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BIOSYNTHESIS OF DIHYDROXYCOUMARINS IN *DAPHNE ODORA* AND *CICHORIUM INTYBUS**

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Abstract—The biosynthesis of daphnin and daphnetin-8-glucoside in *Daphne odora* and of cichoriin in *Cichorium intybus* was studied using labelled cinnamic acids as precursors, and it is suggested that these compounds are produced mainly via *p*-coumaric acid, but not via caffeic acid.

INTRODUCTION

A NUMBER of studies using labelled precursors have shown that the naturally-occurring coumarins are derived from the corresponding cinnamic acids, as in the case of coumarin itself (I),[1-4] umbelliferone (II),[5-7] herniarin (III)[3-5] and scopoletin (IV).[8] It has been suggested that the dihydroxycoumarin, esculetin (V), is derived from caffeic acid,[9] but no tracer experiments have yet been carried out. This coumarin has been obtained in a cell-free system containing the *cis*-isomer of caffeic acid and an enzyme of the phenolase type.[10]

Another natural dihydroxycoumarin, daphnetin (VI), has two adjacent hydroxyl groups like esculetin, and may therefore be derived from caffeic acid. However, one of its hydroxyl groups is at 8- instead of 6-position, so that its metabolic pathway may differ from that of esculetin. The authors have studied a transglucosylation reaction between coumarin glucosides by an enzyme preparation of *Daphne odora* which contains daphnetin and its two monoglucosides, daphnin (VII), and daphnetin-8-glucoside (VIII),[11,12] and similar experiments are now in progress with *Cichorium intybus*[13,14] using esculetin and its two monoglucosides, esculin (IX) and cichoriin (X).

The main concern of the present study is to determine, by feeding these plants with labelled cinnamic acids, whether the glucosides of daphnetin and esculetin are really derived from caffeic acid.

* Part III in the series "Metabolism of Coumarins in *Daphne odora*".

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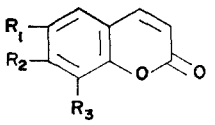

(I)	Coumarin	$R_1 = R_2 = R_3 = H$
(II)	Umbelliferone	$R_1 = R_3 = H$, $R_2 = OH$
(III)	Herniarin	$R_1 = R_3 = H$, $R_2 = OCH_3$
(IV)	Scopoletin	$R_1 = OH$, $R_2 = OCH_3$, $R_3 = H$
(V)	Esculetin	$R_1 = R_2 = OH$, $R_3 = H$

(VI)	Daphnetin	$R_1 = H$, $R_2 = R_3 = OH$
(VII)	Daphnin	$R_1 = H$, $R_2 = OGl$, $R_3 = OH$
(VIII)	Daphnetin-8-glucoside	$R_1 = H$, $R_2 = OH$, $R_3 = OGl$
(IX)	Esculin	$R_1 = OGl$, $R_2 = OH$, $R_3 = H$
(X)	Cichoriin	$R_1 = OH$, $R_2 = OGl$, $R_3 = H$

RESULTS

With D. odora

The content of daphnin and daphnetin-8-glucoside in *Daphne* plants was determined (Table 1), as a preliminary to feeding experiments. Three radioactive cinnamic acids (cinnamic, *p*-coumaric and caffeic acids) were then separately fed to *Daphne* cuttings and

TABLE 1. CONTENT OF DAPHNIN AND DAPHNETIN-8-GLUCOSIDE IN THREE ORGANS OF *Daphne odora* (21 JANUARY)

	Daphnin	Daphnetin-8-glucoside
Leaves	26·8* (9·11)	1·3 (0·44)
Stem	18·5 (6·29)	0·9 (0·31)
Inflorescence	75·9 (25·8)	23·6 (8·02)

* μmoles/g fresh wt. Values in parentheses; mg/g fresh wt.

after 20 hr and 4 days metabolism, daphnin (from all the organs) and daphnetin-8-glucoside (from the inflorescence) were isolated. The results (Tables 2–4) show that *p*-coumaric acid

TABLE 2. UPTAKE OF LABELLED SUBSTANCES AND THE DISTRIBUTION OF RADIOACTIVITY AFTER 20 hr METABOLISM IN *D. odora*

Compounds fed	μc/m-mole	Uptake (μc)	Radioactivity of the extract (mμc) Total	Leaf	Stem	Inflorescence
Cinnamic acid	53	1·27	706	57 (8)	627 (89)	22 (3)
p-Coumaric acid	56	1·03	501	81 (16)	385 (77)	35 (7)
Caffeic acid	51	1·36	773	163 (21)	539 (70)	71 (9)

The values in parentheses are per cent of the total radioactivity of the extract.

TABLE 3. THE DETAILED RESULTS OF THE EXPERIMENTS IN TABLE 2

Organs	Cinnamic acids fed	Compounds isolated	Specific radioactivity (mμc/m-mole)	Dilution value ($\times 10^{-2}$)	Incorporation (%)
Leaves	Cinnamic	Daphnin	2·4	220	0·61
	p-Coumaric	Daphnin	2·5	220	0·49
	Caffeic	Daphnin	1·7	300	0·17
Stem	Cinnamic	Daphnin	9·2	58	0·08
	p-Coumaric	Daphnin	22·9	24	0·36
	Caffeic	Daphnin	4·3	120	0·04
Inflorescence	Cinnamic	Daphnin	15·5	34	12·8
	p-Coumaric	Daphnin	47·4	12	27·8
	Caffeic	Daphnin	2·9	180	0·80
	Cinnamic	Daphnetin 8-glucoside	10·9	49	2·8
	p-Coumaric	Daphnetin 8-glucoside	32·3	17	5·9
	Caffeic	Daphnetin 8-glucoside	3·0	170	0·26

is the best precursor. Cinnamic acid is also utilized though with lower efficiency, but there was no significant incorporation of label from caffeic acid. It is therefore unlikely that the conversion of *p*-coumaric acid to caffeic acid is an obligatory step in the synthesis of the glucosides.

Two isotope competition experiments were carried out (Table 5). In both experiments, the conversion of labelled compounds (phenylalanine and cinnamic acid) into the glucosides was apparently higher in the plants which had been treated with caffeic acid than in the plants supplied with *p*-coumaric acid. It should however be noted that caffeic acid is rather inefficient in isotope competition experiments as compared with cinnamic acid, and moreover, cinnamic acids in general seem to lower glucoside formation. This may be attributed to some inhibitory action of the relatively high amounts of exogeneously supplied cinnamic acids, especially of caffeic acid, on one or more steps in the biosynthesis.

With C. intybus

The distribution of esculetin and its two monoglucosides (esculin and cichoriin) among the various parts of this plant was examined. Cichoriin is present in large amount in the head, both at bud stage and in full-bloom, and esculin is found in much lower amount as compared with cichoriin and occurs only in the style and stamen of the head. In subsequent experiments therefore cichoriin was isolated from the head (see Experimental).

TABLE 4. THE EFFECT OF A LONGER METABOLISM IN *D. odora*

Compounds fed	Specific radioactivity (mμc/m-mole)	Dilution value ($\times 10^{-2}$)	Incorporation (%)
Cinnamic acid	46	12	13·0
p-Coumaric acid	117	4·8	45·0
Caffeic acid	10	51	2·5

Experimental conditions were the same as in Tables 2 and 3, except 4 days metabolism was adopted. Daphnin was isolated in each case from the inflorescence.

TABLE 5. RESULTS OF ISOTOPE COMPETITION EXPERIMENTS

Non-labelled compound trapped	Radioactive compound* uptake (μc)	Specific radioactivity (mμc/m-mole) Daphnin†	D-8-G†
Experiment A			
Cinnamic acid	0·25	47·5	
p-Coumaric acid	0·26	7·8	
Caffeic acid	0·42	23·4	
Umbelliferone	0·47	81·5	
None (control)	0·45	93·4	
Experiment B			
p-Coumaric acid	0·43	8·6	2·9
Caffeic acid	0·58	15·4	8·9
None (control)	0·83	56·7	34·9

* Phenylalanine in Experiment A and cinnamic acid in Experiment B.
† Isolated from the inflorescence. D-8-G; daphnetin-8-glucoside.

Similar experiments to those with *D. odora* were carried out using the cuttings. The results summarized in Tables 6 and 7 show that *p*-coumaric acid again is readily utilized for cichoriin formation, but caffeic acid is not. As compared with *D. odora*, cinnamic acid was poorly converted.

TABLE 6. UPTAKE OF LABELLED SUBSTANCES AND THE DISTRIBUTION OF RADIOACTIVITY AFTER 18·5 hr METABOLISM IN *C. intybus*

Compounds fed	μc/m-mole	Uptake (μc)	Radioactivity of the extract (mμc) Total	Stem	Heads*
Cinnamic acid	53	0·96	832	738	94 (19)
p-Coumaric acid	56	0·56	429	377	52 (12)
Caffeic acid	51	0·44	306	238	68 (22)

* Values in parentheses are per cent of the total.

TABLE 7. SPECIFIC RADIOACTIVITY AND DILUTION VALUE OF CICHORIIN

Compounds fed	Specific radioactivity (mμc/m-mole)	Dilution value ($\times 10^{-2}$)
Cinnamic acid	3·4	136
p-Coumaric acid	250	2·24
Caffeic acid	4·6	110

DISCUSSION

General

From these results it is clear that the major route to natural dihydroxycoumarins, both to daphnetin and esculetin, is via *p*-coumaric acid, but not via caffeic acid as has been previously suggested.[9] Others have also shown that *p*-coumarate is a more important metabolic intermediate than caffeate in the synthesis of 'chlorogenates',[15,16] and flavonoids.[17,18]

Further steps from *p*-coumaric acid to the coumarin glucosides may involve ring cyclization, hydroxylation (at 6- and 8-position of esculetin and daphnetin respectively) and glucosylation. At least in *D. odora*, umbelliferone may not be the subsequent intermediate, because it is not efficient in competition experiments (Table 5). In *Hydrangea macrophylla* it has been shown that *p*-coumaric acid is glucosylated to form *p*-glucosyloxycinnamic acid,[5-7] so that the latter compound may be formed before ring cyclization also in our plants. Use of this glucoside in feeding experiments would not give any clear results, because the tissue contains a highly active glucosylase.[11,12]

Tables 3 and 7 show that the dilution value even of the best precursor, *p*-coumaric acid, is in the order of 10^{-3} in *D. odora* and 224 in *C. intybus* after 20 hr metabolism, and a longer metabolism in the former plant does not lower this value greatly (Table 4). It has been reported that this compound is readily converted to scopolin in *H. macrophylla* with such a low dilution as 85 in a day metabolism.[5] This high dilution may be explained by assuming that the radioactive products are greatly diluted by endogenous glucosides present in the tissue in a high amount (Table 1). This explanation would not contradict the fact that the incorporation rate of *p*-coumaric acid into daphnin is remarkably high (27·8 and 45·0 per cent after 20 hr and 4 days metabolism, respectively; Tables 3 and 4).

Some Aspects of the Metabolism of the Glucosides in D. odora

The authors reported that a transglucosylase from the flowers of this plant can convert daphnin to daphnetin-8-glucoside, but not vice versa, namely the formation of the former glucoside may precede that of the latter in metabolic sequence.[11,12] The formation *in vivo* of daphnetin-8-glucoside from daphnin would be indicated if the specific radioactivities of the two glucosides in the inflorescence are different (Table 3); indeed the incorporation of label from both *p*-coumaric and cinnamic acids into daphnin is clearly higher than that into daphnetin-8-glucoside.

The distribution of radioactivities in three different organs of the plant are shown in Table 2. In the plants supplied with *p*-coumaric acid, only 7% of radioactivity of this precursor (or any compounds derived from it) occur in the inflorescence. The specific radioactivity of daphnin and the incorporation rate of the precursor into this glucoside is however distinctly higher in the inflorescence than in leaves and stem. It is therefore unlikely that the leaves and stem can actively synthesize the glucosides at least at flowering or that the radioactive product in the inflorescence is formed in other organs and translocated therefrom.

EXPERIMENTAL

Materials. The experiments were carried out using the plants at flowering: *D. odora* from January till March and *C. intybus* in July.

Feeding experiments. In the experiments with *D. odora* (Tables 2–4), three cuttings consisting of leaves (total wt. 5·1–5·3 g), stem (2·5–2·8 g) and a head-like inflorescence (2·5–3·0 g) were picked from the plant

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immediately before flowering. The cut end of the stem was washed with water to remove the mucilage exuded, and solution of 2-^{14}C-cinnamic acids (cinnamic, *p*-coumaric and caffeic acids; 0·035 M dissolved in 0·1 M phosphate buffer pH 6·8) was allowed to absorb for 5 hr. Because of the mucilage, only a part of the solution could be taken up. The stem was then inserted into water and the whole plant kept at 20° under the illumination of 20,000 lx with mercury lamps. The feeding to *C. intybus* was carried out with the cuttings consisting of stem (*ca.* 10 cm in height) and 7–8 heads in bud (heads in bloom are not suitable because they blossom early morning and wither before noon). The cut end of four cuttings (total weight of heads 2·8–3·1 g) was inserted into 3 ml of labelled cinnamic acids solution dissolved in 0·2 M phosphate buffer pH 7·0, and the whole plant placed for 18·5 hr under the same experimental conditions as in *D. odora*.

Isotope competition experiments. The experiment A in Table 5 was carried out using three inflorescences in a pre-flowering stage, from which leaves and almost all the stem were detached. The short stalk of the stem was immersed in a saturated solution of each trapping compound (0·2 ml) and stood for 5 hr until the solution was completely absorbed. A solution of 2-^{14}C-phenylalanine (0·5 μc/μmole, 0·02 M) was then administered up to the end of metabolic period of 20 hr. In the experiment B, *ca.* 1 ml of 0·01 M non-labelled *p*-coumaric acid or caffeic acid was continuously fed for 20 hr to three cuttings with inflorescences in full-bloom. Radioactive cinnamic acid solution (0·025 M, *ca.* 1 ml) was then supplied until the completion of metabolism (20 hr). In both experiments the plants were placed under the same experimental conditions, as in Tables 2–4, and all the compounds were dissolved in 0·1 M phosphate buffer pH 6·8.

Isolation of the glucosides. Daphnin was isolated from three organs of *D. odora* (stem, leaves and inflorescence), daphnetin-8-glucoside from the inflorescence of this plant and cichoriin from the heads of *C. intybus*. The tissues were extracted with 50 vol. of 25% EtOH and then with equal volume of H_2O at 100°. The combined extracts were brought to dryness and extracted with boiling EtOH. After EtOH was replaced by H_2O, the solution was thoroughly washed with EtOAc, and the concentrate of aq. phase then chromatographed on Whatman No. 1 paper with water-saturated *n*-BuOH for 48 hr. The corresponding bands were eluted with 50% EtOH and the eluate concentrated to *ca.* 2 ml. In the competition experiment A with *D. odora*, further chromatography with *n*-BuOH–HOAc–H_2O (4:1:2) (BAW) was needed to remove a trace amount of radioactive phenylalanine. The crystals obtained from the concentrate were recrystallized from water more than three times.

Estimation of daphnin and daphnetin-8-glucoside in the tissue. A measured volume of the combined extract mentioned before was chromatographed in BAW in the first direction and with 5% HOAc in the second. The area of daphnin and daphnetin-8-glucoside was cut out and the paper strip eluted with 50% EtOH. After EtOH was replaced by H_2O, a part of the solution was used for fluorometric estimation of daphnetin-8-glucoside,[11,12] and another part allowed to hydrolyse in 0·2 N HCl for 2 hr at 100° to liberate daphnetin, which was extracted with EtOAc and determined spectrophotometrically at 330 nm in 50% EtOH ($\epsilon = 1{\cdot}24 \times 10^4$). The amount of daphnin was obtained by subtracting the molar value of daphnetin-8-glucoside from that of daphnetin.

The specific radioactivity measurement. The purified sample was dissolved in a definite volume of 50% EtOH (in general 5 mg in 1 ml), and the amount was determined spectroscopically (ϵ in 50% EtOH: daphnin; $1{\cdot}28 \times 10^4$ at 314 nm, daphnetin-8-glucoside; $1{\cdot}55 \times 10^4$ at 326 nm, cichoriin; $1{\cdot}02 \times 10^4$ at 343 nm) with a part of solution, then the radioactivity measured using residual solution with a liquid scintillation spectrophotometer.

Radioactive compounds. Cinnamic acids (cinnamic; 53 μc/mole, *p*-coumaric; 56 μc/m/mole, caffeic; 51 μc/m-mole) were synthesized from 2-^{14}C-malonate and the corresponding aldehydes according to ordinary procedures. Phenylalanine (2-^{14}C-, 0·5 μc/μmole) was commercially obtained.

Key Word Index—Daphne odora; Thymeleaceae; *Cichorium intybus*; Compositae; biosynthesis; coumarins; daphnetin; aescutetin.