Phytochemistry, 1963, Vol. 2, pp. 137 to 144. Pergamon Press Ltd. Printed in England

BIOSYNTHESIS OF THE COUMARINS IV. THE FORMATION OF COUMARIN AND HERNIARIN IN LAVENDER*

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(*Received* 31 *October* 1962)

Abstract—Evidence was found for the existence in *Lavandula officinalis* Chaix of 2-glucosyloxy-4-methoxy-*cis*-cinnamic acid†, which yields 7-methoxycoumarin (herniarin) on hydrolysis *in vitro*. Tracer experiments showed that D-glucose, L-phenylalanine, and cinnamic acid are common precursors of coumarin and herniarin, but that *o*- and *p*-hydroxycinnamic acids are selectively converted to coumarin and herniarin, respectively. Data obtained were consistent with the occurrence of herniarin biosynthesis according to the partial sequence: Cinnamic acid→*p*-coumaric acid→*p*-methoxycinnamic acid→2-glucosyloxy-4-methoxy-*trans*-cinnamic acid→2-glucosyloxy-4-methoxy-*cis*-cinnamic acid. Umbellic acid (2,4-dihydroxy-*trans*-cinnamic acid) and umbelliferone were utilized to a minor degree. An improved preparation of umbelliferone-2-^{14}C, and syntheses of umbellic acid-carboxy-^{14}C, *p*-methoxycinnamic acid-α-^{14}C,‡ and 2-glucosyloxy-4-methoxy-*trans*-cinnamic acid-α-^{14}C are described.

STUDIES in recent years have yielded significant information about the biosynthetic pathway to coumarin in *Melilotus*[1-5] and *Hierochloe*[6,7] species. In common with other phenylpropanoid compounds found in higher plants, coumarin can arise via the shikimic acid pathway, with L-phenylalanine and *o*-coumaryl-glucoside (2-glucosyloxy-*trans*-cinnamic acid) being intermediates in the synthesis. It is now clear that *ortho*-hydroxylation is a feature of the lactone ring formation in the biosynthesis of coumarin, and that *para*-hydroxylated intermediates are not precursors of coumarin.[8,9]

With the sole exception of coumarin itself, all coumarins produced by higher plants do possess *para*-hydroxylation. The author has drawn attention to the noteworthy fact that the co-occurrence of coumarin and these 7-hydroxylated coumarins is a rare phenomenon.[9] It was suggested that the two classes of coumarin are formed by independent routes, probably involving *ortho*- and *para*-hydroxylation, respectively, of cinnamic acid, which is known to be a precursor of coumarin. Although existing evidence has lent support to this idea,[10-12] no quantitative tracer studies have been done to permit a more rigorous delineation of the intermediates lying between phenylalanine and the 7-hydroxycoumarins.

Lavender (*Lavandula officinalis* Chaix) is one of the few species which elaborate both coumarin and a 7-hydroxylated coumarin, in this case 7-methoxycoumarin (herniarin).

* Issued as N.R.C. No. 7220. For Part III of this series see Reference 7.

† Abbreviations: *cis*-GMC and *trans*-GMC are the *cis*- and *trans*-isomers respectively of 2-glucosyloxy-4-methoxycinnamic acid.

‡ α = carbon atom adjacent to the carboxyl group.

[1] F. WEYGAND and H. WENDT, *Z. Naturforsch.* **14b**, 421 (1959).
[2] G. W. SCHAEFFER, F. A. HASKINS and H. J. GORZ, *Biochem. Biophys. Research Commun.* **3**, 268 (1960).
[3] T. KOSUGE and E. E. CONN, *J. Biol. Chem.* **234**, 2133 (1959).
[4] T. KOSUGE and E. E. CONN, *J. Biol. Chem.* **236**, 1617 (1961).
[5] J. R. STOKER and D. M. BELLIS, *J. Biol. Chem.* **237**, 2303 (1962).
[6] S. A. BROWN, G. H. N. TOWERS and D. WRIGHT, *Can. J. Biochem. and Physiol.* **38**, 143 (1960).
[7] S. A. BROWN, *Can. J. Biochem. and Physiol.* **40**, 607 (1962).
[8] F. WEYGAND, H. SIMON, H.-G. FLOSS and U. MOTHES, *Z. Naturforsch.* **15b**, 765 (1960).
[9] S. A. BROWN, *Z. Naturforsch.* **15b**, 768 (1960).
[10] W. W. REID, *Chem. & Ind. (London)* 1439 (1958).
[11] H. REZNIK and R. URBAN, *Naturwissenschaften* **44**, 13 (1957).
[12] J. B. HARBORNE and J. J. CORNER, *Biochem. J.* **81**, 242 (1961).

It was chosen for the present study as a convenient species in which to compare the biosynthesis of coumarin and the 7-hydroxylated coumarin. A part of the present work has been published as a preliminary report.[13]

"Bound" coumarin and herniarin in lavender

Coumarin in *Melilotus*,[14] and possibly in *Hierochloe*,[7] exists to a major degree in a "bound" form, as coumarinyl glucoside (2-glucosyloxy-*cis*-cinnamic acid). On hydrolysis with β-glucosidase this glucoside yields the free phenolic *cis*-acid, which lactonizes spontaneously to coumarin. The process occurs rapidly in disrupted cells.[4] Coumarinyl glucoside also exists in lavender, as can be shown by paper chromatography, and by the release of additional coumarin upon emulsin treatment of a lavender extract from which free coumarin has been removed. Evidence for an analogous "bound herniarin" has now been obtained. The yield of herniarin could be increased several-fold, after extraction of the free compound, by the emulsin hydrolysis, and it appears that herniarin exists in lavender predominantly in a bound form. Chromatography of the unhydrolysed extract in 1% aqueous acetic acid effected a partial purification of the "bound herniarin". Spraying of the developed chromatogram with a 0·1% emulsin solution, and incubation for 1–2 hr revealed a band with a purple fluorescence characteristic of herniarin, which had run just ahead of coumarinyl glucoside.

It has not yet been possible to isolate the new glucoside in pure form. Treatment of an aqueous lavender extract with lead acetate at an acidic, and then at a slightly alkaline pH led to a selective recovery of the glycoside in the second precipitate, from which it was easily released by decomposition with sulphuric acid in the cold. Column chromatography on cellulose powder freed it from fluorescent contaminants, but not from coumarinyl glucoside. The latter compound could be largely separated after chromatography of the eluate on large paper sheets in 1% acetic acid, but some free sugar remained with the herniarin-yielding glycoside. When the eluate from the paper was hydrolysed with emulsin, a chromatogram of the hydrolysate revealed a distinct increase in the glucose spot as compared to the unhydrolysed control.

It seems quite certain, therefore, that the compound in question is 2-glucosyloxy-4-methoxy-*cis*-cinnamic acid [*cis*-GMC]. Its presence implies the formation, at some stage, of an *ortho*-hydroxylated precursor, and indicates that the lactone ring of 7-hydroxylated coumarins, too, can be synthesized via *ortho*-hydroxylation. One other substituted coumarinyl glucoside is known; it is found in *Coronilla glauca* and yields psoralen on hydrolysis.[15]

Experiments with carbon-14

In Table 1 are collected the results of a series of experiments in which ten ^{14}C-labelled compounds have been compared as precursors of herniarin, and in some cases coumarin, in lavender. L-Phenylalanine, the first compound tested, was incorporated with moderate dilution of ^{14}C into herniarin as well as coumarin, an expected result in view of the known conversion of this amino acid to scopoletin, another 7-hydroxylated coumarin.[10] Also as expected, glucose was used with lower efficiency as a precursor of both coumarins. *o*-Coumaric and *p*-coumaric acids were utilized with a high degree of selectivity. The former was used for the synthesis of coumarin some 150–200 times as efficiently as it was used for

[13] S. A. BROWN, *Science* **137**, 977 (1962).
[14] F. A. HASKINS and H. J. GORZ, *Crop Science* **1**, 320 (1961).
[15] A. STOLL, A. PEREIRA and J. RENZ, *Helv. Chim. Acta* **33**, 1637 (1950).

herniarin synthesis, and the latter was selectively utilized for herniarin synthesis by a slightly lesser factor. Cinnamic acid, like phenylalanine, was a precursor of both coumarins, and shoots were as effective as plants with roots. The roots are thus unnecessary for conversion of these precursors to coumarin or herniarin. Nevertheless, plants with roots were used in most other experiments, as the 6- to 7-day metabolic periods attainable in this way produced higher specific activities in these coumarins.

These findings show unequivocally that herniarin, unlike coumarin, is not synthesized by way of the *o*-coumaric acid ⟶ *o*-coumaryl glucoside pathway. They confirm the theory

TABLE 1. CONVERSION OF ^{14}C-LABELLED COMPOUNDS TO COUMARINS BY LAVENDER

Experiment and duration of feeding	Compound administered	Specific activity (μc/mmole)	Dose (μmole/g) extd. dry wt	Compound isolated*	Specific activity (μc/mmole)	^{14}C-dilution Absolute	^{14}C-dilution Relative
1 (6 days)	L-Phenylalanine-G-^{14}C	33·3		C	0·166	201	
				H	0·31	108	
2 (6 days)	Glucose-G-^{14}C	100	28	C	0·015	6,700	
				H	0·047	2,100	8·5
	p-Coumaric acid-α-^{14}C	100	35	C	0·0022	45,000	
				H	0·405	247	1
	o-Coumaric acid-carboxy-^{14}C	100	26	C	0·309	324	
				H	0·0012	83,000	340
	o-Coumaryl glucoside-α ^{14}C	100	23	C	0·755	132	
				H	0·0004	250,000	1,000
3 (6 days)	*p*-Coumaric acid-α-^{14}C	68	21	C	0·0015	45,000	
				H	0·114	596	1
	o-Coumaric acid-carboxy-^{14}C	95	22	C	0·350	272	
				H	0·0023	41,000	69
4 (2 days)	Cinnamic acid-α-^{14}C†	85	24	C	0·15	570	
				H	0·194	440	
	Cinnamic acid-α-^{14}C	85	18	C	0·09	940	
				H	0·188	450	
5 (2 days)	*p*-Coumaric acid-α-^{14}C†	100	26	H	0·0776	1,300	1
	2-glucosyloxy-4-methoxy-*trans*-cinnamic acid-α-^{14}C†	57	33	H	2·32	24·6	0·019
	p-Methoxycinnamic acid-α-^{14}C†	62·5	24	H	0·173	360	0·28
6 (7 days)	*p*-Coumaric acid-α-^{14}C	73	35	H	0·078	940	1
	Umbellic acid-carboxy-^{14}C	52	30	H	0·0177	3,000	3·2
	Umbelliferone-2-^{14}C	100	40	H	0·0468	2,100	2·2
	p-Methoxycinnamic acid-α-^{14}C	63	38	H	5·09	12·3	0·013
7 (7 days)	*p*-Coumaric acid-α-^{14}C	73	25	H	1·35	54	1
	Umbellic acid-carboxy-^{14}C	52	31	H	0·603	87	1·6
	Umbelliferone-2-^{14}C	100	31	H	0·585	170	3·2
	p-Methoxycinnamic acid-α-^{14}C	100	25	H	28·3	3·5	0·065

* C = Coumarin, H = Herniarin.
† Shoots only fed.

that coumarin and herniarin arise via *ortho-* and *para*-hydroxylation, respectively, of a common precursor, which is most probably some form of cinnamic acid.

The low dilution of ^{14}C in *trans*-GMC [2-glucosyloxy-4-methoxy-*trans*-cinnamic acid] suggests that this compound is the immediate precursor of *cis*-GMC. The *trans-cis* inversion necessary for this step would be analogous to that postulated in the formation of coumarinyl glucoside.[4,7] In addition, during the conversion of *p*-coumaric acid to *cis*-GMC an *ortho*-hydroxylation, glucoside formation, and an *o*-methylation of the *para*-hydroxyl group must occur. Glucoside formation must obviously follow the *ortho*-hydroxylation, but the order of the other steps remained in question. In an attempt to elucidate this problem, *p*-methoxycinnamic acid-α-^{14}C and 2,4-dihydroxy-*trans*-cinnamic acid-carboxy-^{14}C (umbellic acid) were synthesized and compared with *p*-coumaric acid as precursors of herniarin. The results, listed under Experiments 5–7, clearly demonstrate the very high efficiency with which *p*-methoxycinnamic acid was converted to herniarin. Umbellic acid and umbelliferone, while moderately well utilized, were both poorer precursors than *p*-coumaric acid, and 25–50 times as poor as *p*-methoxycinnamic acid. Their conversion to herniarin must be via a minor pathway. The dilutions of ^{14}C in coumarin, which have been omitted from Experiments 5–7 in the interests of brevity, were all high, falling in the 10,000–100,000 range. An examination of the relative dilutions in Table 1 (in those cases where a direct comparison with the standard *p*-coumaric acid was made) reveals the following order of precursor efficiencies for herniarin synthesis:

trans-GMC > *p*-Methoxycinnamic acid > *p*-Coumaric acid > Umbellic acid > Umbelliferone > Glucose

The data in Table 1 are entirely consistent with the scheme of herniarin biosynthesis shown in Fig. 1. This scheme is presented with several reservations. Although all of the first six compounds except 2-hydroxy-4-methoxycinnamic acid have been shown to be precursors of herniarin or *cis*-GMC, it remains to be established that several of these compounds are naturally occurring intermediates. It is rather surprising, in view of the relatively large concentrations of *o*-coumaryl glucoside present both in lavender and other species, that the analogous *trans*-GMC has not been found. It is readily hydrolysed to the aglycone, which is easily identifiable on a paper chromatogram by phenol reagent sprays,

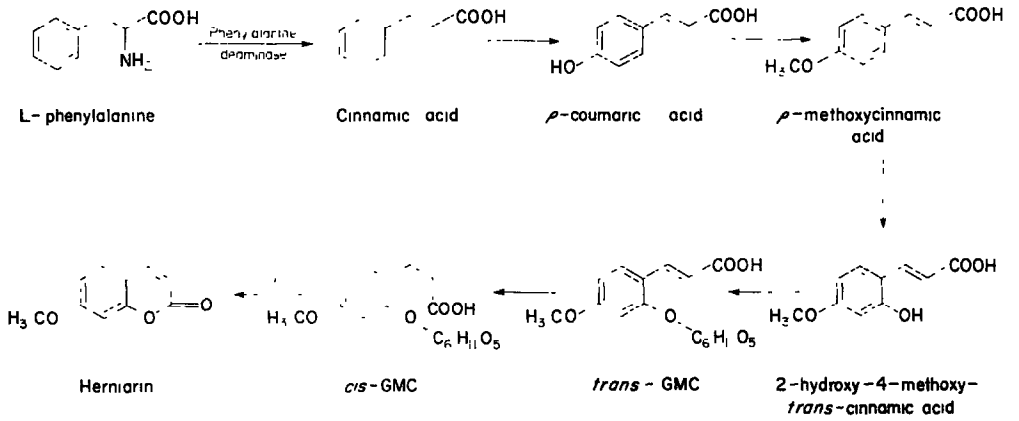

FIG. 1. PROPOSED BIOSYNTHETIC ROUTE TO HERNIARIN.

but in spite of this neither the glucoside nor the aglycone has been detected in the present work on lavender extracts. If they are naturally formed intermediates they must have very high turnover rates, or else exist in the form of derivatives, such as esters. *p*-Methoxycinnamic acid is also apparently absent, perhaps for the same reasons. The lack of a free phenolic hydroxyl, in addition, would make this compound difficult to identify on a chromatogram, as it is inert to phenol reagents. It has been shown to occur in other plant species.[16]

In view of these reservations the pathway proposed in Fig. 1 must not be regarded as conclusively established. Additional evidence for its validity is currently being sought.

EXPERIMENTAL

Cultivation of plants

Lavender seeds were allowed to germinate in moist Vermiculite. The seedlings were transplanted into pots of coarse quartz sand irrigated from below with "California" nutrient medium,[17] and grown in a greenhouse, or in a growth chamber under artificial illumination of at least 16,000 lux.

Administration of labelled compounds

In Experiments 4 and 5, Table 1, compounds were fed to plants through the cut stems. In the other experiments root feeding was adopted. The sand was gently washed free of the roots, which were blotted free of excess water, and a rough fresh weight was taken. (Plants in the fresh weight range 25–50 g were used.) The roots were then inserted into a small polyethylene bag, which contained the radioactive compound dissolved in water (usually 10 ml). Insoluble acids were fed as the sodium salts. The upper part of the bag was gathered closely around the stem, closed with twisted fine wire, and the bag placed in a beaker, which was wrapped in aluminium foil to exclude light from the roots. The plant was allowed to absorb the solution under illumination, the closed bag ensuring 100 per cent humidity of the air in contact with the roots. A distilled water rinse approximating the original volume was added after the absorption had apparently ended. The entire process required only a few hours. The roots were then rinsed with a large volume of water, an aliquot of which was analysed for ^{14}C to permit calculation of the uptake, which was usually incomplete. The plant was then returned to quartz sand for the desired period. Usually the treatment produced no noticeable effect except a darkening of the roots. Occasionally a few leaves became necrotic, and these were removed before harvesting.

Preparation of ^{14}C-labelled compounds

All melting points are corrected.

L-Phenylalanine-G-^{14}C and D-glucose-G-^{14}C, as well as the malonic acid-2-^{14}C used in syntheses to be described, were purchased from commercial sources. Previous papers have contained references to procedures suitable for the synthesis of cinnamic acid-α-^{14}C,[18] *p*-coumaric acid-α-^{14}C,[19] and *o*-coumaric acid-carboxy-^{14}C.[6,20]

[16] W. KARRER, *Konstitution und Vorkommen der organischer Pflanzenstoffe*, p. 381. Birkhäuser Verlag, Basel (1958).
[17] C. ELLIS and M. W. SWANEY, *Soilless growth of plants*. 2nd ed. revised and enlarged by T. EASTWOOD. Reinhold Publishing Corp., New York (1947).
[18] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. and Physiol.* 33, 948 (1955).
[19] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. and Physiol.* 34, 769 (1956).
[20] In a preliminary communication[18] this compound was erroneously described as *o*-coumaric acid-α-(or -2-)^{14}C.

p-Methoxycinnamic acid-α-^{14}C was synthesized from anisaldehyde and malonic acid-2-^{14}C by the procedure used previously for ferulic acid-α-^{14}C.[21]

o-Coumaryl glucoside-α-^{14}C was prepared[22] by a method based on the procedure of Helferich and Lutzmann.[23] To a mixture of helicin (284 mg, 1 mmole) and malonic acid-2-^{14}C (208 mg, 2 mmole) in dry pyridine (1·5 ml) was added 0·04 ml of aniline. The reaction mixture was heated at 60° for 6 hr. The reaction was interrupted for the addition of non-radioactive malonic acid (104 mg, 1 mmole), and heating was continued for a further 7 hr. Dropwise addition of the cooled reaction mixture to 100 ml of absolute ether, with vigorous stirring, precipitated crude *o*-coumaryl glucoside, which was quickly filtered in a sintered glass funnel. The product was dissolved in hot water, decolorized with charcoal, and allowed to crystallize. The purified *o*-coumaryl glucoside-α-^{14}C was obtained as colourless needles, m.p. 241·5–243·5° (decomp.). The radiochemical yield was 40 per cent.

2-Glucosyloxy-4-methoxy-*trans*-cinnamic acid-α-^{14}C was synthesized by an analogous procedure. 2-Glucosyloxy-4-methoxybenzaldehyde was prepared from 2-hydroxy-4-methoxybenzaldehyde[24] and acetobromoglucose by the method described for helicin by Robertson and Waters.[25] The tetra-acetyl glucoside (m.p. 160–162°, 4·5 g) was dissolved with some heating in about 300 ml of absolute methanol and the solution cooled to room temperature. Two millilitres of 0·91 N barium methoxide in methanol was added and the solution placed in a refrigerator for 1 hr. An equivalent volume of sulphuric acid was added, the resulting mixture filtered, and the product collected by evaporation of the methanol. It was twice recrystallized from water after a preliminary decolorization with charcoal. The purified 2-glucosyloxy-4-methoxybenzaldehyde weighed 2·67 g, m.p. 196–198°. (Found: C, 53·3; H, 6·02. $C_{14}H_{18}O_8$ required: C, 53·5; H, 5·73 per cent). The product (314 mg, 2 mmole) was condensed with malonic acid-2-^{14}C under conditions similar to those described for helicin. One mole of the labelled malonic acid was added at first, and after 8 hr a second mole of inactive compound. Total reaction time was 16 hr. After recrystallization from water *trans*-GMC, slightly yellow needles, melted at 194–196°. (Found: C, 53·49; H, 5·68. $C_{16}H_{20}O_9$ required: C, 53·93, H, 5·67).

Umbelliferone-2-^{14}C has been synthesized by Weygand and co-workers[8] by condensation of 2,4-dihydroxybenzaldehyde with malonic acid-2-^{14}C. In our hands this reaction gave unsatisfactory results, and as an alternative a cyano-acetate condensation[26] was employed. A mixture of sodium and potassium cyano-acetates was prepared from K^{14}CN by a procedure based on that of Phelps and Tillotson.[27] Chloracetic acid (218 mg) was mixed with anhydrous sodium carbonate (274 mg) in arm A of the vessel shown in Fig. 2. In arm B was placed K^{14}CN (176 mg, 500 μc). Water (*ca.* 0·25 ml) was added to arm A, and when the reaction had ceased 0·14 ml of water was added to arm B to dissolve the cyanide. Both arms were heated in an oil bath to 70°, and the contents of A tipped into B. The resulting solution was boiled for 5 min over a small hotplate in such a way that some of the condensate collected in A and could be used as a rinse. The reaction mixture in B was carefully neutralized with concentrated sulphuric acid, no attempt being made to remove the salt which precipitated. 2,4-Dihydroxybenzaldehyde (276 mg) was then introduced into A with the aid of 0·5 ml of water containing 500 mg of sodium hydroxide, and A was warmed to

[21] F. VORSATZ, *J. prakt. Chem.* **145**, 265 (1936).
[22] *o*-Coumaryl glucoside-α-^{14}C was synthesized by Dr. C.-H. Yang.
[23] B. HELFERICH and H. LUTZMANN, *Ann. Chem. Liebigs* **537**, 11 (1939).
[24] H. A. OFFE and H. JATZKEWITZ, *Chem. Ber.* **80**, 469 (1947).
[25] A. ROBERTSON and R. B. WATERS, *J. Chem. Soc.* **131**, 2729 (1930).
[26] W. BAKER and A. LAPWORTH, *J. Chem. Soc.* **125**, 2333 (1924).
[27] I. K. PHELPS and E. W. TILLOTSON Jr., *Am. J. Sci.* **26**, 267 (1908).

effect solution. The vessel was flushed with nitrogen, stoppered, and repeatedly tipped to mix the two solutions. After the mixture had stood overnight at room temperature,[28] acidification with 7 ml of 3 N hydrochloric acid yielded 351 mg of yellow crystals of crude 2-cyano-3-(2,4-dihydroxyphenyl)-acrylic acid. The yield was 86 per cent based on the aldehyde, 63 per cent based on the cyanide.

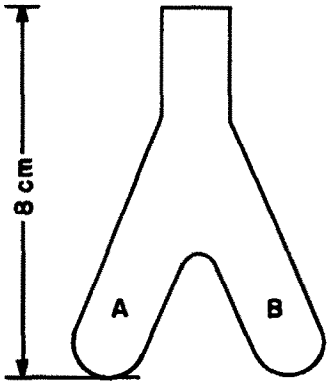

FIG. 2. REACTION VESSEL FOR CYANOACETATE CONDENSATION.

(The reaction of 2,4-dihydroxybenzaldehyde with a slight excess of sodium cyano-acetate prepared from crystalline cyano-acetic acid, under the same conditions, gave similar yields. Commercially available cyano-acetic acid-2-^{14}C could thus be utilized for the preparation of umbelliferone-3-^{14}C.)

The condensation product was heated under reflux with 4% HCl for 0·5 hr. The umbelliferone-3-carboxylic acid-2-^{14}C which separated was collected after cooling. The yield was 333 mg, 95 per cent. The product was purified by sublimation (240°, 1 Torr.).

Umbelliferone-3-carboxylic acid was decarboxylated in an evacuated sealed tube large enough to accommodate the liberated carbon dioxide. The tube was heated for 1 hr in a tube furnace at 260–265°. The umbelliferone in the reaction mixture was purified as described by Weygand *et al.*[8] except that it was sublimed at 150°, 1 Torr. The yield of chromatographically pure umbelliferone-2-^{14}C, m.p. 232–234°, was 79 per cent.

Umbellic acid-carboxy-^{14}C was prepared[29] from umbelliferone-2-^{14}C. Umbelliferone (100 mg) was dissolved in 3·5 ml of water containing 165 mg of potassium hydroxide, and the solution heated in an oil bath maintained at 83°. The temperature of the solution rose to 70° in 10 min, and was held within $\pm 1°$ of this temperature for 20 min. After being cooled to room temperature the solution was acidified to yield a light brown crystalline precipitate. This was dissolved in 5% $NaHCO_3$ solution and reprecipitated by acidification. The yield was 92·4 mg, 83 per cent. Only one radioactive spot was detected on a chromatogram of the product in 1% aqueous acetic acid. The product also had R_f values identical to those reported for umbellic acid in water-saturated butanol, butanol–acetic acid–water (4 : 1 : 5), and 2% aqueous acetic acid.[30] It melted with decomposition above 210°.

Isolation of the coumarins

The lavender plant was cut into boiling ethanol, disintegrated in a VirTis homogenizer or Servall Omni-Mixer, the mixture filtered, and the residue washed with hot 80% ethanol.

[28] The conditions used by Baker and Lapworth[26] for this reaction—one hour's heating at 50°—were found to give excessive resin formation.
[29] E. POSEN, *Ber. deut. chem. Ges.* **14**, 2744 (1881).
[30] M. FUJITA and T. FURUYA, *Chem. Pharm. Bull. (Tokyo)* **6**, 511 (1958).

Removal of the ethanol *in vacuo* left an aqueous residue which was treated for 48 hr with 0·1% emulsin, and then continuously extracted with ether to remove the coumarins. The ether-soluble material was submitted to sublimation over a 50–75° range, 1 Torr. The coumarin and herniarin in the sublimate were purified by gas–liquid phase chromatography.[31] They were present in quantities of the order of 1–1·5 and 0·2–0·25 mg/g fresh weight, respectively.

Partial purification of "bound" herniarin

An aqueous residue prepared as described in the last section from 300 g of fresh lavender plants was treated with a saturated solution of neutral lead acetate until no more precipitate formed, and the mixture was filtered. The filtrate was adjusted to pH 7–8 by the addition of N NaOH, and the second precipitate was removed by filtration. Each precipitate was suspended in water and decomposed by the addition of dilute sulphuric acid with vigorous stirring. The precipitate of lead sulphate was removed and each filtrate was again treated with lead acetate as above. The two precipitates at pH 7–8, which contained most of the "bound" herniarin, were combined and decomposed with sulphuric acid as before.

An aliquot of the filtrate from this treatment was chromatographed on a 2·2×40 cm column of Whatman cellulose powder, developed with 80% ethanol. Five-ml fractions were collected and examined by paper chromatography in 1% aqueous acetic acid. Fractions 30–38 contained a mixture of "bound" herniarin and coumarinyl glucoside, but no fluorescent impurity. A partial separation of the two glucosides was achieved by chromatography of the combined fractions on 46×57 cm sheets of Whatman No. 3MM paper in 1% acetic acid.

Acknowledgements—Analyses were done by Messrs. J. Dyck and M. Mazurek. Expert technical assistance was contributed by Mr. J. P. Shyluk throughout. The author is grateful to Dr. C.-H. Yang, formerly of this laboratory, for preparing the *o*-coumaryl glucoside-α-^{14}C used in these studies.

[31] S. A. BROWN and J. P. SHYLUK, *Anal. Chem.* **34**, 1058 (1962).