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BIOSYNTHESIS OF COUMARIN: THE ISOMERIZATION STAGE

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Abstract—The *trans–cis* isomerization of *o*-coumaroyl glucoside to coumarinoyl glucoside, which is involved in the biosynthesis of coumarin, has been shown to be non-enzymic in *Melilotus officinalis*. The reaction is catalysed by light.

INTRODUCTION

THE biosynthetic pathway from phenylalanine (I) to coumarin (VI) in plants, expecially in *Melilotus* species and *Hierochloe odorata*, has been well established using tracer techniques.[1–4] The pathway is depicted in Fig. 1.

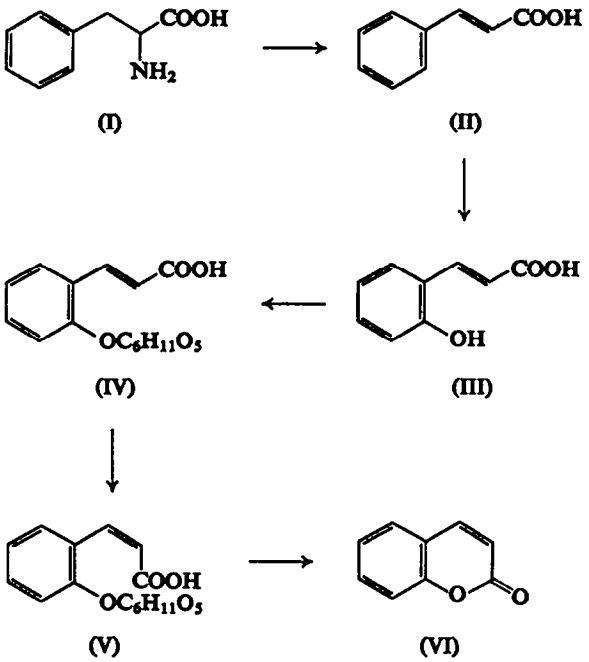

FIG. 1. THE BIOSYNTHESIS OF COUMARIN.

[1] T. KOSUGE and E. E. CONN, *J. Biol. Chem.* **234**, 2133 (1959).
[2] F. WEYGAND and H. WENDT, *Z. Naturforsch.* **14b**, 421 (1959).
[3] S. A. BROWN, G. H. N. TOWERS and D. WRIGHT, *Can. J. Biochem. Physiol.* **38**, 143 (1960).
[4] J. R. STOKER and D. M. BELLIS, *J. Biol. Chem.* **237**, 2303 (1962).

The present paper is concerned with the isomerization of *o*-coumaroyl glucoside (IV) to coumarinoyl glucoside (V). That this isomerization is catalysed by light, especially u.v. light, has been well established.[5,6] At present this is the only method available for the synthesis of coumarinoyl glucoside. However, whether this is the only way in which the isomerization is brought about in plants or whether an isomerase enzyme is involved is not quite clear.

Stoker[7] published results which showed that *M. alba* plants fed *trans*-cinnamic acid-3-^{14}C in the dark produced ^{14}C-labelled coumarin. The amount of radioactivity in the coumarin was less than when plants were exposed to sunlight during the feeding experiments. Homogenates of *M. alba* leaves were also found to convert the β-glucoside of *o*-coumaric acid-2-^{14}C to coumarin in the dark. These results are all indicative of the presence of an isomerase enzyme system in *M. alba*.

Haskins, Williams and Gorz[8] from less direct evidence deduced that the isomerization of *o*-coumaroyl glucoside was probably entirely light catalysed in *M. alba*. These workers compared the relative amounts of the *cis* (V+VI) and *trans* (III+IV) isomers in excised leaves maintained at 0° and at 29° while exposed to u.v. light. There was no significant difference in the amounts of *cis* glucoside under these two sets of conditions and it was assumed that no isomerase was present, since an enzyme would be expected to act at 29° but to be almost inactive at 0°. Further evidence was provided by comparing the relative amounts of the two isomers in excised leaves which were either fresh or steam heated and then exposed to u.v. light. Although the amount of *cis* isomer was smaller in steamed leaves than in fresh leaves the authors claimed that this was possibly due to wrinkling of the steamed leaves which thereby reduced the area of surface exposed to light.

Because of these two conflicting views on the isomerization of *o*-coumaroyl glucoside in *Melilotus* species the present authors have further investigated the problem.

RESULTS

The results given in Table 1 show that steam distillation of a solution of *o*-coumaric acid-2-^{14}C causes the continuous conversion of small amounts of *o*-coumaric acid to coumarin.

TABLE 1. STEAM DISTILLATION OF *o*-COUMARIC ACID-2-^{14}C*

Aliquots of distillate† (50 ml)	Radioactivity in distillate (dpm per 50 ml aliquot)	Per cent *o*-coumaric acid distilled as coumarin
1	$1{\cdot}52 \times 10^4$	0·55
2	$1{\cdot}05 \times 10^4$	0·38
3	$1{\cdot}46 \times 10^4$	0·53
4	$7{\cdot}98 \times 10^3$	0·29
5	$1{\cdot}16 \times 10^4$	0·42
6	$1{\cdot}28 \times 10^4$	0·46

* 1·00 mg *o*-coumaric acid-2-^{14}C ($4{\cdot}52 \times 10^8$ dpm/mmole).
† Eighteen aliquots were collected, all giving essentially the same type of results.

[5] H. LUTZMANN, *Ber. Deut. Chem. Ges.* **73b**, 632 (1940).
[6] F. A. HASKINS and H. J. GORZ, *Biochem. Biophys. Res. Commun.* **6**, 298 (1961).
[7] J. R. STOKER, *Biochem. Biophys. Res. Commun.* **14**, 17 (1964).
[8] F. A. HASKINS, L. G. WILLIAMS and H. J. GORZ, *Plant Physiol.* **39**, 777 (1964).

This is presumably due to the formation of an equilibrium mixture containing about 99·5 per cent of the *trans* isomer; removal of the steam-volatile *cis* isomer causes more coumarin to be formed. Variations in the amounts of radioactivity found in equal volumes of distillate are probably due to different rates of heating and to fluctuations in the volume of solution in the distillation flask. That the radioactivity was associated with coumarin was confirmed by

TABLE 2. CHROMATOGRAPHIC MOBILITIES AND COLOUR OF COUMARIN AND THE RADIOACTIVE COMPOUND IN THE STEAM DISTILLATE

	R_f value in		Fluorescence under 350 nm lamp after treatment with		
	Solvent 1	Solvent 2	N/1 HCl	Ammonia vapour	N/1 NaOH
Coumarin	0·90	0·95	None	None	Yellow–green
Radioactive compound in distillate	0·95	0·95	None	None	Yellow–green

Solvent 1—Benzene–glacial acetic acid–water (2:2:1), upper phase. Solvent 2—Chloroform–formic acid (19:1). Whatman No. 1 paper used with descending technique.

paper chromatography followed by scanning of the developed chromatograms for radioactivity. Radioactive spots were found which corresponded in position and fluorescence to coumarin (Table 2). Because of the formation of coumarin during steam distillation this method for the isolation of coumarin was abandoned in favour of solvent extraction followed by chromatography.

TABLE 3. ISOMERIZATION OF *o*-COUMARIC ACID-2-^{14}C ON STORAGE IN THE DARK AT pH 8·5

Time of removal of aliquot* (hr)	Radioactivity in aliquot taken (dpm × 10^{-3})	Percentage of sample as coumarin
0	1·16	0·21
3	1·81	0·33
6	1·99	0·36
12	2·45	0·44
24	4·28	0·78
36	4·58	0·83
48	4·28	0·78

* Incubation at 25° of *o*-coumaric acid-2-^{14}C (2 mg; $4{\cdot}52 \times 10^8$ dpm/mmole) in sodium bicarbonate solution (10 ml) neutralized to pH 8·5. 1·0 ml aliquots taken each time.

During the experimental work it was observed that aqueous solutions of *o*-coumaric acid-2-^{14}C at pH 8·5, when acidified, extracted with ether and chromatographed, yielded radioactive spots corresponding in position to coumarin. Consequently the storage of such solutions was further examined and the results of these experiments are given in Table 3.

The conversion of *o*-coumaric acid to coumarin is appreciable even in such a short period as 24 hr, the time involved in feeding experiments. This will make difficult the assessment of results of experiments in which *o*-coumaric acid-2-^{14}C is to be administered to shoots of *Melilotus officinalis*. Consequently control experiments were performed using labelled precursor mixed with ground-up heat-killed plant material, to obtain some indication of the amount of non-enzymic isomerization at the pH of the cell sap. The results of such experiments in which *o*-coumaric acid-2-^{14}C was administered to cut shoots of *M. officinalis*, and the control experimental results, are shown in Table 4. Although much more radioactivity was found in the coumarin isolated from shoots exposed to light there was no significant difference between test and control experiments in the dark.

TABLE 4. CONVERSION OF *o*-COUMARIC ACID-2-^{14}C TO COUMARIN IN *M. officinalis* SHOOTS

Pretreatment of plant material*	Quantity of coumarin isolated (mg)	Radioactivity in the coumarin (dpm × 10^{-3})	Specific activity of coumarin (dpm/mmole × 10^{-4})
In darkness			
None	2·75	1·49	7·85
None	2·34	1·53	9·55
Heated at 100°	2·79	1·39	7·55
Heated at 100°	2·97	1·85	9·30
In light†			
None	3·11	291	137
None	3·20	210	104

1·0 ml of 0·0068 M solution of *o*-coumarate-2-^{14}C; 4·52 × 10^8 dpm/mmole.

* Five experiments were performed in each series, only two typical results are quoted.

† A Philips MB/U 250 lamp was used.

The results in Table 4 indicate that an enzyme is not involved in the isomerization of *o*-coumaroyl glucoside to coumarinoyl glucoside in *M. officinalis*. Further evidence to support this was obtained from experiments with homogenates and dialysed homogenates of *M. officinalis* (Tables 5 and 6).

DISCUSSION

It has previously been shown[9] that the steam distillation technique of Duncan and Dustman[10] does not cause any detectable conversion of *o*-coumaric acid to coumarin as determined by u.v. absorption studies on the distillate. However, when the method is used for the steam distillation of ^{14}C-labelled *o*-coumaric acid then radioactive coumarin was found in the distillate. Consequently steam distillation should not be used as a method for the isolation of coumarin under these circumstances.

[9] J. R. STOKER, Unpublished results.

[10] I. J. DUNCAN and R. B. DUSTMAN, *Ind. Eng. Chem. Anal. Edition* **9**, 471 (1937).

TABLE 5. CONVERSION OF *o*-COUMARIC ACID-2-^{14}C TO COUMARIN IN HOMOGENATES OF *M. officinalis*

Pretreatment of homogenate	pH of buffer	pH of homogenate	Total activity isolated as coumarin (dpm × 10^{-2})
None	6·0	6·1	9·70
None	7·0	6·9	2·41
None	8·0	7·7	2·36
Heated at 100°	6·0	6·1	5·09
Heated at 100°	7·0	6·9	3·79
Heated at 100°	8·0	7·7	3·27
Dialysed	6·0	6·1	8·12
Dialysed	7·0	6·9	10·6
Dialysed	8·0	7·7	3·44
Dialysed then heated at 100°	6·0	6·1	5·07
Dialysed then heated at 100°	7·0	6·9	6·07
Dialysed then heated at 100°	8·0	7·7	4·24

1·0 ml of 0·0031 M solution of the β-glucoside of *o*-coumaric acid-2-^{14}C; 4·40 × 10^8 dpm/mmole. Phosphate buffer (0·1 M).

TABLE 6. CONVERSION OF *o*-COUMARIC ACID-2-^{14}C TO COUMARIN IN HOMOGENATES OF *M. officinalis*

Pretreatment of homogenate	pH of buffer	pH of homogenate	Total activity isolated as coumarin (dpm × 10^{-2})
None	7·2	7·3	3·91
None	8·0	7·8	1·49
Heated at 100°	7·2	7·3	2·85
Heated at 100°	8·0	7·8	2·84
Dialysed	7·2	7·3	5·39
Dialysed	8·0	7·9	7·12
Dialysed then heated at 100°	7·2	7·3	6·54
Dialysed then heated at 100°	8·0	7·9	5·46

1·0 ml of 0·0031 M solution of the β-glucoside of *o*-coumaric acid-2-^{14}C; 4·40 × 10^8 dpm/mmole. Tris buffer (0·1 M).

The isomerization of *o*-coumaric acid at pH 8·5 in the dark complicated the interpretation of the results of feeding experiments. However, comparison of the amounts of radioactivity associated with the coumarin in test and control experiments shows no significant differences. This indicates that an isomerase enzyme system is not involved in the isomerization of *o*-coumaroyl glucoside to coumarinoyl glucoside in *Melilotus officinalis* shoots. Similar findings were obtained in the homogenate experiments. This confirms the original results of Haskins, Williams and Gorz.[8] The earlier results of Stoker[7] indicating the presence of an

isomerase were almost certainly artefacts due to the steam distillation technique which was used in the work.

Although a few *cis–trans* isomerase enzymes have been found[11–13] these usually involve substrates such as maleoylacetoacetate in which conjugation is less than in *o*-coumaroyl glucoside. Consequently photochemical isomerization will be more difficult in such cases. The retinal pigments, which exhibit a large amount of conjugation and with which an isomerase is associated, are an exceptional case since of necessity isomerization must be able to occur in both light and darkness.

EXPERIMENTAL

Plant Material

Melilotus officinalis plants were grown from seed in the greenhouse. Artificial illumination was provided when necessary to maintain 16 hr of light per day.

Radioactive Compounds

The β-glucoside of *o*-coumaric acid-2-^{14}C was synthesized from helicin and malonic acid-2-^{14}C according to the method of Helferich and Lutzmann.[14] *o*-Coumaric acid-2-^{14}C was prepared by hydrolysis of the glucoside with emulsin followed by extraction and chromatography in the dark.

Steam Distillation Technique

o-Coumaric acid-2-^{14}C (1 mg; $4{\cdot}52 \times 10^8$ dpm/mmole) was placed in a 50 ml flask with water (10 ml) and N/1 sulphuric acid (1 ml). The solution was steam distilled and 50-ml aliquots of distillate collected. During distillation an attempt was made to keep the volume of liquid in the distillation flask constant. The distillates were extracted with ether (4 × 10 ml). After evaporation aliquots of the ether solution were chromatographed using benzene–acetic acid–water (2:2:1) upper phase and chloroform–formic acid (19:1). Other aliquots were counted for radioactivity.

Administration of Labelled Compounds

The *o*-coumaric acid-2-^{14}C was dissolved in the calculated quantity of 5% $NaHCO_3$ just prior to use. Shoots (*ca.* 10 cm) were cut from plants and the stems trimmed under water. The cut ends of the shoots were immersed in the aqueous *o*-coumarate solutions (1 ml of 0·0068 M *o*-coumarate-2-^{14}C; $4{\cdot}52 \times 10^8$ dpm/mmole). As the solutions were taken up by the shoots water was added to keep the cut ends immersed. Metabolic periods of 24 hr were used either in light or darkness.

Isolation of Coumarin

After the metabolic period the plant material was ground up with sand (5 g) and distilled water (5 ml), and 1 ml of β-glucosidase solution (1 mg/ml) added. After 1 hr at 25°, acetone (10 ml) and N HCl (1 ml) were added and the mixture was shaken for 1 hr and filtered and diluted with water (50 ml). The solution was extracted with ether (5 × 20 ml), and the extract

[11] R. HUBBARD, *J. Gen. Physiol.* **39**, 936 (1956).
[12] S. W. EDWARDS and W. E. KNOX, *J. Biol. Chem.* **216**, 489 (1955).
[13] L. LACK, *J. Biol. Chem.* **236**, 2835 (1961).
[14] B. HELFERICH and H. LUTZMANN, *Ann. Chem.* **537**, 11 (1939).

chromatographed on Kieselgel G plates developed with benzene. The area corresponding in position to coumarin was scraped off the plate and shaken with ether (25 ml) for 3 hr. The ether was filtered and rechromatographed on Whatman No. 1 paper using benzene–acetic acid–water (2:2:1). The area corresponding to coumarin was cut out, eluted with ether and evaporated to dryness. The residue was dissolved in absolute ethanol (1 ml) and aliquots used for measurement of radioactivity and for u.v. spectrophotometric analysis.

Homogenate Experiments

Plant material (10 g) was ground up in a cold mortar with 20 ml of the required buffer (0·1 M phosphate at pH 6·0, 7·0 and 8·0; also 0·1 M Tris at pH 7·2 and 8·0). The buffered homogenates were strained through muslin and cooled in ice. Aliquots (2 ml) of each buffered homogenate were transferred to each of two 10 ml test tubes. One tube was heated 3 min at 100° to inactivate the enzymes. To each tube was added a solution of the β-glucoside of *o*-coumaric acid-2-^{14}C (1 ml of 0·0031 M; $4{\cdot}40 \times 10^8$ dpm/mmole) and 1 ml of β-glucosidase solution (1 mg/ml). The tubes were incubated with shaking in the dark at 25° for 1 hr. The coumarin was extracted as described above.

Dialysed homogenates were prepared by dialysis at 4° using two quantities of 2 l. of buffer for each 20 ml of homogenate.

Determination of Radioactivity

Aliquots of the alcoholic coumarin solutions were counted in toluene containing 0·6% PPO and 0·05% POPOP using an I.D.L. 6012 Liquid Scintillation Counter. *n*-Hexadecane-1-^{14}C was used as an internal standard in all measurements.

Chromatograms were scanned for radioactivity using a Panax RCMS-2 Scanner.

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