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BIOSYNTHESIS OF ACACETIN IN *ROBINIA PSEUDACACIA*: INCORPORATION OF MULTIPLE LABELLED *p*-METHOXYCINNAMIC ACID

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Abstract—After hydrolysis of the flavonoid glycosides from leaves of *Robinia pseudacacia* L. the following aglycones were identified: acacetin, apigenin, kaempferol and quercetin. In some samples of older leaves, the synthesis of acacetin and apigenin was stimulated strongly by cutting, whereas no increase in the amounts of kaempferol and quercetin was observed. This stimulation was not found in younger leaves. When older leaves were incubated for 64 hr with *p*-methoxycinnamic acid-[β-^{14}C-methyl-^{14}C-methyl-T] the T/^{14}C ratio in acacetin was only 23 per cent of that in the precursor, a result which showed that demethylation takes place in the leaves. However, when younger leaves were incubated with *p*-methoxycinnamic acid-[β-^{14}C-methyl-T] for shorter periods of time (8, 20 and 30 hr) the T/^{14}C ratio in acacetin was almost the same as that in the precursor. The result proves intact incorporation of *p*-methoxycinnamic acid into acacetin.

INTRODUCTION

IN AN earlier investigation we tried to determine at what stage in the biosynthesis of 4'-methoxyflavonoids the methylation of the 4'-hydroxy group occurs.[1] However, during incorporation of *p*-methoxycinnamic acid-[-β-^{14}C-methyl-^{14}C-methyl-T] and 4-methoxy-2',4'-dihydroxychalcone-[β-^{14}C-methyl-^{14}C-methyl-T] into the 4'-methoxyisoflavones, formononetin and biochanin A, in garbanzo seedlings (*Cicer arietinum* L.) a rapid demethylation of the precursors occurred so that it was not possible to draw definite conclusions as to the stage in the biosynthesis at which methylation step occurs.[1]

In this paper, we present evidence that in young leaves of *Robinia pseudacacia* L., demethylation of *p*-methoxycinnamic acid or its metabolites is less rapid than in *Cicer* seedlings and that this acid can be incorporated into acacetin (4'-methoxy-5,7-dihydroxyflavone) without prior demethylation.

RESULTS

Flavonoids in Leaves of Robinia pseudacacia

The main flavone glycoside of the leaves of *R. pseudacacia* is acaciin (acacetin-7-rutinoside).[2,3] Acacetin also occurs as a 7-xylosylrhamnosylglucoside.[4] In our experiments we were interested only in the aglycones present in the leaves. The mixture of the crude glycosides[4] was hydrolysed with 2 N H_2SO_4 and the ether extract of the hydrolysate separated by paper and thin-layer chromatography in several solvent systems (see Experimental). Four different flavonoids were detected on the chromatograms. By chromatographic and spectroscopic

[1] W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **22b**, 627 (1967).
[2] S. HATTORI, *Acta Phytochim. Japan* **2**, 99 (1925); *Chem. Zentr.* **97**, 955 (1926).
[3] G. ZEMPLÉN and L. MESTER, *Magy. Kem. Folyóirat* **56**, 2 (1950); *C.A.* **45**, 7977e (1951).
[4] K. FREUDENBERG and L. HARTMANN, *Liebigs Ann. Chem.* **587**, 207 (1954).

comparisons with reference substances and by degradative studies they were identified as acacetin, apigenin, kaempferol, and quercetin. The 3-rhamnosyl-galactoside-7-rhamnoside of kaempferol (robinin) had previously been isolated from flowers of *R. pseudacacia*,[5] while the occurrence of apigenin and quercetin in this plant has not been described before.

Stimulation of Flavonoid Synthesis by Cutting the Leaves

In preliminary experiments with D,L-phenylalanine-[1-^{14}C] two incubation techniques were compared.

A. The radioactive solution was taken up through the stalks of the intact leaves.
B. The leaves were cut into 3–4-mm broad strips and incubated with labelled phenylalanine in 0·025 M potassium phosphate buffer (pH = 5·9, the isoelectric point of phenylalanine)[6] under efficient aeration and illumination (5 Osram L-Fluora tubes, 40 W).

With both techniques, the incorporation of phenylalanine into acacetin and apigenin was of the order of 0·01 per cent and kaempferol and quercetin also contained significant radioactivity.

However, the *amounts* of acacetin and apigenin isolated per gram of leaves incubated according to method B were respectively 52 and 12 times greater than from method A. In contrast, there was no observable increase in the amounts of kaempferol and quercetin. The strong stimulation of flavone synthesis by cutting the leaves was confirmed in a further experiment with *p*-methoxycinnamic acid as precursor (Table 1).

TABLE 1. QUANTITY OF FLAVONOIDS ISOLATED FROM FRESH LEAVES OF *Robinia* AFTER INCUBATION OF INTACT OR CUT LEAVES WITH LABELLED PHENYLALANINE OR *p*-METHOXYCINNAMIC ACID

	mg Flavonoid/100 g fresh leaves		
	A	B	Date leaves picked
Acacetin	0·75*	39·2*	10 July 1968
	0·62†	39·7†	27 Aug. 1968
	34·0†	—	19 May 1969
	—	34·4†	29 May 1969
	—	40·5†	24 June 1969
Apigenin	0·053*	0·63*	10 July 1968
	0·07†	0·71†	27 Aug. 1968

A: intact leaves; B: cut leaves.
* Incubation with phenylalanine.
† Incubation with *p*-methoxycinnamic acid.

The above-mentioned experiments were carried out in July/August 1968, but later experiments with younger leaves collected in May/June 1969 gave different results. Leaves from the same two trees then contained a much higher concentration of flavonoids and no significant stimulation of flavone synthesis by cutting was observed (Table 1).

[5] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 70, Academic Press, London (1967).
[6] M. H. ZENK, *Z. Pflanzenphysiol.* 53, 404 (1965).

Incorporation of Multiple Labelled p-*Methoxycinnamic Acid into the Flavonoids of* Robinia

A first experiment was carried out with leaves picked in August 1968. One hundred grams of fresh leaves were incubated according to method B with 59·9 μc *p*-methoxycinnamic-acid-[β-^{14}C-methyl-^{14}C-methyl-T] for 64 hr. Acacetin, apigenin, kaempferol and quercetin were isolated and purified to constant specific radioactivity. While kaempferol and quercetin had no detectable radioactivity, both acacetin and apigenin were radioactive. The radioactive acacetin was degraded to phloroglucinol and anisic acid with KOH and anisic acid was further degraded by Schmidt reaction and nitration to 2·4·6-trinitroanisole (see Experimental). The results are shown in Table 2.

The ratio methyl-T/2-^{14}C in acacetin (1:3·12) is only 23 per cent of the ratio methyl-T/β-^{14}C in the *p*-methoxycinnamic acid (1:0·72) fed. It must be concluded, therefore, that a partial demethylation of the cinnamic acid or its metabolites occurred prior to the conversion to acacetin. The ratio methyl-^{14}C/2-^{14}C in acacetin (1:2·97) was 44 per cent of the corresponding ratio in the precursor. A comparison of the T/^{14}C ratios in the methoxy groups of *p*-methoxycinnamic acid and acacetin reveals a relative increase of ^{14}C-radioactivity in the methyl group of acacetin, which could arise by partial reincorporation of the liberated methyl group via the C_1-pool.[1]

TABLE 2. INCORPORATION OF MULTIPLE LABELLED *p*-METHOXYCINNAMIC ACID INTO ACACETIN IN OLDER LEAVES

p-Methoxycinnamic acid†	β-^{14}C*	Methyl-^{14}C	Methyl-T
Activity fed (dpm × 10^{-7})	4·22	3·23	5·87
Relative ratio of activities	0·72	0·55	1·0
Acacetin	**2-^{14}C**	**Methyl-^{14}C**	**Methyl-T**
Activity isolated (dpm)	5570	1867	1787
Relative ratio of activities	3·12	1·05	1·0

* The incorporation of β-^{14}C in this experiment was in the order of 1·3 × 10^{-2} per cent.

† Incubation time 64 hr.

This conclusion is corroborated by the fact that a significant amount of radioactivity was present in apigenin (incorporation rate 0·5 × 10^{-2} per cent, dilution 1000), which was located mainly in C-2, as was shown by alkaline degradation of apigenin to *p*-hydroxybenzoic acid, *p*-hydroxyacetophenone and phloroglucinol.[7]

It was hoped that by using a shorter incubation time and younger leaves the extent of demethylation reaction would be reduced. In the following year three incorporation experiments were therefore carried out with *p*-methoxycinnamic acid-[β-^{14}C-methyl-T] using leaves picked in June from the same two trees as before but with incubation times of 8, 20 and 30 hr. Furthermore, bacterial contamination was kept at minimum by treatment of the leaves with 70 per cent ethanol prior to cutting and by sterilization of all solutions and equipment. After 20 hr a sample of the solution was plated on agar. Only very weak growth of micro-organisms was observed and no radioactive CO_2 was detected when the centrifugate of the incubation mixture of the 30-hr experiment was incubated with *p*-methoxycinnamic acid-[methyl-^{14}C] for 20 hr at 25°.

[7] H. GRISEBACH and W. BILHUBER, *Z. Naturforsch.* **22b**, 746 (1967).

For the final purification of the acacetin samples from the short-time incubation experiments the trimethylether was prepared. The results of the experiments shown in Table 3 clearly demonstrate that practically no demethylation has taken place in the 8-hr experiment and that an intact incorporation of the methoxycinnamic acid into acacetin has occurred under these conditions. Even after 20 and 30 hr the bulk of the precursor must have been incorporated without prior demethylation as indicated by the only slight decrease of the T/^{14}C ratios.

TABLE 3. INCORPORATION OF *p*-METHOXYCINNAMIC ACID-[β-^{14}C-METHYL-T] INTO ACACETIN IN YOUNGER LEAVES

p-Methoxycinnamic acid	Methyl-T		β-^{14}C
Activity fed in each experiment (dpm $\times 10^{-7}$)	6·84		2·21
Methyl-T/β-^{14}C		3·1	
Acacetin trimethylether	8 hr	20 hr	30 hr
T	3550	8350	5560
^{14}C (dpm)	1188	3290	2050
T/^{14}C	3·0	2·5	2·7

The incorporation in all experiments was of the order of 1–2 $\times 10^{-2}$ per cent.

DISCUSSION

Whereas, in experiments with older leaves of *Robinia* and a long incubation period, demethylation of *p*-methoxycinnamic acid or its metabolites is markedly observed, the experiments with younger leaves and shorter incubation times clearly demonstrate that this acid can be incorporated intact into acacetin. This result proves for the first time that the 4′-methoxy group of a flavonoid can originate from the corresponding cinnamic acid. Hess had earlier shown that ferulic and sinapic acid are best incorporated into the correspondingly methylated anthocyanins in *Petunia hybrida*.[8] However, only part of the radioactivity could be found in the methoxyl groups. The intact incorporation of *p*-methoxycinnamic acid into acacetin is in accord with the "Zimtsäurestarthypothese" of Hess,[9] in which it is assumed that the substitution pattern in ring B of flavonoids is already determined at the cinnamic acid stage and a specific enzyme selects a certain cinnamic acid from a given cinnamic acid pool for flavonoid biosynthesis. It must be remembered, however, that in the case of isoflavonoid biosynthesis hydroxychalcones can serve as very good precursors for 4′-methoxylated isoflavones.[1,10]

It is interesting to note that the strong stimulation of flavonoid biosynthesis in leaves by cutting was observed only with leaves picked in July/August 1968 and not with leaves from the same trees picked in May/June 1969. Furthermore, only the formation of acacetin and apigenin was stimulated and there was no increase in the amount of kaempferol and quercetin.

[8] D. HESS, *Planta* **60**, 568 (1964).
[9] D. HESS, *Biochemische Genetik*, p. 89 ff., Springer-Verlag, Berlin (1968).
[10] H. GRISEBACH and G. BRANDNER, *Experientia* **18**, 400 (1962).

This effect cannot entirely be explained by the known stimulation of phenylalanine ammonia lyase[11] under wounding conditions because one would expect such a reaction to effect all four flavonoids. Our data rather suggest further regulatory processes to be involved.[12]

EXPERIMENTAL

Plant Material

Leaves were picked from two trees of *Robinia pseudacacia* L. in the neighbourhood of the Botanical Garden, Freiburg, Germany.

Chromatography

Paper chromatography was performed on Schleicher Schüll 2043 b and Whatman 3 MM with the solvent system (v/v) benzene/acetic acid/water = 125:72:3. For TLC, silica gel G (Merck AG., Darmstadt) was used with the following solvent systems (v/v). (1) Benzene/dioxane/acetic acid = 90:25:4; (2) Benzene/ethyl acetate/methanol = 6:4:1; (3) Benzene/ethyl acetate/acetic acid = 90:5:2·5; (4) Toluene/ethyl formiate/formic acid = 5:4:1; (5) Toluene/ethyl acetate/acetic acid = 9:2:0·5.

Acacetin

Acacetin was synthesized according to a modified method by Seshadri *et al.*[13] 18·6 g (0·065 mol) anisic acid anhydride[14] and 4·3 g (0·026 mol) phloracetophenone were refluxed in 220 ml of dry acetone under N_2 with 21 g of dry K_2CO_3 for 30 hr. The solvent was removed *in vacuo* and the residue extracted with warm H_2O. After filtration the residue was first heated for ½ hr with 5 per cent aqueous K_2CO_3 and then warm 10 per cent acetic acid was added to the slurry. The precipitate was boiled for 4 hr with 5 per cent aqueous K_2CO_3 under N_2. The aqueous extract was combined with the first filtrate and the solution saturated with CO_2. The precipitated reaction product was filtered and washed with water (yield 1·8 g).

The raw product was purified on a polyamide column[15] (Ultramidpulver BASF K 228 BM 2, prewashed with warm 5 per cent acetic acid, H_2O, MeOH and benzene) with the solvent system benzene/methanol/ethylmethylketone (9:4:2). Yield: 0·65 g of chromatographically pure acacetin from 1·8 g raw product. λ_{max} 270 (log ϵ 4·30), 327 (4·31) nm.

Degradation of Acacetin

Thirty milligrams of acacetin was refluxed (160°) with 20 ml of 50 per cent KOH for 25 min. The cold reaction mixture was acidified with 20 per cent H_2SO_4 and extracted four times with Et_2O. The etheral extract was washed with H_2O, dried (Na_2SO_4) and the Et_2O removed. The reaction products were separated by TLC with solvent 3. The zones of phloroglucinol and anisic acid were eluted with MeOH. Phloroglucinol was rechromatographed with solvent 4. Both substances were further purified by vacuum sublimation (0·02 torr). Yield: 6·5 mg (41 per cent) of anisic acid and 4 mg (30 per cent) of phloroglucinol. *p*-Anisic acid was degraded by the Schmidt-reaction.[16] In contrast to the standard procedure no organic solvent was used but the acid was dissolved directly in conc. H_2SO_4. The yield of $BaCO_3$ was 90 per cent.

Anisic acid was also converted to 2,4,6-trinitroanisole.[17] From 38 g (0·25 mmol) anisic acid, 32 mg (52 per cent) 2,4,6-trinitroanisole was obtained, melting point 65°. The product was chromatographically pure (TLC, solvent 5).

Apigenin Trimethyl Ether

Ten milligram (0·035 mmol) of acacetin was refluxed with 20 ml of dry acetone, 1 g dry K_2CO_3 and 1 ml CH_3I. The progress of the reaction was followed by TLC with solvent 1 (apigenin trimethyl ether, R_f = 0·23, acacetin, R_f = 0·35). The reaction was complete after 4 hr. After filtration the residue was washed with acetone and the solvent removed *in vacuo*. The product was purified by TLC with solvent systems 1 and 2 and by vacuum sublimation (0·02 torr).

[11] H. Scherf and M. H. Zenk, *Z. Pflanzenphsyiol.* **57**, 401 (1967).
[12] G. Engelsma, *Planta* **82**, 355 (1968).
[13] S. Grover, A. C. Jain, S. K. Mathur and T. R. Seshadri, *Indian J. Chem.* **1** (9), 382 (1963); *C.A.* **60**, 4094f (1964).
[14] Houben-Weyl, *Meth. Org. Chem.* Bd. 8, S. 477, G. Thieme Verlag, Stuttgart (1952).
[15] K. Egger and M. Keil, *Z. Anal. Chem.* **210**, 201 (1965).
[16] F. B. Fisher and A. N. Bourns, *Can. J. Chem.* **39**, 1736 (1961).
[17] C. L. Jackson and R. B. Earle, *Am. Chem. J.* **29**, 104 (1903).

Isolation and Identification of the Flavonoid Aglycones from Robinia

The isolation of the crude glycosides was carried out according to Freudenberg and Hartmann.[4] From 200 g of fresh leaves picked on 5 June 1968 1·55 g of a crude glycoside mixture was obtained. The glycosides were hydrolysed with 2 N H_2SO_4 and the aglycones extracted with Et_2O. The Et_2O extract was washed (H_2O), dried (Na_2SO_4) and concentrated. The extract was applied to Whatman 3 MM and chromatographed with the solvent given above. The zones of acacetin (R_f 0·82), apigenin (R_f 0·47), kaempferol (R_f 0·36) and quercetin (R_f 0·12) were eluted with methanol and the compounds identified by chromatographic and spectral[5,18,19,20] comparisons with reference samples and by degradation with alkali.

Incubations with Radioactive Precursors

All incubations were carried out with cut leaves in K phosphate buffer of pH 5·9 as described in the text.

(1) 100 g of fresh leaves picked on 27 August 1968, 59·9 μc (9·67 mg) *p*-methoxycinnamic acid-[β-^{14}C-methyl-^{14}C-methyl-T].[1]

(2) Three incubations with 50 g fresh leaves picked on 24 June 1969, 40·73 μc (3·24 mg) *p*-methoxycinnamic acid-[β-^{14}C-methyl-T].[1]

Isolation of the Radioactive Flavonoids

The leaves were rinsed (H_2O) and chopped in a Waring Blendor. The homogenate was extracted with boiling 80 per cent EtOH. The solvent of the extract was removed *in vacuo* and the residue extracted several times with light petroleum. The residue was boiled for 3 hr with 4 N H_2SO_4 and the hydrolysate extracted 5× with Et_2O. The flavonoids in the Et_2O extract were separated and purified by TLC with solvent 1 followed by paper chromatography (twice).

For degradation of acacetin and apigenin and for the preparation of the trimethylether of acacetin the flavones were diluted with inactive material.

Counting of Radioactivity

Radioactivity was determined by one of the following two methods.

(1) T and ^{14}C were counted after combustion of the samples in the gas phase according to the method of Simon *et al.*[21]

(2) The samples were dissolved in MeOH and counted in a dioxane "cocktail" (100 g naphthalene, 5 g PPO in 1000 ml dioxane) in a Beckman LS-100 liquid scintillation counter using two channels. T/^{14}C ratios determined in this work are estimated to be accurate to 5–10 per cent.

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