Phytochemistry, 1974, Vol 13, pp 1479 to 1482 Pergamon Press Printed in England

THE BIOSYNTHESIS OF FLAVONOID PIGMENTS: ON THE INCORPORATION OF PHLOROGLUCINOL AND PHLOROGLUCINYL CINNAMATE INTO RUTIN IN *FAGOPYRUM ESCULENTUM*

MIR AMIR ALI and JACQUES KAGAN

Department of Chemistry, University of Illinois at Chicago Circle, Chicago, IL 60607, U S A

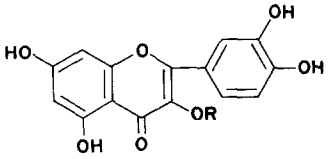
(*Received* 30 *July* 1973)

Key Word Index—*Fagopyrum esculentum*, Polygonaceae, buckwheat, rutin biosynthesis, phloroglucinol, cinnamic acid, phloroglucinyl cinnamate, Fries rearrangement

Abstract—The problem of whether phloroglucinol is a direct biosynthetic precursor of flavonoids was reinvestigated Phloroglucinol-2,4,6-^{14}C was found to be incorporated into rutin in Buckwheat (*Fagopyrum esculentum*) but most of the activity was found in the sugar moiety, the remainder being approximately equally distributed among the A- and B-rings of the aglycone, quercetin This indicates extensive degradation of the added phloroglucinol prior to its utilization in the biosynthesis of the flavonoid. The hypothesis of a bio-Fries rearrangement of phloroglucinyl cinnamate to a chalcone, and hence to flavonoids, was also eliminated by comparing the efficiency of incorporation of ^{14}C-labelled phloroglucinyl cinnamate and those of labelled phloroglucinol and cinnamic acid

INTRODUCTION

ALTHOUGH phloroglucinol itself occurs in the free state in only three species, *Allium cepa*,[1] *Geum urbanum* and *G. rivale*,[2] flavonoid pigments may be viewed as being formally derived from it.[3] Biosynthetic studies[4] suggest, however that the C_6–C_3 unit of flavonoids arises from shikimate, and the phloroglucinol 'A' ring is derived from acetate.



(1) R = H, quercetin
(2) R = rutinose, rutin

The chemical conversion of poly-β-ketoacid derivatives into phenolic compounds is well known[5,6] and cinnamoylphloroglucinol and pinosylvin were obtained from cinnamoyltriacetic acid by Harris and Carney.[7] However, attempts to obtain an enzymatic conversion

[1] K HERRMANN, (1958) *Arch Pharmazie* **291,** 238

[2] BUNOVA, K F (1957) *Shornik Nauch Trudov Leningrad Khim-Farm Inst* **2,** 80, *Chem Abs* **52,** 12099e (1958)

[3] Leading references (a) GEISSMAN, T A and HINREINER, E (1972) *Bot Rev* **18,** 77, (b) KARRER, W (1958) *Konstitution und Vorkommen der Organischen Pflanzenstoffe*, Birkauser, Basel, (c) RICHARDS, J H and HENDRICKSON, J B (1964) *The Biosynthesis of Steroids, Terpenes and Acetogenins*, p 41, W A Benjamin, Inc, New York, (d) HARBORNE, J B and SIMMONDS, N W. (1964) In *Biochemistry of Phenolic Compounds* (HARBORNE, J B ed) p 77, Academic Press, New York

[4] Leading references (a) (1968) *Biogenesis of Natural Products* (BERNFELD, P, ed) Pergamon, Oxford, (b) GRISEBACH, H and BARZ, W (1969) *Naturwissenschaften* **56,** 538

[5] COLLIE, J N and MEYERS, W S (1893) *J Chem Soc* **63,** 122, COLLIE, I (1893) *J Chem Soc* **63,** 329

[6] MONEY, T (1970) *Chem Revs* **70,** 553

[7] HARRIS, T M and CARNEY, R L (1967) *J Am Chem Soc*, **89,** 6734

of cinnamoyltriacetic acid to these products failed using enzyme preparations from *Eucalyptus sideroxylon* [8] Furthermore, although the enzyme preparation did convert the precursor into pinosylvin, this was not a normal constituent of the plant [8 9] A recent report on the enzymatic formation of naringenin from malonyl-CoA and *p*-coumaroyl-CoA *in vitro* did not shed any light on the nature of the intermediate steps.[10]

Watkin *et al* [11] reported short term competitive feeding experiments with phloroglucinol and ^{14}C-labelled carbon dioxide in buckwheat but these were inconclusive In any case, such experiments do not conclusively eliminate the rôle of phloroglucinol in the biosynthesis of quercetin, since its rate of absorption and translocation to the sites of flavonoid synthesis could well have been extremely slow, and long term feeding experiments may have been essential [12] Neish has also made reference to unpublished observations by Watkins and himself, that labelled phloroglucinol was not incorporated into quercetin, but no experimental details were provided.[13]

RESULTS AND DISCUSSION

We recently reported on the conversion of phloroglucinyl cinnamates (3,5-dihydroxyphenyl cinnamates) to cinnamoylphloroglucinols (chalcones) by u.v. light in neutral medium.[14] These results led us to take a critical view of previous investigations, particularly since chalcones are known as the immediate precursor to all flavonoids.[4] The possibility that phloroglucinol and/or phloroglucinyl cinnamates might be precursors to chalcones and hence to flavonoids *in vivo* thus deserved closer scrutiny.

Buckwheat (*Fagopyrum esculentum*) was selected for study,[11 15] and a number of experiments were performed in which ^{14}C-labelled phloroglucinol[16] was fed to the plants through cut stems, and its incorporation into rutin (**2**) investigated [17] The rutin, isolated in experiments in which labelled phloroglucinol was fed and metabolized from 3 to 73 hr, was always found to be radioactive, but with dilution values ranging from 383 to 6560 (Table 1). Most of the experiments were performed when the plants were at the flowering stage, where the rutin content was reported to be at its peak.[18] A few feeding experiments were conducted in plant growth rooms where the light intensity was 2000 lx and yielded more rutin, with greater specific activity than in experiments carried out with plants grown in greenhouse. For example, Experiment 1 (73 hr in the growth room) yielded rutin with a dilution of 780, while Experiment 6 (72 hr in the greenhouse) yielded rutin with a dilution of 4900 Degradation of the labelled rutin (**2**) showed that the activity first went into the

[8] HILLIS, W E and ISHIKURA, N (1969) *Phytochemistry* **8,** 1079
[9] HILLIS, W E and YAZAKI, Y (1971) *Phytochemistry* **10,** 1051
[10] KREUZALER, F and HAHLBROCK K (1972) *FEBS Letters* **28,** 69
[11] WATKIN, J E UNDERHILL, E W and NEISH, A C (1957) *Can J Biochem Physiol* **35,** 219
[12] HEIMBERGER, S I and SCOTT, A I (1973) *J C S Chem Commun* 217
[13] NEISH, A C (1964) In *Biochemistry of Phenolic Compounds*, (HARBORNE, J B, ed) p 341 Academic Press New York
[14] RAMAKRISHNAM, V T and KAGAN, J (1970) *J Org Chem* **35,** 2898, 2901, BHATIA V K and KAGAN J (1970) *Chem Ind (Lond)* 1203
[15] SHIBATA, S and YAMAZAKI, M (1957) *Pharm Bull Tokyo* **5,** 501 GEISSMAN, T A and SWAIN T (1957) *Chem Ind (London)*, GRISEBACH, H and PATSCHKI, L (1961) *Z Naturforsch* **16b,** 645
[16] PATSCHKE L, BARZ, W and GRISEBACH, H (1964) *Z Naturforsch* **19b,** 1110
[17] The interested reader will find the detailed description of this work in ALI, M A's dissertation, University of Illinois at Chicago Circle (1972), available through University Microfilms, Ann Arbor, Michigan entitled "The biosynthesis of flavonoid pigments A search for the bio-Fries rearrangement of a phloroglucinyl cinnamate into a chalcone"
[18] BRAY G A (1960) *Anal Biochem* **1,** 279

sugar. Thus, in the short term experiment 4, involving 3 hr of feeding and metabolism, the rutin had a dilution of 383 but yielded quercetin devoid of activity. Activity in quercetin was also absent in experiments 4 and 6, where the rutin had been obtained with dilutions of 383 and 3860 respectively. In the long term experiment 1 (73 hr), rutin was obtained with a dilution of 780, but in this case it yielded quercetin with a dilution of 2500

TABLE 1 SUMMARY OF EXPERIMENTS

	No plants	Age (weeks)	Amt (mg)	nCi	Precursor‡	Time (hr)	Absorption (%)	Dilution in rutin
1*	10	6	8·8	3352	P	73	97	780
2*	9	6	10·8	4114	P	10	99	6560
4†	10	5	8·6	3377	P	3	91	383
6†	10	5	8·6	3377	P	60	99	3860
10†	14	4–6	13·4	3390	P	72	100	4900
7†	12	4·5	15·3	5680	DPC	48	95	3790
12†	14	4–6	13·2	5290	DPC	95	94·5	3514
11†	14	4–6	7·8	5250	C	62	99	1354

* In plant growth room
† In greenhouse
‡ P = ^{14}C-phloroglucinol, DPC = ^{14}C-phloroglucinyl cinnamate, C = cinnamic acid

The distribution of label in this radioactive quercetin was investigated. Alkali fusion yielded phloroglucinol from Ring A, as well as protocatechuic acid, and *both* were found to be radioactive, with approximately the same activity (72 and 77 dpm respectively). A similar result was observed in Experiment 2, where the rutin had been obtained with even greater dilution of label, to 6560. Degradation of the resulting quercetin yielded phenolic and carboxylic acid fractions which were both radioactive (84 and 188 dpm respectively)

These results demonstrated that the incorporation of the activity from phloroglucinol into rutin was not localized in the A-ring as expected, but was spread over most of the molecule. We conclude, therefore, that in both short-term and long-term experiments the metabolism of phloroglucinol in buckwheat did not lead directly to flavonoids, and that extensive randomization accompanied the eventual incorporation of activity into rutin.

The failure to observe the direct conversion of phloroglucinol into rutin in this system did not necessarily rule out the intermediacy of a phloroglucinyl cinnamate. This might have been formed on an enzyme surface, or in a manner which did not utilize a preformed phloroglucinol molecule. As one example, the possibility of going through an anhydride intermediate was suggested.[4b] We compared, therefore, the incorporation into rutin in buckwheat of phloroglucinyl cinnamate to that of phloroglucinol and of cinnamic acid under similar conditions. Dilution values of 4900, 1350 and 3500 were obtained for the incorporation of radioactive phloroglucinol, cinnamic acid and phloroglucinyl cinnamate respectively (Table 1). The relative values clearly indicated that phloroglucinyl cinnamate was not converted into rutin as efficiently as cinnamic acid, and not much better than phloroglucinol. Consequently, the hypothesis of the formation of chalcones, and hence flavonoids, by a bio-Fries rearrangement of phloroglucinyl cinnamates is not tenable.

EXPERIMENTAL[17]

A 6801–6804 Nuclear-Chicago liquid scintillation counter was used for the radioactivity measurements The samples were dissolved in 10 ml of Bray's mixture[18] and 1 ml of H_2O, mixed and counted at room temperature

[19] COUCH, J F, NAGHSKI, J and KREWSON, C F (1946) *Science*, **106**, 197

The NMR spectra were recorded on Varian A-60A or T-60 spectrometers, and are reported on the δ-scale in ppm down-field from internal tetramethylsilane The m ps were measured on a Koffler microscope-hot stage and were not corrected

Plants materials and feeding experiments The seeds of buckwheat (General Biological Co), were germinated and transplanted into a mixture of soil, sand, peat and fertilizers In earlier experiments, 2–3 week-old seedlings were used and later, 4–6 week-old plants at the flowering stage [10] The labelled compounds were administered in aqueous solution (pH 8 0) to 9 14 plants through cut stems The radioactive rutin was extracted from the plants, purified and hydrolyzed to quercetin by standard techniques [11] Following alkali treatment under N_2 phloroglucinol and protocatechuic acid were isolated and purified chromatographically

Synthesis of 3,5-dihydroxyphenyl cinnamate-α-^{14}C Bromacetic acid-2-^{14}C (0 5 mCi, 55 mCi/mmole) from Amersham/Searle was diluted with 0 504 g of unlabelled acid The mixture (0 138 mCi mmole) was treated with CH_2N_2, and methyl bromoacetate was purified by short path vacuum distillation A soln of this ester and 0 445 g of distilled benzaldehyde in C_6H_6 was slowly added under N_2 to 0 249 g Zn in refluxing benzene After refluxing for 50 min, the mixture was cooled in ice and acidified (20 ml 6 N HCl) The aq phase was extracted with 2 × 30 ml Et_2O, which were combined with the benzene layer This mixture was filtered and conc to yield methyl 3-hydroxy-3-phenylpropionate A soln of this ester and 0 240 g of *p*-toluene sulfonic acid monohydrate in 150 ml of C_6H_6 was refluxed for 4 hr with collection of H_2O in a Dean–Stark condenser, cooled extracted with $NaHCO_3$, H_2O, and concentrated under reduced pressure The residue of methyl cinnamate-α-^{14}C was heated with 20 ml 15% aq NaOH for 15 hr at 88°, cooled and acidified The resulting white solid was washed (H_2O) and recrystallized from hexane-C_6H_6, to yield 0 451 g (63%) of cinnamic acid-α-^{14}C m p 134° 135° 0 105 mCi/mmole, with an NMR identical to that of an authentic sample A solution of 0 179 g of this labelled acid and 0 5 ml of $SOCl_2$ in 20 ml of $CHCl_3$ was refluxed under N_2 for 2 hr The solution was concentrated, 20 ml of dioxane was added and the solvent was again removed to yield a residue free from $SOCl_2$ It was dissolved in 50 ml of dioxane and slowly added under N_2 to a warm solution of 1 7 g of anhyd phloroglucinol and 1 ml of triethylamine in 15 ml dioxane After 4 5 hr at reflux, the mixture was poured over ice The solid formed was filtered and it was purified by silica gel column chromatography EtOAC–$CHCl_3$ (1 19) eluted the monoester, which was recrystallized from aq. EtOH to yield 0 111 g of 3,5-dihydroxyphenyl cinnamate-α-^{14}C m p 196–198°, 0 0975 mCi/mmole NMR (DMSO-d_6) 6 25 (*m*, 3H), 7 72 (*m*, 5H), 7 97 (*d*, 16 Hz 1H), 6 88 (*d*, 16 Hz, 1H) and 9 55 ppm (s 2H disappeared in D_2O)

Acknowledgements—Financial support of this work by the National Science Foundation and the help of Mr J McCorkle, Horticulturist, Department of Biological Sciences, are gratefully acknowledged