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BIOSYNTHESIS OF COUMESTROL IN *PHASEOLUS AUREUS*

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Abstract—Comparative feeding experiments have been performed to investigate the biosynthesis of coumestrol in mung bean seedlings (*Phaseolus aureus* Roxb.). 4′,7-Dihydroxyisoflavone-[4-^{14}C] (daidzein) and 2′,4,4′-trihydroxychalcone-[β-^{14}C] were good precursors for coumestrol, whereas *o*-coumaric acid-[3-^{14}C] was incorporated to a small extent and 3-(4-hydroxyphenyl)-4,7-dihydroxycoumarin-[4-^{14}C] was not incorporated at all. Although 2,2′,4,4′-tetrahydroxychalcone-[6T,βT] was incorporated, this compound does not appear to be a natural intermediate. Dilution analyses with 2′,4′,7-trihydroxyisoflavone suggests that 2′-hydroxylation of daidzein or dihydrodaidzein is a probable step in the biosynthesis of coumestrol.

INTRODUCTION

Coumestrol (I), the simplest naturally occurring coumestan,[1] is found in many leguminous plants and is of particular interest because of its pronounced oestrogenic properties.[2] Earlier communications[3,4] have reported that coumestrol is biogenetically an isoflavonoid and not a coumarin. Feeding experiments in lucerne (*Medicago sativa* L.) have demonstrated that acetate, cinnamic acid and 2′,4,4′-trihydroxychalcone-4′-glucoside are incorporated into coumestrol in the same manner as they are into isoflavones[5] and, in addition, the isoflavone daidzein (4′,7-dihydroxyisoflavone) (II) can act as a precursor.[6] The conversion of 4′,7-dihydroxyisoflavanone and 4′,7-dihydroxyflavanone to coumestrol in mung bean (*Phaseolus aureus* Roxb.) has also been noted.[7] The later transformations in the biosynthetic pathway have, however, not yet been elucidated. Problems still remaining concern the introduction of the carbonyl group and the formation of the furan ring. We now report the results of feeding experiments designed to clarify these points.

RESULTS

Intact mung bean (*Phaseolus aureus* Roxb.) seedlings were used for incorporation experiments, the labelled compounds being administered as sodium salts in aqueous solution via the

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[1] A. L. Livingston, S. C. Witt, R. E. Lundin and E. M. Bickoff, *J. Org. Chem.* **30**, 2353 (1965). The alternative name "coumestone" has been proposed for the coumaronocoumarins by W. D. Ollis, in *Recent Advances in Phytochemistry* (edited by T. J. Mabry, R. E. Alston and V. C. Runeckles), p. 328, Appleton-Century-Crofts, New York (1968).

[2] E. M. Bickoff, A. L. Livingston and A. N. Booth, *Arch. Biochem. Biophys.* **88**, 262 (1960).

[3] H. Grisebach and W. Barz, *Z. Naturforsch.* **18b**, 466 (1963).

[4] H. Grisebach and W. Barz, *Z. Naturforsch.* **19b**, 569 (1964).

[5] H. Grisebach and G. Brandner, *Z. Naturforsch.* **16b**, 2 (1961).

[6] W. Barz and H. Grisebach, *Z. Naturforsch.* **21b**, 1113 (1966).

[7] H. Zilg and H. Grisebach, *Phytochem.* 7, 1765 (1968).

roots. The labelled compounds tested for precursor efficiency were 3-(4-hydroxyphenyl)-4,7-dihydroxycoumarin-[4-^{14}C] (III), daidzein-[4-^{14}C], 2′,4,4′-trihydroxychalcone-[β-^{14}C] (IV),[8] 2,2′,4,4′-tetrahydroxychalcone-[6T,βT] (V) and *o*-coumaric acid-[3-^{14}C].[9]

The 3-aryl-4-hydroxycoumarin and daidzein were both prepared from 2,4-dihydroxyphenyl-4-methoxybenzylketone-[carbonyl-^{14}C], the product of a Hoesch reaction between resorcinol and *p*-methoxybenzylcyanide-[cyano-^{14}C]. Their preparations followed established procedures for the synthesis of 3-aryl-4-hydroxycoumarins[10] and isoflavones.[11]

The preparation of the tetrahydroxychalcone by the normal chalcone synthesis[12] from resacetophenone and 2,4-dihydroxybenzaldehyde proceeded in only very poor yields, amounts which could, however, be increased to about 7 per cent by the use of tetraacetylglucose as a blocking group for the 4-hydroxyl of resacetophenone. This group was conveniently lost during the subsequent isolation of the chalcone from the reaction mixture. Tritiated 2,4-dihydroxybenzaldehyde was prepared by a modified Wilzbach procedure.[13] The experimentally more convenient method of introducing label by platinum-catalysed exchange with tritiated water[14] was inapplicable in this instance. NMR analysis of the product from heterogeneous exchange with Pt/D_2O showed that label was introduced only into positions 3 and 5 of the aldehyde, positions at which hydrogen may be labile at physiological pH's.[15] Label introduced into these positions by the Wilzbach procedure was removed by base-catalysed exchange. The proportion of label present in the formyl group was assessed by degradation of the methylated aldehyde to 2,4-dimethoxybenzoic acid by alkaline permanganate; the aldehyde contained 76 per cent of its label at C-6 and 24 per cent in the formyl group. As internal standard, DL-phenylalanine-[1-^{14}C] was fed simultaneously with the T-labelled chalcone.

Two sets of experiments were conducted, a preliminary series using 8-day-old seedlings, the roots of which were briefly immersed in aqueous sodium hypochlorite prior to the administration of the labelled substrate and a second series using 10-day-old seedlings which had been grown under aseptic conditions.[16] After an incubation period of 24 hr the plant material was worked up and coumestrol and daidzein were isolated from each extract. These compounds were purified to constant specific activity by the methods described in the Experimental and for each compound the dilution (specific activity of substrate/specific activity of metabolite) and percentage incorporation were measured. The combined results are summarized in the table.

The results from the preliminary experiments clearly indicated daidzein to be the best precursor of coumestrol. This suggested that hydroxylation of daidzein to 2′,4′,7-trihydroxyisoflavone (VI) might be a step involved in the biosynthetic sequence to coumestrol. In consequence, a number of dilution analyses using inactive 2′,4′,7-trihydroxyisoflavone were carried out in the second series of experiments, the results of these are also given in Table 1. The incorporation and dilution data in the second series of experiments were far better than those obtained from the preliminary experiments. Low activity incorporations may be attributable

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[8] H. GRISEBACH and L. PATSCHKE, *Chem. Ber.* **93**, 2326 (1960); **95**, 2098 (1962).

[9] (*a*) K. O. VOLLMER and H. GRISEBACH, *Z. Naturforsch.* **21b**, 435 (1966); (*b*) H. GRISEBACH and H. J. GRAMBOW, *Phytochem.* **7**, 51 (1968).

[10] O. H. EMERSON and E. M. BICKOFF, *J. Am. Chem. Soc.* **80**, 4381 (1958).

[11] R. N. JYER, K. H. SHAH and K. VENKATARAMAN, *Proc. Indian Acad. Sci.* **33A**, 116 (1951).

[12] T. A. GEISSMAN and R. O. CLINTON, *J. Am. Chem. Soc.* **68**, 697 (1946).

[13] H. WOLLENBERG and M. WENZEL, *Z. Naturforsch.* **18b**, 8 (1963).

[14] W. G. BROWN and J. L. GARNETT, *J. Am. Chem. Soc.* **80**, 5272 (1958).

[15] E. S. HAND and R. M. HOROWITZ, *J. Am. Chem. Soc.* **86**, 2084 (1964); G. W. KIRBY and L. OGUNKOYA, *J. Chem. Soc.* (c), 6914 (1965).

[16] W. BARZ, CH. ADAMEK and J. BERLIN, *Phytochem.*, in press.

TABLE 1. INCORPORATION OF LABELLED PRECURSORS INTO COUMESTROL, DAIDZEIN AND 2′,4′,7-TRIHYDROXYISOFLAVONE IN *Phaseolus aureus* Roxb.

Substrate	Preliminary experiments				Aseptic plants*				
	Coumestrol		Daidzein		Coumestrol		Daidzein		2′,4′,7-Trihydroxyisoflavone (percent incorp. by dilution analysis)†
	Dilution	Incorp. (%)	Dilution	Incorp. (%)	Dilution	Incorp. (%)	Dilution	Incorp. (%)	
3-(4-Hydroxyphenyl)-4,7-dihydroxycoumarin-[4-^{14}C]	No incorp.		No incorp.		No. incorp.		No incorp.		—
Daidzein-[4-^{14}C]	454	0·0014	4·4	1·25	59	0·17	20	0·86	0·042
o-Coumaric acid-[3-^{14}C]	No incorp.		No incorp.		3940	0·0014	No incorp.		—
2′,4,4′-Trihydroxychalcone-[β-^{14}C]	945	0·0010	299	0·0049	29	0·026	12	0·13	0·0012
2,2′,4,4′-Tetrahydroxychalcone-[6T,βT]	T No incorp.		T No incorp.		T 87	0·025	T No incorp.		T 0·056
+ DL-Phenylalanine-[1^{14}C] T/^{14}C = 0·026‡	^{14}C 1900	0·0034	^{14}C 2400	0·021	^{14}C 313 T/^{14}C = 0·093	0·59	^{14}C 415	0·74	^{14}C 0·086 T/^{14}C = 1·84

* See note in Results.

† As a consequence of the experimental procedure used (see Experimental), these figures must be regarded as *minimum* percentage incorporations.

‡ T/^{14}C represents $\frac{\text{specific activity chalcone}}{\text{specific activity phenylalanine}}$, and is corrected for loss of β-T activity on incorporation into coumestrol.

in the preliminary experiments to three causes: (1) the seedlings used were younger, and the isoflavone content was therefore much lower, (2) the brief treatment with sodium hypochlorite may have damaged the plants' metabolism, (3) the contribution from microbial contamination cannot be regarded as negligible.[16]

For convenience, the experiments using aseptically grown plants were conducted in two batches. The first consisted of two experiments, the administration of the two chalcones and the second batch involved the feeding of the 3-aryl-4-hydroxycoumarin, daidzein and *o*-coumaric acid. Although every effort was made to grow the plants under exactly similar conditions, the results of these experiments cannot strictly be compared between batches. The dilution figures do indicate that differences existed between the two batches of plants, since daidzein was diluted to a greater extent than 2′,4,4′-trihydroxychalcone on incorporation into coumestrol, whereas the reverse was true in the case of the preliminary experiments, which were conducted with plants grown in a single batch. Allowance has been made for this difference in the interpretation of the results (see Discussion).

DISCUSSION

Before any feeding experiments had been conducted to study the biosynthesis of coumestans, Bate-Smith suggested[17] that daidzein might be a precursor of coumestrol. He envisaged hydroxylation of daidzein at the 2-position to give, via tautomerism, 3-(4-hydroxyphenyl)-4,7-dihydroxycoumarin, which might then be converted to coumestrol in the manner shown in Fig. 1. Daidzein has been shown to be incorporated into coumestrol in *Medicago sativa* L.,[6] and the present studies have demonstrated that this also occurs in *Phaseolus aureus* Roxb. However, the data clearly indicate that daidzein is converted to coumestrol by a route which does not involve the intermediacy of 3-(4-hydroxyphenyl)-4,7-dihydroxycoumarin. This compound, although metabolized by the plant,* is not incorporated into coumestrol.

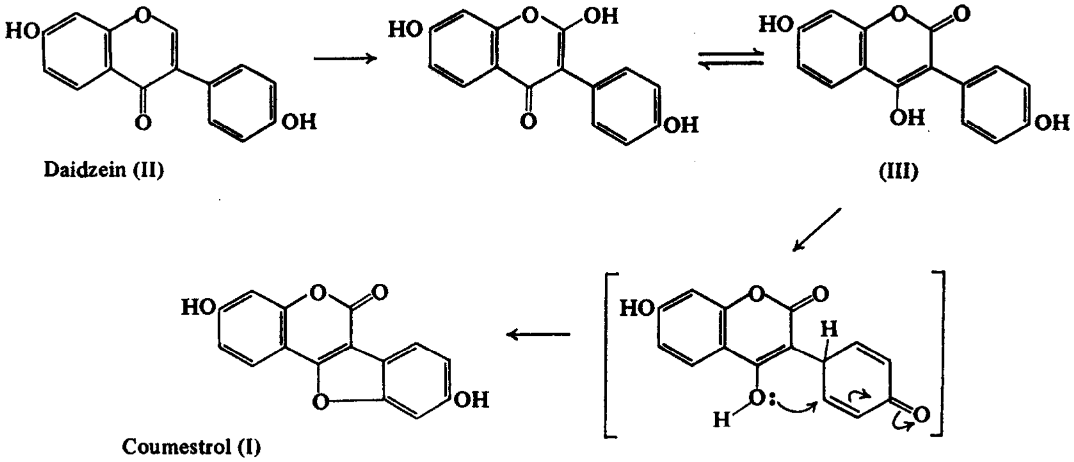

FIG. 1. BIOSYNTHESIS OF COUMESTROL FROM DAIDZEIN AS POSTULATED BY BATE-SMITH.[17]

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* Evidence for the metabolism will be presented in another paper.

[17] E. C. BATE-SMITH, in *The Pharmacology of Plant Phenolics* (edited by J. W. FAIRBAIRN), p. 69, Academic Press, London (1959).

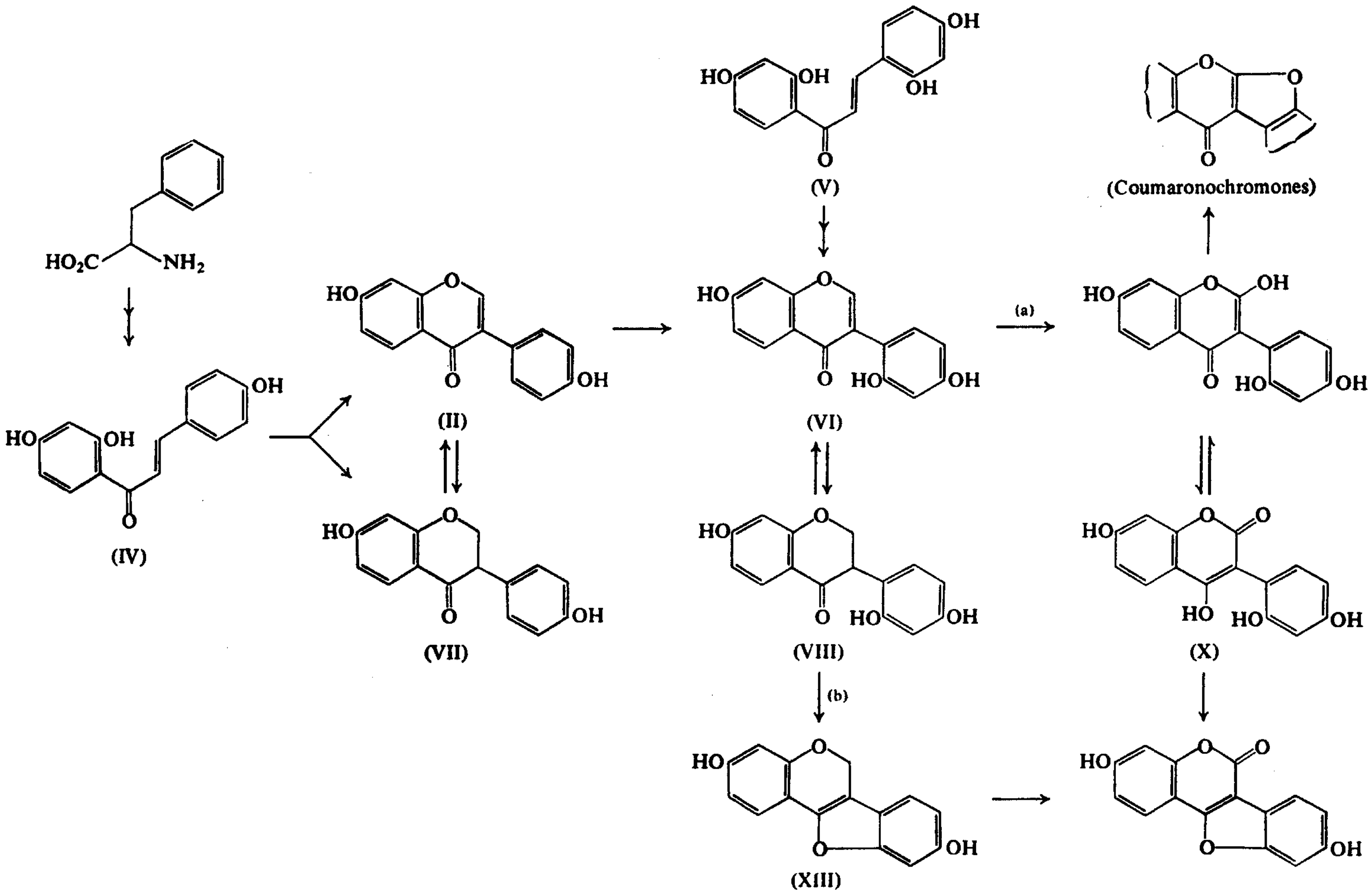

FIG. 2. POSSIBLE ROUTES FOR THE BIOSYNTHESIS OF COUMESTROL IN *Phaseolus aureus* Roxb.

Of the compounds chosen as substrates, daidzein, as measured by the dilution value and incorporation (see note in Results section), is the best precursor of coumestrol. Coupled with the other results, namely (*a*) the very low dilution of label from 2′,4,4′-trihydroxychalcone, (*b*) the higher dilution of label from 2,2′,4,4′-tetrahydroxychalcone and (*c*) the very high dilution of *o*-coumaric acid label, it can be assumed that the 2′-hydroxyl group (isoflavonoid numbering) which appears to be necessary for the formation of the furan ring of coumestrol, is introduced neither before nor at the chalcone stage, but at a later step in the biosynthetic sequence. This may be compared with the recent observation[9b] that the 2′-hydroxyl group of datiscetin (2′,3,5,7-tetrahydroxyflavone) is apparently introduced at the chalcone stage in the biosynthesis. It seems very likely that 2′-hydroxylation of daidzein could be a reaction in the pathway, and the significant incorporation of label from daidzein-[4-^{14}C] into 2′,4′,7-trihydroxyisoflavone as determined by the dilution analyses is useful evidence that such a transformation can occur. At this stage, however, we cannot differentiate between two possible routes regarding this hydroxylation. Since there is probably ready interconversion of isoflavones and isoflavanones *in vivo*[18] hydroxylation of 4′,7-dihydroxyisoflavanone (dihydrodaidzein) (VII) to 2′,4′,7-trihydroxyisoflavanone (VIII) may be the actual pathway involved.

The T/^{14}C ratio of 1·84 observed in 2′,4′,7-trihydroxyisoflavone during the biosynthesis of coumestrol from 2,2′,4,4′-tetrahydroxychalcone-[T] and phenylalanine-[^{14}C] is some seventy times as large as that of the substrate mixture (0·026), whereas coumestrol from this experiment has a T/^{14}C ratio of 0·093. If 2′,4′,7-trihydroxyisoflavone is a precursor of coumestrol, these figures appear incompatible. However, these figures become explicable if we postulate that phenylalanine, via the trihydroxychalcone, is converted quickly and directly to daidzein (or dihydrodaidzein) and then to 2′,4′,7-trihydroxyisoflavone, whereas the tetrahydroxychalcone is transformed much more slowly to 2′,4′,7-trihydroxyisoflavone. If this is the case, the pool of the trihydroxyisoflavone becomes labelled first with ^{14}C and later with tritium. This would explain the high proportion of tritium in the trihydroxyisoflavone after 24 hr compared with coumestrol after this time. These transformations are included in Fig. 2. The trihydroxychalcone, as demonstrated in earlier experiments,[4] is an extremely good precursor of daidzein, the dilution being about one-third that of the same compound on conversion to coumestrol.

Our present results are in accord with two possible sequences (Fig. 2) for the biosynthesis of coumestrol, both involving the 2′-hydroxylation of daidzein to 2′,4′,7-trihydroxyisoflavone (or of dihydrodaidzein to 2′,4′,7-trihydroxyisoflavanone). Route (*a*) involves conversion of the trihydroxyisoflavone to 3-(2,4-dihydroxyphenyl)-4,7-dihydroxycoumarin (X) by 2′-hydroxylation, by analogy with the proposal of Bate-Smith,[17] and then dehydration of this compound to coumestrol. There is insufficient evidence at this stage to suppose that coumaronochromones such as lisetin[19] (XI) are intermediates[20] in coumestan biosynthesis. The coumaronochromones may in fact be synthesized instead of 5-hydroxycoumestans (isoflavonoid numbering), only two of which have so far been isolated,[21] viz. wedelolactone (XII) and desmethylwedelolactone, if we consider both coumestans and coumaronochromones to be derived from the 3-aryl-4-hydroxycoumarins and their 2-hydroxyisoflavone tautomers.

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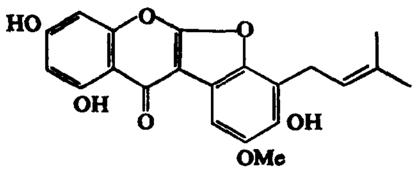
[18] H. Grisebach and H. Zilg, *Z. Naturforsch.* **23b**, 494 (1968).

[19] C. P. Falshaw, W. D. Ollis, J. A. Moore and K. Magnus, *Tetrahedron Suppl.* 7, 333 (1966).

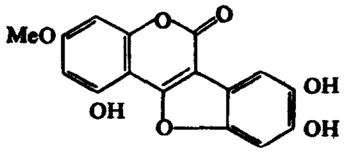
[20] C. P. Falshaw, R. A. Harmer, W. D. Ollis, R. E. Wheeler, V. R. Lalitha and N. V. Subba Rao, *J. Chem. Soc.* (c), 374 (1969).

[21] A. C. Jain, V. K. Rohatgi and T. R. Seshadri, *Tetrahedron* **23**, 2499 (1967).

The 5-hydroxyl substituent is necessary for the 2-hydroxyisoflavone tautomeric form to be significant.[22]



(XI)



(XII)

Route (*b*) is by cyclization of 2′,4′,7-trihydroxyisoflavanone (perhaps via reduction of 2′,4′,7-trihydroxyisoflavone) to 3,9-dihydroxypterocarp-6a-en (XIII) (4′,7-dihydroxy-3,4-dehydropterocarpan according to isoflavonoid nomenclature) which can be easily oxidized at the allylic position to coumestrol. The feasibility of the process has been demonstrated by *in vitro* chemical studies.[23] In addition, the co-occurrence of isoflavonoid compounds, for example coumestans, pterocarp-6a-ens and pterocarpans in *Swartzia madagascarensis*[24] and isoflavones, isoflavanones, rotenoids, pterocarpans and coumestans in *Pachyrrhizus erosus*[25] suggests that such biosynthetic relationships must exist amongst these groups of compounds. Attempts to synthesize labelled 4′,7-dihydroxy-3,4-dehydropterocarpan have so far given only unsatisfactory results.[23]

EXPERIMENTAL

Ultra violet spectra were measured in methanol solution, and i.r. spectra in the form of KBr discs. TLC was carried out using 0·4 mm layers of silica gel (Merck Kiesel-gel G) in: A, toluene–HCO_2Et–HCO_2H, 5:4:1; B, benzene–EtOAc–MeOH, 6:4:1; C, benzene–EtOAc–MeOH–petrol. ether (60–70°), 6:4:1:7; and E, $CHCl_3$–iso–propanol, 10:1. Radiochemicals were purchased from the Radiochemical Centre, Amersham. Radioactive samples were counted with a Beckman LS 100 liquid scintillation spectrometer, in a PPO-naphthalene-dioxan scintillator solution. Efficiencies were measured by internal standardization using toluene-[^{14}C] or *n*-hexadecane-[1,2-T] standards.

Plant Material and Feeding Techniques

Phaseolus aureus Roxb. seeds were obtained from Henselwerk, Magstadt. These were moistened with water and allowed to germinate in the dark at 25° for 48 hr, after which time they were transferred to a growth cabinet and grown as previously described.[26] 8-day-old plants (thirty) were used for the preliminary feeding experiments. The roots were briefly immersed (1 min) in an aqueous solution of NaClO (2·5 % free chlorine), and washed thoroughly with sterilized water before administration of the precursor solutions. Other feeding experiments were carried out using thirty 10-day-old seedlings which had been grown under aseptic conditions.[16] The labelled compounds (1 mg, 2–10 μc) were dissolved in the minimum amount of 0·1 % NaOH, 0·05 M phosphate buffer (pH 8·5, 4 ml) was added, and this solution was then fed to the roots of the seedlings for a period of 24 hr. Sterilized water was added as required during the incubation period. During this time, 80–95 % of the administered activity was absorbed.

Isolation of Metabolites

The plants were homogenized in a Waring blendor with H_2O (50 ml) and the homogenate was allowed to stand at room temp. for 3 hr so that endogeneous glycosidases[27] might hydrolyse glycosides in the extract. The mixture was then poured into boiling ethanol (400 ml). This was boiled for 10 min, cooled and filtered. The

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[22] A. P. Johnson and A. Pelter, *J. Chem. Soc.* (c), 606 (1966).

[23] P. M. Dewick, W. Barz and H. Grisebach, *Chem. Comm.* 466 (1969).

[24] S. H. Harper, A. D. Kemp, W. G. Underwood and R. V. M. Campbell, *J. Chem. Soc.* (c), 1109 (1969).

[25] L. Crombie and D. A. Whiting, *J. Chem. Soc.* 1569 (1963).

[26] W. Barz and B. Roth-Lauterbach, *Z. Naturforsch.* **24b**, 638 (1969).

[27] A. B. Beck, *Australian J. Agri. Res.* **15**, 223 (1964).

residue was twice re-extracted with hot ethanol (200 ml) and the combined extracts were evaporated to dryness. The solid was washed with petrol. ether (60–70°, 5 × 25 ml), suspended in water (50 ml) and extracted with ether (6 × 30 ml). The etheral extract was concentrated, applied to 6 TLC plates and developed in solvent C. Fluorescent bands corresponding to coumestrol and daidzein were separately eluted with acetone.

(*a*) *Coumestrol.* The eluate was further purified by TLC (solvent E), and the coumestrol content measured by means of its u.v. absorption at 343 nm (log ε 4·451). Typically, 6–10 μg were isolated from thirty 8-day-old plants, and 45–60 μg from thirty 10-day-old aseptic plants. Carrier coumestrol (20 mg) was added and the whole was permethylated[6] with MeI/K_2CO_3 in dry acetone. Coumestrol dimethyl ether was purified to constant specific activity by TLC (solvent D), sublimation at 180°/0·05 nm, crystallization from MeOH and a second sublimation.

(*b*) *Daidzein.* The eluate was chromatographed in solvents E and B and its daidzein content measured by its u.v. absorption at 248 nm (log ε = 4·439). Typically, 14–17 μg were isolated from thirty 8-day-old plants, and 50–80 μg from thirty 10-day-old aseptic plants. After the addition of carrier (20 mg), daidzein was purified to constant specific activity by permethylation and TLC as above, sublimation at 145°/0·05 nm, crystallization from aq. MeOH, and a second sublimation.

(*c*) *Dilution analyses with 2′,4′,7-trihydroxyisoflavone.* Since TLC with solvent C, the initial separation step employed with the plant extract, did not separate daidzein and 2′,4′,7-trihydroxyisoflavone, a rather broad band in the daidzein region was eluted with acetone. Inactive 2′,4′,7-trihydroxyisoflavone[28] (1–2 mg) was added to this eluate, which was then rechromatographed on TLC in solvent E. Daidzein and 2′4′,7-trihydroxyisoflavone were separately eluted, daidzein being purified as described above, and 2,′4′,7-trihydroxyisoflavone, after dilution with further carrier (10 mg) by permethylation and repeated TLC (solvent D, $CHCl_3$–isopropanol, 20:1; benzene–EtOAc, 3:1; benzene–EtOH, 92:8) and sublimations (135°/0·05 nm) until constant specific activity was attained.

Labelled Substrates

The preparations of 2′,4,4′-trihydroxychalcone-[β-^{14}C][8] and *o*-coumaric acid-[3-^{14}C][9] have been reported elsewhere.

2,4-*Dihydroxyphenyl-4-methoxybenzylketone*-[*carbonyl*-^{14}C]. Anisyl alcohol (2 ml) was stirred vigorously for 15 min with conc. HCl (5 ml), the mixture was centrifuged and the lower layer of anisyl chloride was removed and dried ($CaCl_2$) for 30 min. Anisyl chloride (500 mg), dry KCN (128 mg), dry $K^{14}CN$ (3·0 mg, 2 mc), dry KI (25 mg) and dry acetone (5 ml) were heated under reflux at 70° with stirring for 18 hr. After cooling, the solids were filtered off, washed with acetone, and the acetone solution was evaporated to a small bulk. This was applied to five TLC plates, which were developed in ether–petrol. ether (60–70°), 1:2. The band at R_f 0·3–0·4, clearly visible to the naked eye, was eluted with Na–dry ether (30 ml) through a column of powdered $CaCl_2$ (about 2 cm), into a dry, two-necked flask. Dry resorcinol (250 mg) and anhydrous $ZnCl_2$ (400 mg) were added, and the solution was saturated with dry HCl at 0°. After standing at 0° overnight, the ether was boiled off, water (20 ml) was added and the mixture was heated under reflux for 2 hr. Extraction with EtOAc (5 × 20 ml) gave a crude product which was purified by TLC (solvent A). The product at R_f 0·8 (yellow fluorescence in u.v.) was eluted with MeOH, and the eluate was evaporated to give a gum (326 mg) which was dried *in vacuo* over $CaCl_2$ and KOH.

Daidzein-[4-^{14}*C*]. The preparation of daidzein-[4-^{14}C] from the above desoxybenzoin is described in another communication.[16]

3-(4-*Hydroxyphenyl*)-4,7-*dihydroxycoumarin*-[4-^{14}*C*]. The above crude desoxybenzoin (57 mg), anhydrous K_2CO_3 (1 g), dry acetone (10 ml) and ethyl chloroformate (0·16 ml) were heated under reflux at 70° for 21 hr. Water (10 ml) was added, the solution was acidified with HCl and then extracted with EtOAc (4 × 20 ml). After removal of solvent, the extract was dissolved in MeOH (10 ml) and heated at 70° for 1 hr with 10% aq. KOH (2 ml). The mixture was cooled, diluted with water (10 ml), acidified with HCl and extracted with EtOAc (3 × 20 ml). 3-(4-Methoxyphenyl)-4,7-dihydroxycoumarin-[4-^{14}C] (12 mg) was obtained from the extract by TLC (solvent A) and was purified repeatedly by TLC using solvent A and benzene–acetic acid–water, 125:72:3. The product was demethylated by heating at 110° for 12 hr with Ac_2O (0·5 ml) and HI (1 ml). The mixture was cooled, diluted with water (10 ml), decolorized by the addition of solid $Na_2S_2O_5$, and extracted with EtOAc (5 × 10 ml). After washing with water (20 ml), the extract was concentrated and purified by paper chromatography (Whatman 3MM) using benzene–acetic acid–water (125:72:3). 3-(4-Hydroxyphenyl)-4,7-dihydroxycoumarin-[4-^{14}C] was eluted from the chromatograms with MeOH, and purified to constant specific activity by paper chromatography as above. Yield 5 mg, spec. activity $1·76 \times 10^9$ dpm/mM (0·793 mc/mM). M.p. 330° (decomp.), ν_{max} 3300, 1680, 1640, 1600, 1570, 1500 cm^{-1}, λ_{max} 320 nm (log ε 4·140).

2,4-*Dihydroxybenzaldehyde*-[6*T*,*formyl*-*T*]. 2,4-Dihydroxybenzaldehyde (40 mg) was labelled with tritium by a modified Wilzbach method,[13] in which the aldehyde, absorbed on finely powdered quartz, was exposed to 5 c of tritium gas for a period of 4 weeks. Labile tritium was removed by dissolving the compound in methanol and evaporating the solution to dryness. This was repeated a number of times. Tritium at positions

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[28] W. B. WHALLEY, *J. Chem. Soc.* 3366 (1953).

3 and 5[15] was removed by heating at 60° with 10% aq. NaOH for 30 min in a stream of N_2. The mixture was acidified and extracted with ethyl acetate. This alkaline exchange was then repeated. The aldehyde was purified to constant specific activity by TLC using the solvents C, E, benzene–EtOH, 92:8, acetone–petrol. ether (60–70°), 1:3, and ether–petrol. ether (60–70°), 3:2. After dilution with inactive aldehyde (90 mg), the whole was sublimed at 100°/0·01 mm. Yield 110 mg, spec. activity $1{\cdot}81 \times 10^9$ dpm/mM (0·815 mc/mM).

2,2′,4,4′-*Tetrahydroxychalcone*-[*6T,βT*]. Resacetophenone-4-tetraacetylglucoside[29] (354 mg) and 2,4-dihydroxybenzaldehyde-[6T,formyl-T] (107 mg) were suspended in EtOH (1 ml) and 100% w/v aq. KOH (10 ml) was added. The mixture was heated at 100° under a stream of N_2 for 3 hr, stoppered and allowed to stand at room temp. overnight. The solution was cooled in ice, acidified with HCl and extracted with ether (3 × 30 ml). The chalcone was separated by TLC (solvent C) and purified to constant specific activity by TLC in the solvents acetone–petrol. ether (60–70°), 4:1, B, and C. Yield 15 mg, spec. activity $1{\cdot}81 \times 10^9$ dpm/mM (0·815 mc/mM). M.p. 330° (decomp.), ν_{max} 3250, 1610, 1580, 1500, 970 cm^{-1}, λ_{max} 388 nm ($\log \epsilon = 4{\cdot}480$). The chalcone could be crystallized from aq. MeOH, but since it is very sensitive to atmospheric oxidation, it was best manipulated in methanolic solution.

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