Biochem. J. (1969) **113**, 537
Printed in Great Britain

Phenol Biosynthesis in Higher Plants

GALLIC ACID

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(*Received* 10 *January* 1969)

The biosynthesis of gallic acid in a number of higher plants was investigated by using L-[U-^{14}C]phenylalanine, (−)-[G-^{14}C]shikimic acid, D-[1-^{14}C]glucose and D-[6-^{14}C]glucose as tracers. The results are compared with those obtained similarly for caffeic acid and are interpreted in terms of the dehydrogenation of 5-dehydroshikimic acid as a normal route of metabolism for gallic acid.

Phenols are one of the major groups of secondary metabolites in plants (Bate-Smith, 1962). Little is known, however, of the factors that control phenol synthesis in plants, and in consequence the possible function of phenols in plants has caused much speculation. Among the commoner phenols in plants are the phenolic acids and their derivatives. Hydroxy- and methoxy-benzoic acids have a limited distribution (Neish, El-Basyouni, Chen, Ibrahim & Towers, 1964), but pre-eminent among them is gallic acid (XII in Scheme 2), which is normally encountered in plant tissues in ester form (Haslam & Haworth, 1964). Closely related to gallic acid, and most probably derived biogenetically from it by oxidative coupling (Schmidt, 1956), is hexahydroxydiphenic acid, which forms an integral structural unit of the ellagitannins. By contrast the hydroxycinnamic acids, i.e. *o*-coumaric acid, *p*-coumaric acid, caffeic acid (IX), ferulic acid and sinapic acid, are almost ubiquitous in their occurrence as esters with glucose or quinic acid or as glycosides (Harborne & Corner, 1961). Significantly 3,4,5-trihydroxycinnamic acid has not been found in Nature, and Bate-Smith (1962) has suggested that ellagic acid, the dilactone of hexahydroxydiphenic acid, is the systematic taxonomic equivalent of the missing acid. In view of the possible biological origin of ellagic acid it would seem more logical to regard gallic acid as the systematic equivalent. Support for this idea comes from subsequent surveys showing gallic acid to be metabolized in plants where the steady-state concentration of hydroxycinnamic acids is either small or zero, e.g. *Acer*, *Bergenia*, *Rhus* and *Quercus* species (Haslam, 1965, and unpublished work).

The present work describes studies aimed at the elucidation of the route of biosynthesis of gallic acid and its relation to that of the hydroxycinnamic acids and to primary routes of metabolism in higher plants. A preliminary account (Dewick & Haslam, 1968) of the work has been published.

EXPERIMENTAL

Paper chromatography was carried out with Whatman no. 2 or 3MM paper in solvent systems *A* [aq. 6% (v/v) acetic acid] and *B* [butan-2-ol–acetic acid–water (14:1:5, by vol.)]. Gallic acid [R_F values 0·50 (solvent *A*) and 0·60 (solvent *B*)] and myricetin [R_F values 0·0 (solvent *A*) and 0·35 (solvent *B*)] were detected by their violet and yellow fluorescence respectively in u.v. light. Quinic acid [R_F values 0·95 (solvent *A*) and 0·45 (solvent *B*)] and shikimic acid [R_F values 0·95 (solvent *A*) and 0·60 (solvent *B*)] were not detectable under u.v. light but were located by a spray of aq. sodium metaperiodate followed by an aq.-ethanol solution of piperazine and sodium nitroprusside (Cartwright & Roberts, 1955). After the paper had been heated at 100° for 10min. both acids appeared as yellow spots on a white background. Caffeic acid was normally present as caffeoyl esters, which showed a blue fluorescence, changing to green (on exposure to NH_3 vapour), under u.v. light.

[1,6-$^{14}C_2$]Shikimic acid was purchased from Calbiochem, Los Angeles, Calif., U.S.A. All other radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks. Radioactive samples were dissolved in methanol or dioxan (1–2ml.) and the volume was adjusted to 10ml. with a toluene scintillator solution [4g. of 2,5-diphenyloxazole and 0·3g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l. of toluene]. Samples were counted, until 20000 counts above background had accumulated, with a Packard Tri-Carb 3003 liquid-scintillation spectrometer. Efficiencies were measured by internal standardization after the addition of 0·02ml. of [1-^{14}C]*n*-hexadecane standard.

Feeding techniques. Leaf discs or shoots (5g.) were placed in contact with an aqueous solution of the substrate (10–15ml., 0·001–0·3mole, 5–50μc) and placed in a weak draught. Distilled water was added as required during the incubation period.

Isolation of metabolites. The plant material was crushed with powdered glass and extracted with water (2×50ml.), which was filtered through iron-free cellulose. The latter

was washed with water (4 × 10 ml.) and the combined filtrates were acidified (18 M-H_2SO_4, 2 drops) and extracted with ethyl acetate (10 × 100 ml.). Removal of the organic solvent gave the crude phenolic extract. The aqueous solution remaining was treated separately.

(*a*) Gallic acid. The crude extract, dissolved in acetone, (1 ml.) was treated with tannase (4 ml.) (Haslam & Stangroom, 1966) in 0·5 M-sodium acetate buffer, pH 6·0 (3 ml.), for 48 hr. The solution was extracted with ethyl acetate (10 × 10 ml.) and the extract separated by chromatography (on Whatman 3MM paper). Spots corresponding to gallic acid were eluted with water (4 × 100 ml.), and the eluates were evaporated to dryness. Gallic acid (500 mg.) was added to the extract and the product was crystallized from water to constant specific radioactivity.

(*b*) Caffeic acid. Caffeoyl esters located on the chromatogram were eluted with water (4 × 50 ml.) as above, diluted with caffeic acid (150 mg.) and heated under N_2 with saturated aq. $NaHCO_3$ solution for 2 hr. After acidification with 2 M-HCl the mixture gave, on cooling, caffeic acid (100 mg.), which was converted by methylation with dimethyl sulphate into dimethylcaffeic acid (see below). The latter was crystallized from aq. acetic acid to constant specific radioactivity.

(*c*) Myricetin. The crude extract in acetone (5 ml.) plus M-HCl (5 ml.) was heated at 90° for 1 hr. and then extracted with ethyl acetate (10 × 10 ml.). The extract was separated and myricetin was extracted with ethanol. The myricetin was degraded to trimethylgallic acid (see below) which was crystallized to constant specific radioactivity from water.

(*d*) Shikimic acid and quinic acid. The aqueous extract was separated by solvent system *B* and bands with R_F values corresponding to those of shikimic acid and quinic acid were eluted with water (100 ml.). The acids were crystallized to constant specific radioactivity from ethanol and then from acetic acid.

Bromopicrin degradation. The substrate (methyl trimethyl-2-nitrogallate, methyl 6-nitroveratrate, or methyl 2,6-dinitroveratrate, 500 mg.) was heated under reflux in acetic acid (4 ml.) and 48% (w/v) HBr (4 ml.) for 3 hr. and then evaporated to dryness. The bromopicrin degradation was then carried out by the method of Birch, Moye, Rickards & Vanek (1962) to yield methylamine hydrochloride, which was heated under reflux for 1 hr. with phthalic anhydride (100 mg.) and sodium acetate (35 mg.) in acetic acid (2 ml.). Water (2 ml.) was added and the solution was boiled for 1 min. and concentrated to 1 ml. Dilution to 5 ml. gave *N*-methylphthalimide (50–100 mg.), m.p. and mixed m.p. 132–134°.

Degradation procedures. (*a*) Gallic acid. The acid was converted into trimethylgallic acid, trimethyl-5-nitropyrogallol and methyl trimethyl-2-nitrogallate by standard procedures (Harding, 1911; Bogert & Plaut, 1915).

(*b*) Myricetin. The flavonol was degraded to trimethylgallic acid by the procedure of Neish, Underhill & Watkin (1957).

(*c*) Caffeic acid. Caffeic acid was degraded by the standard conversion into dimethylcaffeic acid, veratric acid, methyl 6-nitroveratrate, methyl 2,6-dinitroveratrate and 3,4,5-trinitroveratrole (Neish *et al.* 1957; Zincke & Francke, 1896).

(*d*) Shikimic acid and quinic acid. Platinum-catalysed oxidation followed by treatment with acid gave protocatechuic acid (Heyns & Gottschalk, 1961; Davis & Salaman, 1953), which was converted into veratric acid and treated as above.

3,4,5-Trinitroveratrole. Veratric acid (30 mg.) was heated at 100° for 30 min. with fuming nitric acid (1 ml.). The precipitate formed on dilution with water (1 ml.) was crystallized from ethanol to give 3,4,5-trinitroveratrole, m.p. and mixed m.p. 144–145°.

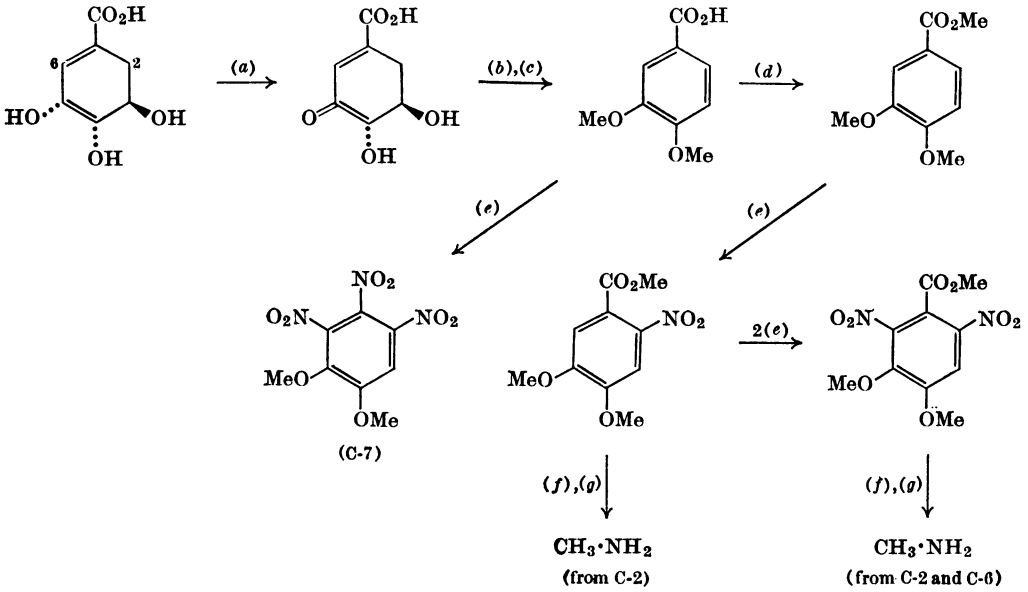
Methyl 6-nitroveratrate. Methyl 6-nitroveratrate (420 mg.) was heated at 100° with fuming nitric acid (5 ml.) for 10 min., then poured into water (40 ml.). The product was crystallized from aqueous methanol to give methyl 2,6-dinitroveratrate (330 mg.), m.p. and mixed m.p. 130–132°.

RESULTS AND DISCUSSION

The concentration of galloyl esters in the leaf tissue of *Rhus typhina* and *Acer saccharinum* was measured during May to October of a growing season by using methods described by Haslam (1965). Fully mature leaves (based on weight and dimensions) contained an approximately constant concentration of galloyl esters from May to September, but a rapid decrease was observed during October before senescence. Very young leaves of both plant species contained smaller amounts of phenols. However, the concentration of phenols/g. of dry or wet leaf tissue was much greater in the very young leaf than in the mature leaf. When D-[U-^{14}C]glucose was administered to *R. typhina* more radioactivity was incorporated into gallic acid in the young tissue than in the mature. These observations suggest ready synthesis of phenols in rapidly developing tissue and a minimal rate of synthesis coupled with a low turnover or transport from the cell in mature tissue, and led to the use in all experiments of very young tissue.

[G-^{14}C]Shikimic acid was obtained by the growth of young shoots of *Ginkgo biloba* in $^{14}CO_2$ under constant illumination in a modification of the method of Weinstein, Porter & Laurencot (1959). The pattern of radioactive labelling was established by two independent procedures. The first was that described by Sprinson (1961) and the second involved a novel conversion into veratric acid, which was subsequently degraded as shown in Scheme 1 to give a measure of the radioactivities at C-2, C-6 and C-7. Somewhat surprisingly (−)-shikimic acid isolated by this method was not uniformly labelled; the carboxyl group always contained approximately one-quarter of the radioactivity.

Gallic acid was isolated from plant tissues after hydrolysis with tannase (Haslam & Stangroom, 1966). Radioactivity at C-7 was determined by nitration of trimethylgallic acid (Harding, 1911), and the combined radioactivity at C-2 and C-6 by successive treatment of methyl trimethyl-2-nitrogallate (Bogert & Plaut, 1915) with hydrogen bromide and barium hypobromite to give bromopicrin. The latter was isolated as *N*-methylphthal-



Scheme 1. Degradation of (−)-shikimic acid. (*a*) Platinum–O_2 (Heyns & Gottschalk, 1961); (*b*) H_3O^+ (Davis & Salaman, 1953); (*c*) dimethyl sulphate–OH^-; (*d*) CH_2N_2; (*e*) HNO_3–acetic acid (Zincke & Francke, 1896); (*f*) HBr–acetic acid, followed by $Ba(OH)_2$–Br_2; (*g*) Zn–acetic acid (Birch *et al.* 1962).

Table 1. *Biosynthesis of gallic acid*

(−)-[G-^{14}C]Shikimic acid contained 0·25 of the radioactivity in the carboxyl group (C-7) and 0·75 in the ring (C-1–C-6).

Species	Substrate	Time of administration (hr.)	Fraction of radioactivity in gallic acid		Incorporation (%)
			C-7	C-1–C-6	
A. saccharinum	(−)-[G-^{14}C]Shikimic acid	33	0·23	0·77	0·4
A. saccharinum	(−)-[G-^{14}C]Shikimic acid, buffered to pH 6	74	0·25	0·75	0·3
R. typhina	(−)-[G-^{14}C]Shikimic acid	65	0·25	0·75	0·85
R. typhina	L-[U-^{14}C]Phenylalanine	93	0·17	0·83	0·03

imide after reduction to methylamine (Birch *et al.* 1962). Caffeic acid was oxidized, after methylation, to give veratric acid, which was degraded as shown in Scheme 1.

The pathway L-phenylalanine → cinnamic acid → *p*-coumaric acid → caffeic acid has been established as a general one for the biosynthesis of the hydroxycinnamic acids (Neish, 1968). A key enzyme in the process is L-phenylalanine ammonia-lyase (EC 4.3.1.5; Hanson & Havir, 1968*a*,*b*), which catalyses the loss of ammonia from L-phenylalanine to give *trans*-cinnamic acid and thus diverts the amino acid from protein to phenylpropanoid biosynthesis. Aromatic hydroxylation (Mason, 1965) then leads to the various hydroxycinnamic acids. Two pathways have been proposed for the biosynthesis of gallic acid. The first (Zenk, 1964), in which the carboxyl group of gallic acid is derived from the β-carbon atom of L-phenylalanine, postulates an extension of hydroxycinnamic acid biosynthesis to 3,4,5,-trihydroxycinnamic acid (X in Scheme 2) followed by β-oxidation, and is similar to other established pathways of $C_6 \cdot C_3 \rightarrow C_6 \cdot C_1$ conversions (Billek & Schmook, 1966). The second proposed route is a direct dehydrogenation of 5-dehydroshikimic acid (V) (Haslam, Haworth & Knowles, 1961; Cornthwaite, & Haslam 1965). Additional evidence for the operation of both of these pathways was obtained in *R. typhina* and *A. saccharinum* after the administration of L-[U-^{14}C]phenylalanine and [G-^{14}C]-shikimic acid (Table 1). A significant observation was, however, made when [G-^{14}C]shikimic acid was

administered to *R. typhina* and it was shown that under these conditions little correlation existed between the biosynthesis of gallic acid and 3,4,5-trihydroxycinnamic acid. The flavonol myricetin (XI) was isolated and degradation of it (by methylation and oxidation to trimethylgallic acid) showed no radioactivity at C-2 (the carboxyl group of the derived trimethylgallic acid). This result is consistent with the accepted pathways of flavonol biosynthesis in which (−)-shikimic acid is converted via L-phenylalanine into the corresponding 3,4,5-trihydroxycinnamic acid, with concomitant loss of the carboxyl group of (−)-shikimic acid, before incorporation into the C_{15} flavonol structure (Grisebach, 1958).

The use of the tracer technique to study metabolic pathways in plants suffers from a number of recognized drawbacks. To establish whether the routes to gallic acid from L-phenylalanine and (−)-shikimic acid are normal or induced metabolic pathways, the isotopic labelling pattern in gallic acid after the administration of D-[1-^{14}C]glucose and D-[6-^{14}C]glucose was investigated. The biosynthesis of (−)-shikimic acid from specifically labelled glucose precursors has been established by Sprinson and his colleagues (Sprinson, 1961) in *Escherichia coli*. From these observations D-glucose labelled with radioactive carbon at C-1 or C-6 would be expected to give labelling predominantly at C-2 and C-6 in (−)-shikimic acid, with negligible incorporation in C-7, the carboxyl group. These predictions were borne out in experiments with plant systems and analogous results were obtained in *R. typhina* and *G. biloba*, such as to suggest similar, if

Table 2. *Biosynthesis of quinic acid (IV) and shikimic acid (VI) in higher plants*

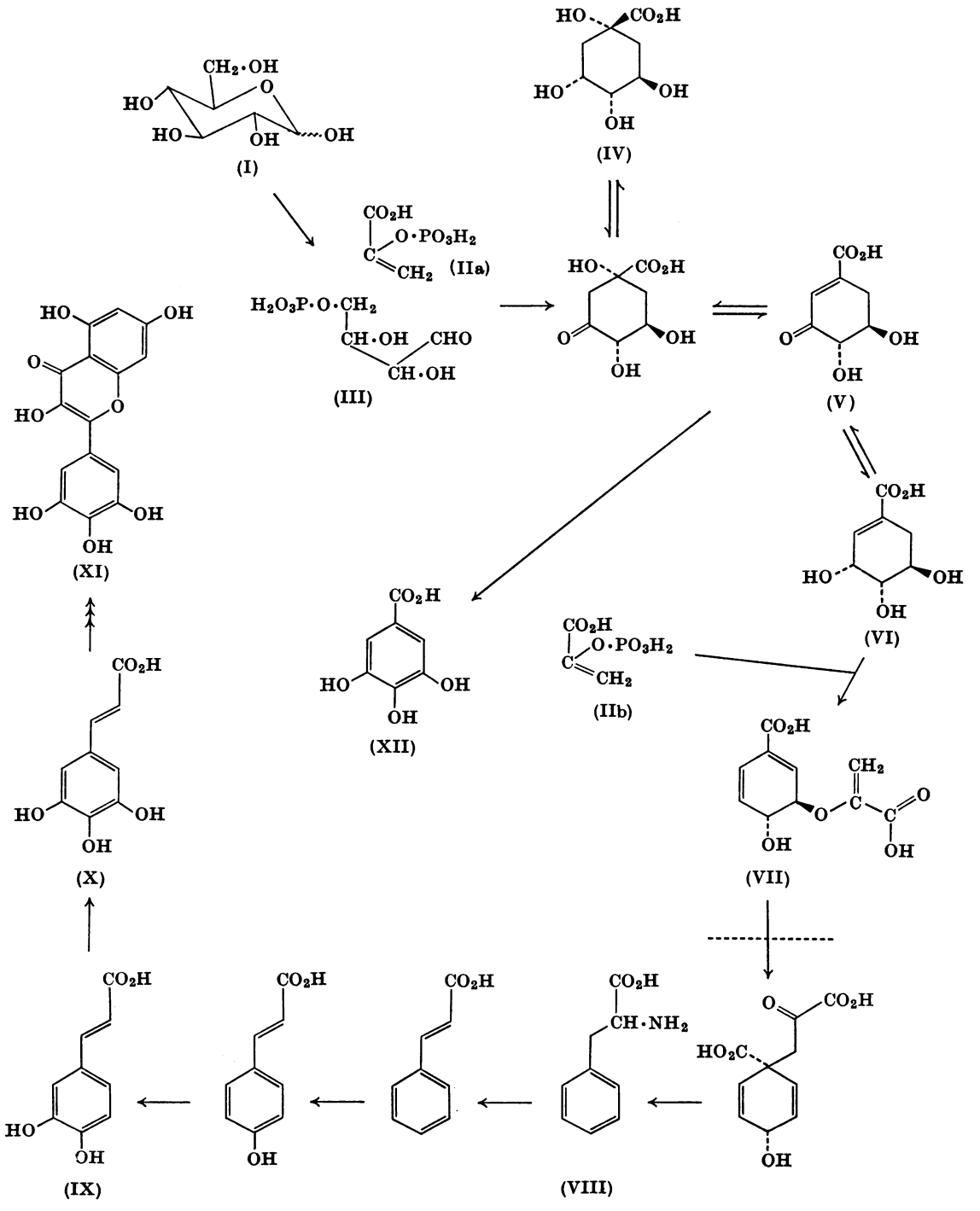
Species	Metabolite	Substrate	Time of administration (hr.)	Fraction of radioactivity in metabolite			Incorporation (%)
				C-7	C-2	C-2+C-6	
R. typhina	Shikimic acid	D-[1-^{14}C]Glucose	56	0·08	0·37	0·74	0·1
G. biloba	Shikimic acid	D-[1-^{14}C]Glucose	70	0·05	0·36	0·84	0·01
G. biloba	Quinic acid	D-[1-^{14}C]Glucose	70	0·08	0·35	0·82	0·08
G. biloba	Shikimic acid	D-[6-^{14}C]Glucose	70	0·06	—	—	0·01

Table 3. *Biosynthesis of gallic acid from D-glucose*

Species	Substrate	Time of administration (hr.)	Fraction of radioactivity in gallic acid		Incorporation (%)
			C-7	C-2+C-6	
A. saccharinum	D-[1-^{14}C]Glucose	24	0·03	—	0·1
A. saccharinum	D-[1-^{14}C]Glucose	70	0·08	0·74	0·02
R. typhina	D-[1-^{14}C]Glucose	56	0·08	0·68	0·02
Acer pseudoplatanus	D-[1-^{14}C]Glucose	72	0·1	0·56	0·01
Bergenia crassifolia	D-[1-^{14}C]Glucose	48	0·03	0·75	0·03
A. pseudoplatanus	D-[6-^{14}C]Glucose	48	0·02	—	0·01

Table 4. *Biosynthesis of caffeic acid in higher plants*

Species	Substrate	Time of administration (hr.)	Fraction of radioactivity in caffeic acid			Incorporation (%)
			C-8 and C-9	C-7	C-1–C-6	
Nicotiana tabacum	D-[1-^{14}C]Glucose	36	0·77	0·23	—	0·01
A. pseudoplatanus	D-[6-^{14}C]Glucose	48	0·41	0·41	0·18	0·005
N. tabacum	(−)-[1,6-^{14}C]-Shikimic acid	90	0·90	0·10	—	0·02
N. tabacum	D-[U-^{14}C]Ribose	100	0·92	0·08	—	0·05
N. tabacum	D-[U-^{14}C]Ribose	125	0·87	0·12	0·02	0·05
Vaccinium vitis-idaea	$^{14}CO_2$	0·25	0·57	0·35	0·08	



Scheme 2. Biosynthesis of gallic acid.

not identical, pathways of D-glucose metabolism in higher plants (Table 2).

Incorporation of radioactivity from carbohydrate precursors into phenolic plant metabolites was invariably low. This may be due to a number of factors such as the difficulty of penetration of the substrate to the site of synthesis or its diversion into other pathways of carbohydrate metabolism. It may nevertheless be an accurate reflection of the low rate of phenol synthesis in plant tissues. Gallic acid produced from D-[1-^{14}C]glucose and D-[6-^{14}C]-glucose shows (Table 3) a pattern of labelling consistent with a normal mode of derivation from (−)-shikimic acid (presumably by dehydrogenation of 5-dehydroshikimic acid) and not from L-phenylalanine via the hydroxycinnamic acids. Thus generally less than 10% of the radioactive label was located in the carboxyl group but up to 75% resided at C-2 and C-6 (combined) in the aromatic ring. In addition, (−)-shikimic acid isolated in the experiment with *R. typhina* showed a very similar pattern of radioactive labelling to that of the gallic acid (Tables 2 and 3). Conversely in *A. pseudoplatanus* caffeic acid isolated concurrently with gallic acid showed a different pattern of radioactive labelling after metabolism of D-[6-^{14}C]glucose (Tables 3 and 4).

These observations on the biosynthesis of gallic acid from carbohydrate precursors should also be considered in the light of further experiments on the biosynthesis of caffeic acid in other plant systems. Although in other respects the biosynthesis of the hydroxycinnamic acids has been thoroughly examined, little information has been obtained on their formation from carbohydrate precursors (Neish, 1968). Our results (Table 4) point to a ready entry of the carbohydrate substrate into only one of the carbohydrate precursors, namely the second molecule of phosphoenolpyruvate (II*b*) that contributes the C_3 side chain of the $C_6 \cdot C_3$ skeleton of the acid (Scheme 2). One rationalization of these observations would be that in plants metabolizing the hydroxycinnamic acids there exists a considerable pool of (−)-shikimic acid. In support of this view is the fact that [1,6-^{14}C]shikimic acid administered as a metabolite is degraded before incorporation into caffeic acid (Table 4).

The consensus of the observations described suggests that during normal modes of metabolism the carbon skeleton and hydroxyl groups of gallic acid are derived directly from shikimate, probably via compound (V) (Scheme 2). The situations examined are those in which a steady state of phenol synthesis obtains and in which, by analogy with mechanisms observed in micro-organisms, the synthesis of L-phenylalanine may be controlled by feedback inhibition at either of two points in the shikimic acid pathway (Cohen, 1965). In gallic acid metabolism, control may be exerted at the chorismate mutase step and intermediates before chorismate will then accumulate. Labelled precursors will then be incorporated into compounds (III) and (II*a*) and the phenol will be produced as a by-product of the pathway. To some extent the situation is analogous to the formation of protocatechuic acid by a mutant strain of *Neurospora crassa* blocked in the conversion of 5-dehydroshikimic acid into shikimic acid (Scheme 2, V → VI) (Gross & Tatum, 1959).

Alternatively in plants producing gallic acid there are frequently no hydroxycinnamic acids in the system and in such cases the cell may not possess the ability to form the enzyme L-phenylalanine ammonia-lyase. Preliminary observations with the root of *Bergenia* species support this suggestion (E. Haslam, unpublished work). Gallic acid would then again be formed as a by-product after the build-up of intermediates to L-phenylalanine.

The authors thank the Science Research Council for a studentship (to P.M.D.) and the Royal Society for a grant for the purchase of radiochemicals.

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