(*E*)-[3-^{3}H]Cinnamic acid (2.45 μCi mg^{-1}) and (*E*)-[3-^{14}C]cinnamic acid (0.97 μCi mg^{-1}) were prepared similarly by the Knoevenagel condensation. (*E*)-[2-^{3}H]Cinnamic acid (*ca.* 10 μCi mg^{-1}) was prepared according to the procedure of Manitto *et al.*[25] from *NN*-dimethyl-DL-phenylalanine.

(*E*)-[2-^{14}C; 4′-^{3}H]*Cinnamic Acid*.—L-Phenylalanine ammonia lyase was isolated from sliced potato tubers (394 g) according to the procedure of Havir and Hanson.[26] The final enzyme solution was obtained in borate buffer (20 ml; 0.1M; pH 9.0; *ca.* 0.300 units) and to this solution were added L-phenylalanine (33 mg), [2-^{14}C]-DL-phenylalanine (5 mg; 50 μCi), and [4′-^{3}H]-L-phenylalanine (5 mg; 250 μCi). The whole was kept at 37 °C for 3 days. Cinnamic acid (67 mg) was added to the solution, which was acidified (5N-hydrochloric acid) and continuously extracted with ether (96 h). Removal of the ether and crystallisation from water gave the cinnamic acid (44 mg, 50%; ^{3}H : ^{14}C 6.6 : 1; *ca.* 0.5 μCi mg^{-1} in ^{14}C).

Feeding Experiments.—(i) Freshly cut flowering stems of *Aesculus* × *carnea* (25—40 g wet weight) with the fruit just ' set ' were immersed in a solution (5 ml) of the cinnamic acid substrate (10—20 mg; 20—150 μCi) in water and containing sufficient sodium hydrogen carbonate to neutralise the acid. When the solution was adsorbed (12—24 h) the cut stems were transferred to water and kept at room temperature for 3—7 days. The plant material was then macerated in a high speed liquidiser with methanol (3 × 100 ml), the plant debris removed by filtration, and the combined methanolic solution [after extraction with light petroleum (b.p. 60—80 °C) (2 × 100 ml)] evaporated to dryness. The residual gum was dissolved in water (75 ml) and extracted with ethyl acetate (6 × 75 ml). Removal of the organic solvent left a gum which was diluted with (−)-epicatechin, procyanidin B-2, and proanthocyanidin A-2 (100 mg each), dissolved in ethanol (5 ml), and chromatographed on a Sephadex LH-20 column (3.5 × 30 cm; 180 × 10 ml fractions). Fractions 5—25 gave, after crystallisation from water, (−)-epicatechin (125 mg), which was converted into the tetramethyl ether (85 mg), m.p. and mixed m.p. 153—154 °C. Fractions 60—90 gave procyanidin B-2 (85 mg), which was either further degraded (*see later*) or converted into the octamethyl ether (52 mg). Fractions 110—160 gave, after crystallisation from acetone–water, proanthocyanidin A-2 (110 mg), which was converted into the nona-acetate (100 mg), m.p. and mixed m.p. 156—157 °C.

(ii) Cut shoots of *Rubus fructicosus* (July; 20 g wet weight) were immersed in solutions of the labelled cinnamic acid (5—10 mg; 10—75 μCi). After metabolism (*ca.* 7 days) (−)-epicatechin and procyanidin B-4 were isolated as described previously [9] and above. Part of the procyanidin B-4 was converted into its deca-acetate, m.p. 171—172 °C, for counting.

(iii) Cut stems of *Salix caprea*, just prior to the emergence of young male catkins from the bud (November—January; *ca.* 20 g wet weight), were treated as above. After metabolism (7 days), (+)-catechin and procyanidin B-3 were isolated as previously described.[9]

Degradation of (−)-*Epicatechin*.—(i) 3′,4′,5,7-*Tetramethoxyflav-2-ene*.(−)-Epicatechin (225 mg) with diazomethane gave 3′,4′,5,7-tetramethyl-(−)-epicatechin (260 mg, 75%), m.p. 153—154 °C, which was treated with toluene-*p*-sulphonyl chloride (147 mg) in anhydrous pyridine (50 ml) at 100 °C for 40 min exactly. The product was dissolved in chloroform (20 ml) and the solution washed with 2N-hydrochloric acid (10 ml) and water (10 ml) and subjected to t.l.c. (silica; 0.2% methanol–chloroform; R_F 0.5), to give after crystallisation (chloroform–methanol) the toluene-*p*-sulphonate ester, m.p. 174—177 °C (lit.,[27] 165—168 °C) (102 mg, 27%). The tosylate dissolved in anhydrous piperidine (5 ml) was refluxed (18 h) under nitrogen. The solvent was removed at 0.05 mmHg, and the solid washed with chloroform (2 × 10 ml); preparative t.l.c. of the chloroform extract (silica; 0.2% methanol–chloroform; R_F 0.8), followed by crystallisation from anhydrous ethanol, gave the flav-2-ene (57 mg, 84%) as fine needles, m.p. 120—121 °C (lit.,[27] 119 °C).

(ii) *Veratric acid*. The radioactive flav-2-ene (50 mg) and inactive flav-2-ene (150 mg) were added to a solution of sodium periodate (950 mg) and potassium carbonate (400 mg) in t-butyl alcohol–water (10 ml; 1 : 1 v/v). Potassium permanganate solution (0.8%) was added to the refluxing solution over 2 h (sufficient to maintain a permanent pink coloration). The solution was then cooled and sodium hydrogen sulphite solution added to destroy the pink colour, followed by acid (50% sulphuric acid; 20 ml, at 0 °C). The resultant mixture was continuously extracted with ether (24 h). Evaporation of the extract and crystal-

[25] P. Manitto, D. Monti, P. Gramatica, and E. Sabbioni, *J.C.S. Chem. Comm.*, 1973, 563.

[26] E. A. Havir and K. R. Hanson, *Methods Enzym.*, 1970, **17**, 575.

[27] K. Freudenberg, H. Fitkentscher, and M. Harder, *Annalen*, 1925, **441**, 157.

lisation from water (charcoal) gave veratric acid (120 mg), m.p. and mixed m.p. 181 °C.[28]

Veratric acid was converted into 3,4,5-trinitroveratrole, methyl veratrate, methyl 6-nitroveratrate, and methyl 2,6-dinitroveratrate as previously described.[29]

Degradation of Procyanidin B-2.—Procyanidin B-2 (87 mg; *ca.* 2 000 disint. min^{-1} mg^{-1}) was dissolved in ethanol (3 ml) containing toluene-α-thiol (0.75 ml) and glacial acetic acid (0.5 ml) and refluxed under nitrogen for 24 h. (−)-Epicatechin (100 mg) and (2*R*,3*S*,4*S*)-4-benzylthioflavan-3,3′,4′,5,7-pentaol (100 mg) were added, and the volatile solvents were removed at 30 °C. The residual oil was applied to a Sephadex LH-20 column [2.0 × 55 cm; propan-1-ol–chloroform (1 : 4); 300 fractions of 8 ml]. Fractions 125—160 gave (2*R*,3*S*,4*S*)-4-benzylthioflavan-3,3′,4′,5,7-pentaol, which was converted [9] into its tetramethyl ether (80 mg), m.p. 57—58 °C. Fractions 210—260 gave (−)-epicatechin, which was converted into its tetramethyl ether (78 mg), m.p. 153—154 °C. This was degraded as above after dilution with inactive ether (180 mg).

(2*R*,3*S*,4*S*)-4-Benzylthio-3′,3′,4′,5,7-pentamethoxyflavan (80 mg) was dissolved in ethanol (2 ml) and treated with Raney nickel (ethanolic slurry; 7 ml) at room temperature for 24 h. Removal of the catalyst and solvent and preparative t.l.c. gave tetramethyl-(−)-epicatechin (30 mg), m.p. 152—153 °C. This was diluted with inactive material (120 mg) and degraded as above.

Procyanidin B-4 and procyanidin B-3 were degraded in analogous fashion [9] by using toluene- α-thiol and acetic acid.

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