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FURTHER STUDIES ON THE BIOSYNTHESIS OF FLAVONOIDS IN *DATISCA CANNABINA*

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Abstract—The incorporation of the pairs dihydrogalangin-[G-^{3}H]/5,7,2′-trihydroxyflavanone-7-glucoside-[β-^{14}C] and galangin-[G-^{3}H]/5,7,2′-trihydroxyflavanone-7-glucoside-[β-^{14}C] into datiscetin and galangin in *Datisca cannabina* was investigated. Dihydrogalangin is a good precursor for both datiscetin and galangin, whereas galangin is not a precursor for datiscetin. The chalcone–flavanone isomerase(s) from *D. cannabina* has no specificity for ring B substitution (flavonoid numbering), but the enzyme(s) does not catalyse the isomerisation of either chalcones with resorcinol-type substitution in ring A or chalcone glucosides. Studies on the metabolism of *o*-coumaric acid-[3-^{14}C] prove that this acid is metabolised by the plants and that the bulk of the acid is not trapped in a metabolically inactive pool. Experimental evidence is presented for the light sensitivity of flavonol methyl ethers during thin layer chromatography.

INTRODUCTION

EARLIER work from this laboratory showed that in *Datisca cannabina* L. 2,2′,4′,6′-tetrahydroxychalcone-[β-^{14}C] (I) is a much better precursor for datiscetin (3,5,7,2′-tetrahydroxyflavone) than is cinnamic acid-[β-^{14}C] (II), and that o-hydroxycinnamic acid-[β-^{14}C] (III) is only very poorly incorporated into datiscetin.[1] These results indicated that the 2′-hydroxyl group in datiscetin is not introduced until after formation of the chalcone–flavanone. However, cinnamic acid was a good precursor for galangin (3,5,7-trihydroxyflavone) in the same plant whereas the incorporation of I and III into galangin was negligible.[1]

Since we had found earlier that dihydroflavonols are good precursors of flavonols[2,3] it was of interest to compare the incorporation of dihydrodatiscetin and dihydrogalangin into datiscetin and galangin with the incorporation of 5,7,2′-trihydroxyflavanone (the cyclization product of chalcone, I) into the same products. However, by analogy to the ready aerial oxidation of dihydromorin (3,5,7,2′,4′-pentahydroxyflavanone) to morin,[4] dihydrodatiscetin should be a very labile substance and it was not possible to synthesize it from datiscetin by PEW-reduction.[4] Therefore, in the present work only dihydro-galangin could be tested as precursor for datiscetin and galangin. Furthermore, results on the metabolism of *o*-coumaric acid in *D. cannabina* and on the specificity of the chalcone-flavanone isomerase of this plant are reported.

[1] H. GRISEBACH and H. J. GRAMBOW, *Phytochem.* **7**, 51 (1968).
[2] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **21b**, 45 (1966).
[3] L. PATSCHKE and H. GRISEBACH, *Phytochem.* **7**, 235 (1968).
[4] W. R. CARRUTHERS, R. H. FARMER and R. A. LAIDLAW, *J. Chem. Soc.* 4440 (1957).

RESULTS

Synthesis of Dihydrogalangin-[G-^{3}H] *and Galangin-*[G-^{3}H]

Dihydrogalangin was obtained from galangin[5] by a modified PEW-reduction.[6] The synthetic substance was identical in chromatographic and spectroscopic (u.v., i.r.) properties with authentic pinobanksin.[7] Dihydrogalangin-[^{3}H] was prepared by a modified Wilzbach procedure[8] and purified to constant specific activity. Galangin-[^{3}H] was obtained from dihydrogalangin-[^{3}H] by oxidation with sodium bisulfite.[9] Comparison of the specific activities of galangin and dihydrogalangin showed that 8·5% of the total radioactivity of dihydrogalangin-[^{3}H] was located at C-2 and C-3.

Incorporation of Dihydrogalangin-[^{3}H] *and Galangin-*[^{3}H] *into the Flavonoids of Datisca*

In two parallel experiments the plants were incubated for 48 hr with the mixture dihydrogalangin-[^{3}H]/5,7,2′-trihydroxyflavanone-7-glucoside-β-[^{14}C] and galangin-[^{3}H]/5,7,2′-trihydroxyflavanone-7-glucoside-β-[^{14}C]. Datiscetin and galangin were isolated and purified as described previously.[1] The results recorded in Table 1 show that dihydrogalangin is a good precursor for datiscetin and galangin whereas galangin is not a precursor for datiscetin.

TABLE 1. INCORPORATION OF DIFFERENT PRECURSORS INTO DATISCETIN AND GALANGIN IN *Datisca cannabina*

Precursor or compound	Dilution		Incorporation		Ratio
	^{14}C $\times 10^{-3}$	^{3}H $\times 10^{-3}$	^{14}C % $\times 10^2$	^{3}H % $\times 10^2$	T/^{14}C
Dihydrogalangin-[^{3}H] / Flavanone-[^{14}C]					7
Datiscetin*	23	33	140	39	5
Galangin*	126	41	10	13	22
Galangin-^{3}[H] / Flavanone-[^{14}C]					7
Datiscetin	41	5800	49	0·2	0·05
Galangin	116	12	10	51	68

The values are calculated on the basis of radioactivity taken up by the plants.
* The tritium values are corrected for loss of protons at C-2 and C-3 of dihydrogalangin.

Metabolism of o-*Coumaric Acid in Datisca*

The significance of the result that *o*-coumaric acid is incorporated into datiscetin to only a very small extent[1] is open to question if (1) this acid does not penetrate the cell, or (2) the acid is transformed very rapidly into compounds which are not further metabolized. To clarify this point *o*-coumaric acid-[3-^{14}C] (13,4 μc) was again fed to 3 *Datisca* plants via the roots and after 48 hr the plants were worked up as described in the Experimental. About 80% of the radioactivity taken up by the plants was in the ethanol-soluble fraction and the rest was distributed between the petrol fraction (4%), and the solid residue (6%). The radioactivity in the respiratory CO_2 was not determined. Although no complete analysis

[5] J. KALF and R. ROBINSON, *J. Chem. Soc.* 1968 (1925).
[6] J. C. PEW, *J. Am. Chem. Soc.* **70**, 3031 (1948). H. GRISEBACH and W. BARZ, *Z. Naturforsch.* **21b**, 47 (1966).
[7] G. G. LINDSTEDT, *Acta Chem. Scand.* **4**, 448, 772, 1042 (1950).
[8] H. WOLLENBERG and M. WENZEL, *Z. Naturforsch.* **18b**, 8 (1963).
[9] H. PACHÉCO, *C. R. Acad. Sci., Paris* **251**, 1077 (1960).

of the compounds derived from *o*-coumaric acid was carried out, the following conclusions can be drawn: (1) *o*-coumaric acid is rapidly metabolized by the plants because after 48 hr only about 0·6% of the original activity was present in *o*-coumaric acid reisolated after hydrolysis of the extract; (2) various radioactive bands appeared on the chromatograms. One of these bands was identified as salicylic acid[10] (0·03% incorporation); (3) no labelled coumarin or esters[11] of *o*-coumaric acid with quinic acid or sugars could be found.

Specificity of the Chalcone—Flavanone Isomerase from Datisca

Because of the unusual substitution pattern of the flavonoids in *Datisca* it was of interese to test the substrate specificity of the chalcone-flavanone isomerase[12] from this plant. The optical test[12] was carried out with partially purified enzyme from the youngest leaves of 20-cm high plants. The results are summarized in Table 2. No specificity for the substitution pattern in ring B (flavonoid numbering) is observed for the compounds tested, whereas the chalcone lacking the hydroxyl group at 6′ and chalcone-glucosides do not function as substrates for the isomerase.

On polyacrylamide-gel plates the enzyme gives rise to at least 3 bands which are enzymatically active with 'kaempferol-chalcone'.[13] Isomerase activity with galangin- and datiscetin-calcone could not be tested on the plates because only very small amounts of these chalcones were available.

TABLE 2. SUBSTRATE SPECIFICITY OF CHALCONE-FLAVANONE ISOMERASE FROM *Datisca cannabina*

Substrate	Spec. activity (conversion of μmole chalcone/mg protein/min)
4,2′,4′,6′-Tetrahydroxy-chalcone ('kaempferol-chalcone')	9·0
2,2′,4′,6′-Tetrahydroxy-chalcone ('datiscetin-chalcone')	5·5
2′,4′,6′-Trihydroxychalcone ('galangin-chalcone')	6·6
4,2′,4′-Trihydroxychalcone	0
'Kaempferol-chalcone'-4′-glucoside	0
'Datiscetin-chalcone'-4′-glucoside	0
'Galangin-chalcone'-4′-glucoside	0

DISCUSSION

Dihydrogalangin but not galangin is a good precursor for datiscetin, while 5,7,2′-trihydroxyflavanone (or the corresponding chalcone) is incorporated into datiscetin to about the same extent as dihydrogalangin.* On the basis of these and earlier results[1] two pathways for the biosynthesis of datiscetin can be visualized:

(a) Cinnamic acid → 5,7-dihydroxyflavanone (or chalcone) → dihydrogalangin → dihydrodatiscetin → datiscetin;

(b) 5,7-Dihydroxyflavanone (or chalcone) → 5,7,2′-trihydroxyflavanone (or chalcone) → dihydrodatiscetin → datiscetin. Then the substrate specificity of the 2′-hydroxylase

* It is unknown how much tritium is lost upon hydroxylation of dihydrogalangin in the 2′-position. This reaction could involve an 'NIH-shift' of the substituted tritium.[14]

[10] K. O. VOLLMER and H. GRISEBACH, *Z. Naturforsch.* **21b**, 435 (1966).
[11] J. B. HARBORNE, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 146, Academic Press, London (1964).
[12] E. MOUSTAFA and E. WONG, *Phytochem.* **6**, 625 (1967).
[13] K. HAHLBROCK, E. WONG, L. SCHILL and H. GRISEBACH, *Phytochem.* **9**, 949 (1970).

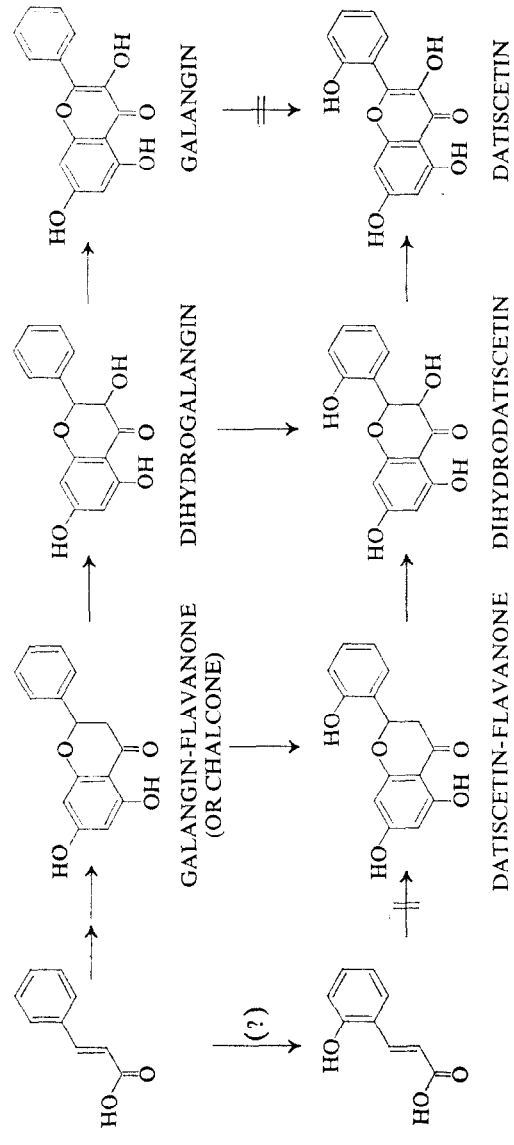


FIG. 1. POSTULATED BIOGENETIC RELATIONSHIP OF FLAVONOIDS IN *Datisca cannabina*.

involved should be low, a situation which could result in a 'metabolic grid'[15] for this flavonoid pathway (Fig. 1). It also follows from the incorporation studies that the conversion dihydrogalangin → galangin readily occurs and that galangin must be subject to rapid turnover.[16] The latter conclusion stems from an experiment where only a small percentage of administered galangin could be reisolated from the plants.

The studies with *o*-coumaric acid-[3-^{14}C] prove that this acid is metabolized by *Datisca* and that the bulk of the acid is apparently not trapped in a metabolically inactive pool. Therefore *o*-coumaric acid can be excluded as precursor for datiscetin. Otherwise the very low incorporation of this acid compared with cinnamic acid or the 'datiscetin-chalcone'[1] would be difficult to understand.

No specificity of the chalcone–flavanone isomerase(s) in *Datisca* for ring B (flavonoid numbering) substitution could be found, whereas the enzyme(s) does not catalyse the isomerization of a chalcone with a resorcinol-type substitution in ring A. The same specificity for ring A substitution has been found previously with the isomerase from parsley.[13] Chalcone glucosides were not substrates for the isomerase(s), a finding which is in agreement with results obtained with isomerases from other plants.[13] Polyacrylamide electrophoresis indicated the presence of three active bands possibly isoenzymes of the isomerase. This question was not investigated further.

APPENDIX

Stability of Flavonoid-Methylethers

Methyl ethers of flavonoids have been used extensively for purification by TLC or paper chromatography. To our knowledge no warning has appeared in the literature that these substances are light sensitive, although Waiss *et al.*[17,18] have reported the photooxidative cyclisation of quercetin pentamethyl ether.

When the tetramethyl ethers of datiscetin and kaempferol, and the trimethyl ether of galangin were separated by TLC (solvent system: toluol–ethyl formate–formic acid [5:2:1]) in normal daylight a series of blue fluorescent products with lower R_f-values than the parent substances were regularly observed. When the methylethers were eluted from the plates, recrystallised, and rechromatographed in the same manner, the blue fluorescent zones appeared again. Working in the dark almost completely prevented the formation of these products.

From a methanolic solution of galangin trimethyl ether irradiated for 20 min with an analytical u.v. lamp (λ_{max} 350 nm) 3 crystalline decomposition products could be isolated by TLC: (IV) yellow, m.p. 247–261°, (V) colourless, m.p. > 330°, (VI) light-yellow, m.p. 205°.

The relative intensities of the fragment ions in the mass spectrum of VI are recorded in Table 3 together with the corresponding ions of galangin trimethyl ether (VII) and datiscetin tetramethyl ether (VIII). Taken together with the results of Waiss *et al.*[18] on the photochemistry of quercetin pentamethyl ether, the mass spectral data of VI are in agreement with the following structure for this compound.

(1) The molecular ion of VI is 2 mass units lower than that of VII.

(2) In the mass spectrum of VI there is only a weak ion *m/e* 77 (phenyl) to observe. This finding requires that in this compound ring B is bound more strongly to the benzopyrone than in VII.

(3) The retro-dien fragment produced in dimethoxybenzo-γ-pyrones[19] at *m/e* 180 and 181 is present in the spectrum of VII and VI which proves that no structural change in ring A has occurred in the conversion of VII to VI.

[4] G. Guroff, J. W. Daly, D. M. Jerina, J. Renson, B. Witkop and S. Udenfriend, *Science* **157**, 1524 (1967).

[15] J. D. Bu'Lock, *The Biosynthesis of Natural Products*, p. 82, McGraw-Hill, London (1965).

[16] Compare: W. Barz, *Z. Naturforsch.* **24b**, 234 (1969).

[17] A. C. Waiss and J. Corse, *J. Am. Chem. Soc.* **87**, 2068 (1965).

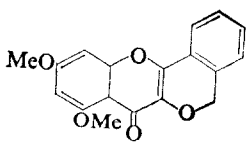
[18] A. C. Waiss, R. E. Lundin, A. Lee and J. Corse, *J. Am. Chem. Soc.* **89**, 6213 (1967).

[19] G. Spiteller, *Massenspektrometrische Strukturanalyse organischer Verbindungen*, p. 176, Verlag Chemie, Weinheim (1955).

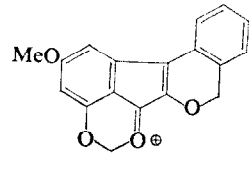
TABLE 3. MASS SPECTRA (RELATIVE INTENSITIES) OF GALANGINTRIMETHYLETHER (VII), ITS PHOTOPRODUCT (VI), AND DATISCETIN-TETRAMETHYLETHER (VIII)

m/e	VII	VI	VIII	*m/e*	VII	VI	VIII
342			89,5 M	237	1,8	16,4	
341			59,4 M-1	235	2,4 M-phenyl		
327			11,7 M-CH_3	202		100	
323			12,2 M-19	181	8,6	2,4	37,2
313	23,8		8,7 M-COH	180	4,4	1,3	3,5
312	84,6 M		21,5	168	2,6	4,3	2,3
311	100 M-1	19,2	100 M-OCH_3	157			11,6
310	7,7	100 M		155	3,3	7,9	
309		11,2 M-1		152	5,9	7,8	3,5
297	7,7 M-CH_3			151		12,2	7,5
296	3,3	6,1	8,7	149		6,5	2,9
295	4,8		4,1	142	9,9		2,6
293	34,4 M-19	10,4 M-17	24,9	141		8,8	
283	9,2 M-COH		4,1	137	7,5	4,0	9,9
282		13,0 M-CO		135	2,9		12,2
281	12,5 M-OCH_3	16,4 M-COH	6,4	131			6,4
280		5,6		106	11,6		12,9
279		10,4 M-OCH_3		105	27,2	2,4	5,9
269	3,5		2,6	93	3,7	4,8	5,6
268		14,0		92			14,2
267		86	2,8	91	5,3		20,4
266		8,3		90	3,3	13,5	
265	7,3	42	2,6 M-phenyl	89	13,9	10,5	7,0
264		9,1		77	41 phenyl	5,2 phenyl	23,4 phenyl
253	5,1		4,1	76		7,0	
251	6,2	7,8					

Mass spectra were taken on an Atlas CH4 instrument.



(VI)



(IX)

(4) According to Müller[20] the fragment *m/e* M-19 of VII has structure IX which represents one of the four possible mesomeric forms. This ion should correspond to a fragment *m/e* M-17 of VI which is present in the spectrum of this compound.

The structures of compounds IV and V were not investigated.

EXPERIMENTAL

Dihydrogalangin (Pinobanksin)

Galangin (1 g) and Na_2CO_3 (8·5 g) were thoroughly mixed, heated to 120° and 100 ml of hot H_2O added. MeOH-saturated aqueous Na_2CO_3 (1:1) was then added to dissolve the galangin completely. Under N_2 33 g $NaHSO_3$ was then added during 2 min and the solution kept boiling for another 45 min. The solution was then rapidly cooled to 50° and acidified (conc. H_2SO_3 ~ 13 ml). The precipitate was collected, and the mother liquor extracted 4× ethyl acetate. The residue from the extraction was combined with the precipitate.

[20] H. P. MÜLLER, *Diplomarbeit*, Freiburg i. Br. (1969).

The reaction product was first crystallised from EtOH–H_2O and was then chromatographed on a cellulose column (2 × 30 cm), attached to a uvicord (LKB) with increasing concentrations of HOAc in H_2O. With 30% HOAc, 200 mg chromatographically pure dihydrogalangin (λ_{max} 293 nm)[7] was obtained. The later fractions yielded another 200 mg of a mixture of galangin and dihydrogalangin. On paper chromatograms the synthetic dihydrogalangin had R_f-values identical with those of pinobanksin[7] in the following solvent systems I, 15% HOAc; R_f 0·68; II, 60% HOAc R_f 0·87; III, benzene–light petrol (60–70°) MeOH–H_2O (50:50:1:50, by vol.), R_f 0·14; IV, benzene–HOAc–H_2O (125:72:3, by vol.), R_f 0·66.

Dihydrogalangin-[G-^{3}H]

Dihydrogalangin (50 mg) absorbed on quartz powder was kept in contact with 5 C ^{3}H for 4 weeks. The product was eluted from the quartz powder with 200 ml of acetone, and after removal of the solvent the residue was recrystallised from EtOH–H_2O. The product was then purified on paper (Whatman 3MM) in the solvent systems V, 40% EtOH, IV and again two times in V. The specific activity (4,73 × 10^{10} dis/min/mmole; 21,3 mc/mmole) remained constant in the last two steps. For the experiments the labelled compound was diluted (×2) with unlabelled dihydrogalangin.

Galangin-[G-^{3}H]

Galangin-[^{3}H] obtained by oxidation of dihydrogalangin-[^{3}H] with $NaHSO_3$[9] was purified 2× by paper chromatography with 30% HOAc. It had a specific activity of 43,3 × 10^9 dis/min/mmole.

Feeding of Labelled Compounds

The compounds were fed as salts in aqueous solutions. For each experiment two 13-week-old *Datisca* plants with roots were used. The plants were kept in a phytochamber at 23° and a 14 hr light period (16,000 lx, xenon burner, Osram XQO) during the experiment. The activity of the precursors and the uptake by the plants is recorded in Table 4.

TABLE 4. ACTIVITY AND UPTAKE OF THE LABELLED PRECURSORS (COMPARE TABLE 1)

Precursor	mmole × 10^3	dis/min × 10^{-6}	Uptake by the plants (dis/min × 10^{-6})
5,7,2′-Trihydroxy-flavanone-7-glucoside-[β-^{14}C]	1·46	4·56	0·92
Dihydrogalangin-[^{3}H]	1·12	24·3	16·0
5,7,2′-Trihydroxy-flavanone-7-glucoside-[β-^{14}C]	1·46	4·56	1·05
Galangin-[^{3}H]	1·12	24·3	13·8

The isolation of the flavonoids was carried out as described[1] with the only difference that the glycosides were hydrolysed with the glycosidases present in the plant[21] by allowing a homogenate of the plates in water to stand for 8 hr.

Metabolism of o-*Coumaric Acid*-[3-^{14}C]

3 *Datisca cannabina* plants (30-cm high) were incubated with the Na salt of *o*-coumaric acid-[3-^{14}C] (10·6 μmole, 13·43 μc = 2·98 × 10^7 dis/min) in H_2O for 48 hr. After that time, the aqueous solution remaining contained 3·1 × 10^6 dis/min (10%). The plants were chopped in a Waring blendor for 10 min in 100 ml of 70% EtOH. The ethanolic solution was filtered, and the residue extracted 2× 100 ml of boiling 70% EtOH. The remaining solid residue contained ~ 1·5 × 10^6 dis/min (6%) of the activity taken up by the plants. The combined EtOH solutions were evaporated to dryness and extracted on the boiling water bath first with light petrol (b.p. 60–70°) (1·1 × 10^6 dis/min, 4%) and then with EtOH (2·19 × 10^7 dis/min, 80%). Part of the EtOH fraction was evaporated to dryness and the residue hydrolysed for 2 hr at 100° with 2 N HCl in MeOH (1:1).

When the hydrolysate was chromatographed on paper with 10% HOAc no radioactivity was found in the zones of datiscetin and coumarin. The glycoside fraction (unhydrolysed ethanolic extract) was separated on paper with benzene–HOAc–water (6:7:3, by vol., upper phase).

Besides various other radioactive bands two zones which showed a blue fluorescence in u.v. light were present on the chromatograms which could be the quinic or glucose esters of cinnamic acids.[11,22] These

[21] J. FARRE-BOUVIN, M. MASSIAS and J. MASSICOT, *C. R. Acad. Sci., Paris Ser. D* **268**, 2495 (1969).
[22] L. BIRKOFER, C. KAISER, W. NOUVERTNÉ and U. THOMAS, *Z. Naturforsch.* **16b**, 249 (1961).

zones contained only very little radioactivity. The eluates from these zones were hydrolysed for 30 min with boiling 2 N HCl–MeOH (1:1) and the hydrolysis products separated on paper with *n*-BuOH–HOAc–H_2O (4:1:2·2, by vol.). No *o*-coumaric acid, quinic acid or glucose could be detected on the chromatograms. The absence of *o*-coumaric acid was confirmed by the u.v.-spectrum of this hydrolysate.

Another portion of the hydrolysate of the EtOH extract was separated by TLC on silica gel with benzene–dioxane–HOAc (90:25:4, by vol.) and on polyamide plates with $CHCl_3$–methylethylketone–MeOH (30:13:2, by vol.). Radioactivity was present in the zones of *o*-coumaric and salicylic acid. This was confirmed by twice chromatographing on paper the hydrolysate together with carrier substances using the solvent system benzene–HOAc–H_2O (6:7:3, organic phase, *o*-coumaric acid R_f 0·4; salicylic acid, R_f 0·75).

Chalcone-flavanone Isomerase

All preparations were carried out at 4°. 11·4 g young *D. cannabina* leaves were ground in a mortar with 23 ml of 0·2 M tris-HCl buffer, pH 7·6, containing 0·025 M EtSH. The resulting suspension was filtered through cheese-cloth and centrifuged for 5 min at 40,000 *g*. Addition of ammonium sulfate to the supernatant up to 80% saturation yielded an enzymatically active protein. The precipitate was dissolved in 5 ml of 0·2 M tris-HCl buffer, pH 7·6, and used for the enzyme determinations. 1 ml of this solution was dialysed against 0·03 M tris-HCl buffer, pH 8·5, for 6 hr and used for the analytical gel electrophoresis. The enzyme assay and the analytical gel electrophoresis were carried out as described previously.[13]

Determination of Radioactivity

Radioactive samples were counted with a Beckman LS 100 liquid scintillation spectrometer, in a PPO-naphthalene-dioxan and PPO-toluene scintillator solution. Efficiencies were measured by internal standardization using toluene-[^{14}C] or *n*-hexadecane-[1,2-T] standards.

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