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THE FORMATION OF 7-OXYGENATED COUMARINS IN HYDRANGEA AND LAVENDER[1]

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Abstract—Tracer experiments involving the feeding of ^{14}C-labelled *cis-p*-coumaric acid derivatives to intact *Hydrangea macrophylla* and *Lavandula* (Munstead) plants have provided strong evidence against the oxidative cyclization theories proposed for the biosynthesis of 7-oxygenated coumarins. In contrast, *trans-p*-coumaric acid and its β-D-glucoside were very well incorporated into umbelliferone in *Hydrangea* while *trans-o*-coumaric acid and its β-D-glucoside were very poorly incorporated. Evidence was obtained that "free" umbelliferone present in the plant extracts is almost entirely an artefact. The primary site of synthesis of umbelliferone appears to be the leaves.

INTRODUCTION

AS EARLY as 1942, Haworth made the suggestion that *p*-coumaric acid was a general precursor for coumarin synthesis in plants.[2] Verification of this proposal in the cases of umbelliferone (7-hydroxycoumarin) and herniarin (7-methoxycoumarin) has only been possible since the development of isotopic tracer techniques. Haworth was apparently troubled by the necessity of a mechanicistically unfavourable attack *meta* to a phenol to establish the lactone ring, and suggested an initial attack of a hydroxyl moiety *para* to the phenolic group to yield the dienone (II). Lactonization onto the β-position of the dienone and subsequent dehydration of lactone (III) would restore aromaticity and produce the coumarin. A more recent elaboration of this concept proposed a direct oxidative cyclization of *cis-p*-coumaric acid (I) to give a spirolactone (IV) which might rearrange to the 7-oxygenated coumarin system (V) (Fig. 1).[3] Demonstration of the feasibility of this scheme *in vitro* was taken as strong support for such spirolactones being intermediates in the formation of natural oxygenated coumarins.[4] However, rearrangement of the spirolactone (IV) with aqueous acid produces almost entirely 6-hydroxycoumarin[4] while treatment with base produces the related spiro hydroxy acid and some 6-hydroxycoumarin.[5] It is noteworthy that no 6-mono-oxygenated coumarin is known in nature.[6] Tracer studies with ^{18}O on the mould coumarin novobiocin, also favoured an oxidative cyclization step in the formation of the lactone ring, either via a spirolactone or a direct carboxyl attack.[7]

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[1] Part of this work has been reported in a preliminary communication: D. J. AUSTIN and M. B. MEYERS, *Tetrahedron Letters* 765 (1964).

[2] R. D. HAWORTH, *J. Chem. Soc.* 448 (1942).

[3] H. GRISEBACH and W. D. OLLIS, *Experientia* 17, 4 (1961); A. I. SCOTT, *Proc. Chem. Soc.* 207 (1962).

[4] A. I. SCOTT, P. A. DODSON, F. A. MCCAPRA and M. B. MEYERS, *J. Amer. Chem. Soc.* 85, 3702 (1963).

[5] M. B. MEYERS, *Proc. Chem. Soc.* 243 (1963).

[6] W. KARRER, *Konstitution und Vorkommen der Organischen Pflanzenstoffe* pp. 531–564. Birkhäuser, Basel (1958).

[7] C. A. BUNTON, G. W. KENNER, M. J. T. ROBINSON and B. R. WEBSTER, *Tetrahedron* 19, 1001 (1963).

Phenols are not predominantly *ortho-para* directing towards certain free radical hydroxylating reagents such as the metal ion-ascorbic acid system.[8] Since it is recognized that enzymic aromatic hydroxylation is possibly free radical in nature,[9] it would seem that the difficulties of hydroxylation *meta* to the phenol group of *p*-coumaric acid may well have been overestimated. In fact, *cis-p*-coumaric acid has been hydroxylated *in vitro* exclusively in the *meta*-position. This has been accomplished by treatment of the *cis*-acid with molecular oxygen in the presence of a copper (I and II)-ascorbic acid system in dilute aqueous solution (pH 4–6) at room temperature.[5,10] Many phenol oxidases and laccases contain copper which is essential for activity and also require ascorbic acid as a co-factor.[11]

For coumarins with either no oxygen substituents or more than one, oxidative cyclization does not appear to be necessary. In the accepted scheme for the biosynthesis of coumarin itself, *ortho*-hydroxylation of *trans*-cinnamic acid is followed by glucosidation. After isomerization of the side-chain double bond, the glucosyl group is removed and the lactone ring forms spontaneously.[12] *p*-Coumaric acid is not an intermediate in this pathway nor can umbelliferone be converted to coumarin. 6,7-Dihydroxycoumarin forms spontaneously

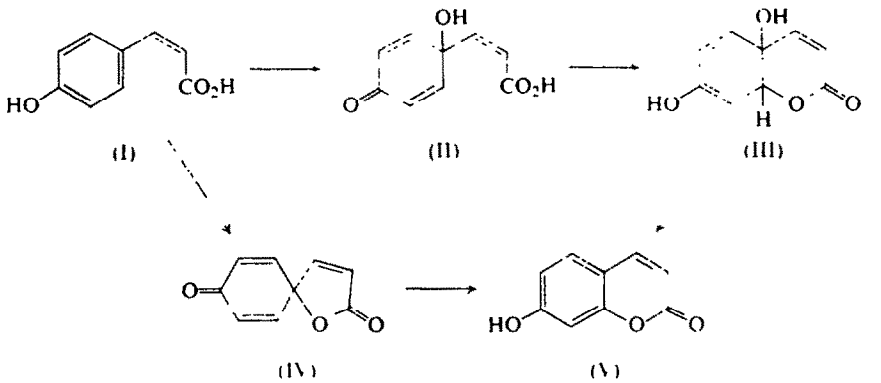

FIG. 1. PROPOSED OXIDATIVE CYCLIZATION MECHANISMS.

from *trans*-3,4-dihydroxycinnamic acid *in vitro* on treatment of the acid with manganous ions and oxygen in daylight.[13]

It would therefore seem unnecessary on chemical or biological grounds to produce the effect of *meta* hydroxylation by an initial attack *para* to a phenol group by hydroxyl or carboxyl groups.

RESULTS AND DISCUSSION

Since Brown had already established that *p*-coumaric acid is an effective precursor for herniarin in Lavender,[14] this plant was chosen for a preliminary examination of the oxidative

[8] R. O. C. NORMAN and G. K. RADDA, *Proc. Chem. Soc.* 138 (1962); J. H. GREEN, B. J. RALPH and P. J. SCHOFIELD, *Nature* **198**, 754 (1963).

[9] D. H. BÜHLER and H. S. MASON, *Arch. Biochem. Biophys.* **92**, 424 (1961).

[10] M. B. MEYERS, Unpublished results.

[11] S. BOUCHILLOUX, *Plant Phenolics and their Industrial Significance*, p. 1. Plant Phenolics Group of North America (1963).

[12] (*a*) T. KOSUGE and E. E. CONN, *J. Biol. Chem.* **236**, 1617 (1961), (*b*) J. R. STOKER and D. M. BELLIS, *J. Biol. Chem.* **237**, 2303 (1962), (*c*) S. A. BROWN, *Can. J. Biochem. Physiol.* **40**, 607 (1962).

[13] W. L. BUTLER and H. W. SIEGELMAN, *Nature* **183**, 1813 (1959); C. F. VAN SUMERE, F. PARMENTIER and M. VAN POUCKE, *Naturwiss.* **46**, 668 (1959).

[14] S. A. BROWN, *Phytochem.* **2**, 137 (1963).

cyclization hypotheses. The COOH-^{14}C-labelled compounds were administered by root feeding to *Lavandula* (Munstead). The results (average of three feedings) show that the relative incorporation of *trans-p*-coumaric acid, *cis-p*-coumaric acid and the spirolactone (IV) were in the ratio 5:2·5:1. In contrast to Brown's findings, the absolute incorporation of the *trans* acid was very low (~ 0·02 per cent) and therefore the difference in incorporation could not be accepted as definitive.

Some difficulty was experienced in separating herniarin from coumarin which accompanies it in *Lavandula* but the required purification was achieved by treatment of the mixture with boiling hydrobromic acid to demethylate herniarin, followed by fractional sublimation of the umbelliferone-coumarin mixture. The umbelliferone ultimately derived from the spirolactone feeding was converted to umbellic acid and the acid decarboxylated. The evolved CO_2 contained over 90 per cent of the recovered activity demonstrating that no appreciable randomization of the label had taken place. The plants fed with spirolactone wilted and exhibited leaf necrosis, and the observed incorporation of spirolactone into

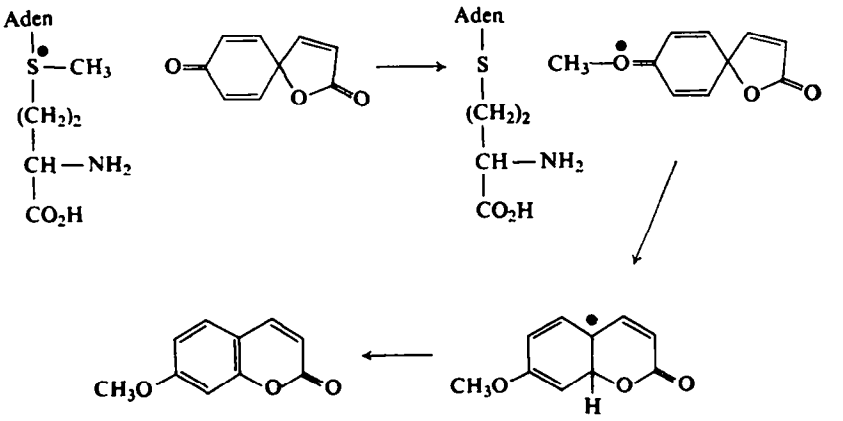

FIG. 2. POSSIBLE DETOXICATION OF SPIROLACTONE (ADEN = ADENOSYL).

hernariarin may well be a detoxication mechanism assisted by the presence of enzymic methylating systems[15] (Fig. 2). Scott *et al.* have shown that the transitory spirolactone derived from *p*-methoxyphloretic acid rearranges under quite mild conditions to a coumarin.[4]

The main studies were carried out on the biosynthesis of umbelliferone in *Hydrangea macrophylla* ser. (var. 'Blue Wave').[6] Billek and Kindl[16] have established that *trans-p*-coumaric acid is a "good" precursor for umbelliferone in hydrangea and this has recently been confirmed by Brown, Towers and Chen.[17]

The one-year-old plants were fed via wicks inserted through the stems with aqueous solutions of β-^{14}C-labelled *trans-p*-coumaric acid, *trans-p*-D-glucosyloxycinnamic acid, *cis-p*-coumaric acid, *trans*-cinnamic acid, spirolactone (IV), *trans-o*-coumaric acid, and *trans-o*-D-glucosyloxycinnamic acid; the acids being administered as their sodium salts. After a suitable period the stems and leaves were extracted and, following hydrolysis with

[15] H. T. WILLIAMS, pp. 358–359, 561–562. *Detoxication Mechanisms*, Chapman and Hall, London (1959).
[16] G. BILLEK and H. KINDL, *Monats.* 93, 85 (1962).
[17] S. A. BROWN, G. H. N. TOWERS and D. CHEN, *Phytochem.* 3, 469 (1964). (We thank Dr. S. A. Brown for making this manuscript available to us before its publication.)

emulsin, the umbelliferone was recovered and purified by thin-layer chromatography, fractional sublimation and recrystallization. The results are summarized in Table 1.

TABLE 1. CONVERSION OF β-^{14}C-LABELLED COMPOUNDS TO UMBELLIFERONE BY *Hydrangea macrophylla* (VAR. BLUE WAVE)

Compound fed (cpm/mmole × 10 ^{6})	Cpm fed × 10 ^{4}	% Incorporation (Dilution) 1 day	3 days	5 days
trans-p-Coumaric acid (1·60)	1·97	4·0 (53)	4·2 (57)	4·9 (51)
		2·6 (49)	2·7 (41)	5·1 (35)
trans-p-Glucosyloxycinnamic acid (2·17)	2·73	2·5 (85)	4·1 (41)	3·2 (29)
cis-p-Coumaric acid (1·60)	1·95	0·49 (319)	0·33 (112)	0·65 (217)
		—	0·26 (778)	1·16 (72)
Spirolactone (IV) (1·19)	1·40	0·062 (1140)	0·094 (2430)	0·146 (1470)
trans-Cinnamic acid (1·82)	2·35	0·24 (119)	—	0·33 (118)
o-Coumaric acid (1·08)	1·39	0·10 (850)	0·12 (802)	0·14 (804)
trans-o-Glucosyloxycinnamic acid (8·26)	9·75	0·025 (3850)	0·025 (4900)	0·024 (5000)

Trans-p-coumaric acid and its glucoside are readily incorporated into umbelliferone with almost equal efficiency. This may be explained in terms of either rapid hydrolysis of the glucoside by an endogenous glucosidase or an equally fast glycosylation of the aglycone. The latter explanation is perhaps more consistent with the fact that phenols and aromatic acids are known to be rapidly converted into glucosides and glucose esters when fed to plants [12a, 18] and is also in agreement with the finding that 7-glucosyloxycoumarin (skimmin) is the predominant "bound" form of umbelliferone in *Hydrangea macrophylla* (see following paper) and *Skimmia japonica*.[19] A third possibility is that the *o*-hydroxylating enzyme is not sensitive to glycosylation at the *para* position.

Cis-p-coumaric acid was only about one-seventh as efficient as the *trans* acid. This is in direct contradiction to the requirements of the oxidative cyclization theories which involve a *cis*-cinnamic precursor for the coumarin system. The increase in the incorporation of the *cis-p*-coumaric acid at the end of 5 days is possibly due to its isomerization to the *trans* form.

The spirolactone (IV) was utilized poorly for the synthesis of umbelliferone being less than one-fortieth as efficient as *trans-p*-coumaric acid. Since *p*-coumaric acid isolated from the plants fed with this precursor was appreciably radioactive (Table 2) the spirolactone may

TABLE 2. INCORPORATION OF RADIOACTIVITY INTO *p*-COUMARIC ACID IN HYDRANGEA

β-^{14}C-labelled compound fed	% Incorporation after 1 day	3 days	5 days
trans-p-Coumaric acid	8·1	—	12·4
	18·9	6·9	12·5
trans-p-Glucosyloxycinnamic acid	7·4	1·3	11·2
cis-p-Coumaric acid	8·9	15·8	19·2
	12·9	13·2	22·4
Spirolactone IV	6·7	3·6	5·9

[18] J. B. HARBORNE and J. J. CORNER, *Biochem. J.* **81**, 242 (1961).
[19] E. SPÄTH and O. NEUFELD, *Rec. Trav. Chim.* **57**, 535 (1938).

not undergo direct rearrangement to umbelliferone as postulated in the Grisebach-Ollis theory,[3] but rather hydrogenolysis to *cis-p*-coumaric acid. The combined evidence of the hydrangea and lavender feedings certainly seems to rule out the spirolactone as an active intermediate in 7-oxygenated coumarin biosynthesis.

It has been suggested that a phosphorylated dienone intermediate (Fig. 3) may be involved in 7-oxygenated coumarin biosynthesis,[4] from *trans-p*-oxygenated coumaric acids. While this hypothesis is not inconsistent with the results reported in this paper, the fact that *meta*-hydroxylation of *p*-coumaric acid can occur would appear to make it unnecessary.

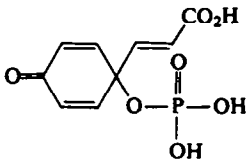

FIG. 3. POSTULATED PHOSPHORYLATED DIENONE INTERMEDIATE.

The incorporation of *trans*-cinnamic acid into umbelliferone suggests that the synthesis of *p*-coumaric acid in hydrangea is accomplished by deamination of phenylalanine and subsequent *para*-hydroxylation of the resulting *trans*-cinnamic acid. This route is an alternative to the direct deamination of tyrosine which is apparently carried out only in grasses.[20]

The very small incorporation of *trans-o*-coumaric acid and the virtual non-incorporation of its β-D-glucoside are in accord with Brown's findings for herniarin biosynthesis in lavender[14] and tend to confirm that *para* hydroxylation of *trans*-cinnamic acid precedes *ortho*. There is, however, a significant discrepancy between the results reported here and those given by Brown, Towers and Chen.[17] They found that both *o*-coumaric and *p*-coumaric acids are equally efficient precursors for umbelliferone when fed to cut shoots of *Hydrangea macrophylla*. This difference may be ascribed to variation in the utilization of *o*-coumaric acid between varieties of *H. macrophylla*. In any event, the *para*-hydroxylation of *o*-coumaric acid must be considered to be unlikely as a natural pathway since *o*-coumaric acid has not been detected in hydrangea extracts.[21]

TABLE 3. SHORT VS. LONG TERM INCORPORATION OF β-^{14}C-*trans-p*-COUMARIC ACID INTO UMBELLIFERONE IN HYDRANGEA

	1 day	21 days
Incorporation (Dilution) (%)	0·92 (44)	2·85 (36)
Dose (μ moles)	30	31
Activity (cpm/mmole × 10^{-6})	1·11	1·60
Fresh weight of fed stems and leaves (g)	8·0	11·5
Incorporation into *p*-coumaric acid (%)	12	2·1

The results of a comparative feeding experiment are given in Table 3. The rise in the incorporation of *trans-p*-coumaric acid into umbelliferone after 21 days shows that the "bound" hydroxycoumarin, once formed, is resistant towards further metabolism in

[20] S. A. BROWN, D. WRIGHT and A. C. NEISH, *Can. J. Biochem. Physiol.* 37, 25 (1959).
[21] R. K. IBRAHIM, Ph.D. Thesis, Department of Botany, McGill University, 1961.

marked contrast to coumarin itself which is rapidly metabolized to melilotic acid and other compounds.[12]

A comparison of the incorporations of *trans-p*-coumaric acid at two dose rates (12 and 30 μM) demonstrates that the absolute incorporation falls on increasing the dose rate. This may be taken as an indication of saturation of the hydroxylation enzymes since the actual *quantity* of *trans-p*-coumaric acid transformed to umbelliferone is quite similar ($\sim$ 0·05 mg). These experiments serve to point out the danger in comparing the incorporations of compounds at non-identical dose rates.

The results given in Table 4 strongly support the idea that essentially *all* of the umbelliferone present in hydrangea is in a "bound" form and the isolable "free" hydroxycoumarin is an artefact of the isolation procedure. The presence of a significant pool of "free" umbelliferone in the plant previous to feeding would cause a much larger dilution of activity in the isolated "free" material. The observed slightly higher dilution in the "free" umbelliferone isolated after the feeding of *trans-p*-coumaric acid is an indication that the production of the "bound" form must take place *before* the formation of any "free" umbelliferone.

TABLE 4. INCORPORATION OF *cis* AND *trans*-β-^{14}C-*p*-COUMARIC ACIDS INTO FREE AND BOUND UMBELLIFERONE AFTER 1 DAY IN HYDRANGEA

	Compound fed (cpm/mmole × 10 ^{6})			
	trans-p-coumaric acid (1·11)		*cis-p*-coumaric acid (2·30)	
Dose (μ moles)	30		18·5	
	Free	Bound	Free	Bound
Incorporation (Dilution) %	0·11 (53)	0·81 (41)	0·033 (368)	0·33 (381)
Ratio by weight of umbelliferone recovered	15	85	9	91
Ratio of radioactivity recovered	12	88	9	91

The direct hydroxylation of *cis*-cinnamic acid in *Melilotus albu* to yield coumarin without the formation of an intermediate "bound" form is not paralleled in hydrangea since, after feeding *cis-p*-coumaric acid, the "free" umbelliferone is not significantly more active than the "bound" (Table 4). This observation can be rationalized by assuming that the *cis* acid must isomerise to the *trans* form before it can take part in the biosynthesis of umbelliferone. Alternatively, *para*-glycosylation of the *cis* acid may occur before hydroxylation *ortho* to the side chain with a concomitant immediate lactonization. The fact that 7-glucosyloxy-coumarin is the predominant "bound" form of umbelliferone in hydrangea does not permit an unequivocal distinction between these and cognate proposals.

The observation that phenylalanine deaminase produces exclusively *trans*-cinnamic acid[22] combined with the apparent reluctance of *cis-p*-coumaric acid to undergo hydroxylation *ortho* to the side chain makes it unlikely that the *cis*-acid is a natural precursor for the 7-oxygenated coumarins or even that it exists in hydrangea under normal conditions.

The data in Table 5 represents an attempt to determine the sites of umbelliferone synthesis and storage. It is clear that the leaves are, on a weight for weight basis, the most active site of synthesis. It can be readily envisaged that rapid translocation of the "bound" form of umbelliferone can account for the observed radioactivity in the stems and roots. These

[22] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).

conclusions accord with those of Haskins and Gorz[23] who adduced evidence that the primary site of coumarin synthesis in *Melilotus alba* is the growing leaves and that the stems and roots are relatively unimportant as synthetic sites. The concentration of umbelliferone is highest by far in the roots and it is reasonable to assume that, after synthesis in the leaves, the "bound" umbelliferone is deposited in the roots.

TABLE 5. INCORPORATION OF β-^{14}C-*trans*-*p*-COUMARIC ACID* INTO UMBELLIFERONE IN VARIOUS PARTS OF HYDRANGEA AFTER 1 DAY

	Leaves	Stems	Roots
Fresh weight (g.)	1·27	4·31	16·03
Umbelliferone isolated (mg.)	0·44	2·07	16·78
% Incorporation (Dilution)	0·58 (36)	0·66 (146)	0·05 (18,300)

* Activity $2{\cdot}30 \times 10^6$ cpm/mmole; dose 12 μM to a single stem.

EXPERIMENTAL

Cultivation of Plants

One-year-old *Lavandula officinalis* Chaix (var. Munstead) plants were donated by the Glasgow Botanical Gardens. These had been grown in soil under natural light in a greenhouse. One-year-old *Hydrangea macrophylla* Ser. (var. Blue Wave) plants were obtained commercially. These were repotted in "Peralite" and fed with a modified Hoagland nutrient media. The plants were grown under fluorescent illumination using a 16-hr "day". The temperature was kept between 17 and 22°. After about two months under these conditions the actively growing plants were suitable for feeding experiments.

Administration of Labelled Compounds

The lavender plants in an immediate pre-flowering stage (average weight about 8 g) were washed and the roots immersed in a solution of *trans*-^{14}C-*p*-coumaric acid [^{14}COOH] (10 mg) and sodium bicarbonate (5·2 mg) in water (10 ml). The solution (shielded from light) was absorbed in 12 hr and an additional 10 ml of water was added, which was also absorbed in a 12-hr period. The plants were then repotted in earth and allowed to metabolize for 6 days. Solutions of *cis*-1^{14}C-*p*-coumaric acid [^{14}COOH] spirolactone [^{14}CO] IV were similarly fed with the exception that no sodium bicarbonate was added when making up the spirolactone solution.

In feeding the hydrangea plants, a leafy stem (average weight between 4 and 6 g) was pierced with a cotton wick dipping into a tube containing 6·1 μM of the labelled compound and an equimolar amount of sodium bicarbonate in water (0·1 ml). No sodium bicarbonate was added to the solution of spirolactone IV. For each plant, two such stems were fed. The absorption of the solution was complete in 3–4 hr and then an additional 0·1 ml of water was added and allowed to be absorbed.

Preparation of Labelled Compounds

Trans COOH-^{14}C and α-^{14}C-*p*-coumaric acid were prepared by a modification of previously reported procedures.[24] Malonic acid (100 mg) was added to an opened vial of sodium

[23] H. J. GORZ and F. A. HASKINS, *Crop Sci.* **2**, 255 (1962).
[24] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. Physiol.* **34**, 769 (1956).

malonate containing 0·1 mc of ^{14}C (about 3 mg). Water (0·5 ml) was added and the solution allowed to stand for one week at room temperature, then transferred to the reaction flask and evaporated to dryness. To the residue was added *p*-hydroxybenzaldehyde (110 mg) followed by pyridine (3 ml) and piperidine (3 drops). The reaction mixture was heated on the steam bath for 2 hr, then cooled and concentrated hydrochloric acid added until the solution was acid, followed by water (50 ml). The aqueous solution was extracted with ether and the ether extracts after washing with brine extracted with aqueous 5% $NaHCO_3$. The bicarbonate solution was reacidified and extracted with ether. Evaporation of the ether produced *trans-p*-coumaric acid, m.p. 208–211°, in about 80 per cent yield. This material was usually further diluted to 300–400 mg with inactive acid.

Cis-p-coumaric acid (COOH or α-^{14}C labelled) was produced by irradiating ethanolic solutions of *trans-p*-coumaric acid in a quartz flask for 24 hr with a mercury vapour lamp. The residue obtained after evaporation of the solvent was dissolved in boiling water (about 10 ml for each gram of residue) and then allowed to stand in the refrigerator overnight. After filtering off the precipitated *trans*-acid, the aqueous solution was thoroughly extracted with ether. Evaporation of the ether gave *cis-p*-coumaric acid, m.p. 127–128° (lit.[25] 126–127°) in about 30–35 per cent yield. By recycling the recovered *trans-p*-coumaric acid several times, more than 85 per cent of starting *trans*-acid could be converted into the *cis* form.

Trans-α-^{14}C-*p*-glucosyloxycinnamic acid was prepared by the following procedure. *Trans*-α-^{14}C-*p*-coumaric acid (100 mg) was dissolved in methanol (25 ml) and after the addition of 3 drops of concentrated sulphuric acid, the mixture was heated under reflux for 4 hr. The solution was poured into water (100 ml) and methyl ester isolated by extraction with ether. The ether extracts, after washing with 5% $NaCO_3$, were evaporated to give methyl *p*-coumarate, m.p. 127–134° (lit.[26] 137°) in quantitative yield. The methyl ester (107 mg), acetobromglucose (675 mg) and silver oxide (500 mg) were suspended in quinoline (2 ml). The mixture was shaken for 30 min, then allowed to stand overnight. After trituration with 25% aqueous acetic acid, the mixture was poured into ethanol (100 ml). Hydrochloric acid was added until the solution was slightly acid and the resulting silver salts were removed by filtration. Evaporation of the filtrate produced methyl tetraacetylglucosyloxycinnamate, m.p. 158–60° (lit.[27] 160–161°). This material was taken up in methanol (25 ml) and saturated aqueous barium hydroxide solution (12 ml) added. After shaking for 18 hr, the solution was adjusted to pH 4 with sulphuric acid. The barium sulphate was removed by centrifugation and the filtrate evaporated. The residue was crystallized from methanol to produce *trans*-α-^{14}C-*p*-β-D-glucosyloxycinnamic acid, 42 mg, m.p. 190–193·5 (lit.[27] 194–195°).

Trans-α-^{14}C-cinnamic acid[28] and *trans*-α-^{14}C-*o*-β-D-glucosyloxycinnamic acid[14] were prepared by methods reported in the literature.

α-^{14}C-*o*-Coumaric acid was prepared by treating an aqueous solution of *trans*-α-^{14}C-*o*-glucosyloxycinnamic acid (12 mg) with emulsin (10 mg) and incubation of the mixture for 3 days at 30°. The *o*-coumaric acid was isolated by ether extraction and diluted with inactive acid (12 mg), then it was dissolved in ethanol and treated with charcoal. Evaporation of the solvent produced α-^{14}C-*o*-coumaric acid, m.p. 210–212° (lit.[29] 215°).

To synthesize the spirolactone IV (2- or 3-^{14}C-labelled), *cis*-^{14}C-*p*-coumaric acid (140

[25] W. A. ROTH and R. STOERMER, *Ber.* **46**, 268 (1913).
[26] T. ZINCKE and F. LEISSE, *Ann.* **322**, 224 (1902).
[27] F. MAUTHNER, *J. Prakt. Chem.* **97**, 222 (1918).
[28] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. Physiol.* **33**, 948 (1955).
[29] S. A. BROWN, G. H. N. TOWERS and D. WRIGHT, *Can. J. Biochem. Physiol* **38**, 143 (1960).

mg) and ammonium chloride (20 mg) were dissolved in warm water (250 ml). The solution was then subjected to electrolysis between platinum electrodes (3 × 4 cm) at 0·2 A and 60–70 V for 8–9 hr, the cathode being protected with a cellophane bag. After cooling, a few millilitres of saturated aqueous sodium bicarbonate solution was added and the solution extracted with ethyl acetate. The organic extracts were washed with brine and evaporated to give an oily residue which was distilled at 120° (0·01 mm Hg). By seeding an ether solution of the distillate, the spirolactone was obtained as stout needles, m.p. 115–116° (lit.[4] 116°) in about 5 per cent yield.

Isolation and Purification Procedures

Lavender. At the completion of the metabolic period 3 plants were washed, weighed and blended in ethanol (600 ml), the mixture heated for 4 hr under reflux. The hot solution was filtered and the residue washed with hot 80% ethanol (500 ml). The filtrate and washings were combined and evaporated to about 100 ml. Cold water (40 ml) was added and after being allowed to stand overnight at 0°, the solution was filtered through celite. The filtrate was reduced in bulk to 80 ml and subjected to constant extraction with ether. This ether extract was found to contain only small amounts of coumarins and was not employed for counting purposes. Emulsin (80 mg) was added to the aqueous residue and the solution allowed to stand at room temperature for 3 days, then re-extracted with ether. Evaporation of the ether gave a residue which was applied as a band on a 1-mm thick Kieselgel G thin layer chromatoplate and eluted with chloroform. A band which contained a mixture of herniarin and coumarin was extracted with methanol and the extracts sublimed and replated in the same manner. The sublimate isolated from the second chromatogram was placed in constant boiling hydrobromic acid (20 ml) and the mixture heated under reflux for 1 hr. The residue obtained after diluting the acid solution with water and then extracting it with ether was sublimed at 95° (0·02 mm Hg) to remove coumarin. The residue was then sublimed at 120–160° (0·02 mm Hg) to produce umbelliferone (about 5–6 mg), m.p. 226·5–229°.

Hydrangea. At the completion of the metabolic period the plant stems were cut below the point of feeding, weighed, and blended in boiling 80% ethanol. The suspension was heated under reflux for 2 hr then filtered through celite. The celite was washed with two portions of hot 80% ethanol (50 ml) and the combined filtrates reduced to about 50 ml under vacuum. Emulsin (50 mg) in water (2 ml) was added and the mixture incubated at 30° for 2 days. After addition of sodium bicarbonate (0·6 g) and heating on the steam bath for 5 min, the mixture was filtered through celite or glass fibre paper. The filtrate was subjected to constant ether extraction for 4 hr. Evaporation of the ether extracts gave a residue which was applied as a band on 0·5 mm thick Kieselgel G chromatoplates. Elution with ethyl acetate-chloroform (1:4) produced a sharp umbelliferone-containing band which was excised and placed overnight in methanol. Filtration and evaporation gave a residue which was sublimed at 120–160° (0·02 mm Hg). The sublimate was weighed and if necessary diluent umbelliferone was added to make up the weight to 3–5 mg. Recrystallization from 1·5 ml of water provided umbelliferone as needles, m.p. 228–232°. In a few of the plant extractions the final sublimation produced a small amount of a crystalline sublimate (< 1 mg) at 100–120°, m.p. 156°, which was identified as 7-hydroxy-8-methoxycoumarin (hydrangetin[17] or collinol[30]) by comparison of its physical properties with published data.

After the first ether extraction to remove coumarins, the aqueous bicarbonate residue

[30] R. F. C. Brown, P. T. Gilham, G. Hughes and E. Ritchie, *Australian J. Chem.* 7, 181 (1954).

was acidified with hydrochloric acid and re-extracted with ether. To the residue obtained on evaporation of the ether extracts was added about 30 mg of diluent *trans-p*-coumaric acid. The mixture was dissolved in boiling water (2 ml) and treated with animal charcoal. Filtration of the solution and cooling produced a crystalline material which was recrystallized from water, giving radioactive *p*-coumaric acid. m.p. 210–214°. Further recrystallizations from water had no effect on either the melting point or the specific activity.

Counting of Radiocactive Compounds

For the lavender experiments, the isolated umbelliferone was counted as a non-self-absorbing layer on lens tissue in a 1 in. diameter aluminium planchet with a Nuclear Chicago windowless gas flow counter.

For the hydrangea experiments, each compound was also counted as a non-selfabsorbing layer. The planchets were counted with an IDL anti-coincidence beta counter equipped with EKCO N610B automatic scaler (<2% S.E.).

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