Phytochemistry, 1975, Vol. 14, pp. 1765–1768. Pergamon Press. Printed in England.

BIOSYNTHETIC STUDIES ON FLAVONES AND *C*-GLYCOSYLFLAVONES: B-RING OXIDATION PATTERNS

JAMES W. WALLACE

Department of Biology, Western Carolina University, Cullowhee, N.C. 28723, U.S.A.

(*Revised Received* 30 *November* 1974)

Key Word Index—*Spirodela polyrhiza*; Lemnaceae; duckweed; flavone; *C*-glycosylflavone; biosynthesis; B-ring oxidation pattern.

Abstract—Data obtained by feeding ^{14}C-labeled amino acids, a flavanone, flavones, and a *C*-glycosylflavone to axenically cultured *Spirodela polyrhiza* indicated that: the B-ring oxidation pattern of flavone-*O*-glycosides was determined at the flavone level of biosynthesis and was essentially irreversible; that the B-ring oxidation pattern of *C*-glycosylflavones was determined prior to *C*-glycosylation, probably at the flavanone stage; and that flavones and *C*-glycosylflavones were not static end products of metabolism but undergo turnover. Specific activities were determined for the *in vivo* incorporation of each of the above kinds of compounds into flavone *C*- and *O*-glycosides of *S. polyrhiza*.

INTRODUCTION

The biosynthesis of flavonoids has been studied very extensively during the last several years and has been the subject of several reviews [1–4]. The following information is pertinent to the present study: phenylalanine has been shown to be a good precursor for ring B and the carbon atoms C 2-4 of ring C of the flavone nucleus, whereas tyrosine and DOPA have been shown to be relatively poor precursors [1, 5]; flavanones have been shown to be precursors for both flavones and *C*-glycosylflavones [6, 7]; flavones have been shown to be *O*-glycosylated [4, 8, 9] but not *C*-glycosylated [8, 9]; and axenically cultured *Spirodela polyrhiza* L. (clone 7003) has been shown to synthesize the flavone *O*-glucosides, apigenin and luteolin-7-*O*-glucosides, as well as the corresponding *C*-glycosylflavones, vitexin and orientin [10].

Published data concerning the biosynthesis of the various flavonoid B-ring hydroxy substitution patterns includes the relative incorporation of various ^{14}C-labeled cinnamic acids into the flavonoids of *Petunia* [11–13] which indicated that the various patterns in anthocyanins were determined at the cinnamic acid stage. In contrast, the relative incorporation of ^{14}C-labeled flavanones, flavanonols, and flavonols with a 4′-hydroxyl group into flavonols with a 3′,4′-dihydroxyl pattern demonstrated that here the B-ring oxidation pattern was determined at the flavanonol level [14].

A subsequent study implied that the B-ring oxidation pattern for flavones may be determined at the flavone level but that the B-ring oxidation pattern of *C*-glycosylflavones occurred prior to that stage [9]. The present paper reports further experiments on this aspect.

Information obtained during the present study using radioactive tracers verifies that during biosynthesis B-ring oxidation for flavones probably occurs at the flavone level while that of *C*-glycosylflavones is probably at the flavanone (or *C*-glycosylflavanone) level. The data also demonstrate that the above biosynthetic steps, as well as the process of *C*-glycosylation, are irreversible. In addition, it supports other reports concerning the turnover of flavonoids.

RESULTS AND DISCUSSION

That fact that phenylalanine, tyrosine, and DOPA are converted to their respective cinnamic

Table 1. Sp.act. of flavonoids from various ^{14}C-precursors fed to *Spirodela polyrhiza* (Clone 7003)

Product	^{14}C-Labeled Precursors Fed*						
	Phenylalanine† (12,000)	Tyrosine† (5800)	DOPA† (2360)	Naringenin‡ (2470)	Apigenin† (2470)	Vitexin† (2030)	Luteolin† (1630)
Apigenin-7-*O*-glucoside§	2470·0	62·8	10·7	397·0	800·0	9·41	4·57
Vitexin	2030·0	54·7	9·9	364·0	4·07	304·0	2·32
Luteolin-7-*O*-glucoside§	1630·0	55·3	9·7	61·9	93·6	1·88	283·0
Orientin	1490·0	48·5	8·8	70·2	2·03	1·77	7·77

* Sp.act. μCi/mM in brackets.
† 10 day culture period.
‡ 3 day culture period; data from Wallace and Grisebach [7].
§ Sp.act. determinations made on flavone aglycones; background radiation subtracted from these figures.

acids (i.e. cinnamic acid, *p*-coumaric acid, and caffeic acid respectively) is well documented [18]. Phenylalanine is known to be a much better precursor than either tyrosine, *p*-coumaric acid, DOPA, or caffeic acid for flavonoids (anthocyanins and flavonols) which have either the 4′- or 3′,4′-dihydroxy B-ring substitution pattern [1]. Data presented in Tables 1 and 2 indicate the same conclusion is valid for the biosynthesis of flavones and *C*-glycosylflavones in *Spirodela polyrhiza*.

Compared to the various aromatic amino acid precursors, [2-^{14}C]-naringenin (5,7,4′-trihydroxyflavanone) was incorporated into apigenin-7-*O*-glucoside and vitexin (8-*C*-glycosylapigenin), to the same degree as phenylalanine (Tables 1 and 2). The lower but still substantial incorporation of the flavanone into luteolin-7-*O*-glucoside and orientin (8-*C*-glycosylluteolin) implies that naringenin is a precursor to the 4′-hydroxylated flavonoids which in turn are precursors to 3′,4′-hydroxylated flavonoids (but see below).

[^{14}C]-Apigenin (5,7,4′-flavone) was incorporated highly into apigenin-7-*O*-glucoside and to a lesser, but still large degree, into luteolin-7-*O*-glucoside demonstrating that it could be both *O*-glycosylated and hydroxylated. In contrast, [^{14}C]-apigenin was incorporated to a very low degree into the *C*-glycosylflavones, vitexin and orientin, as demonstrated earlier [8, 9]. This indicates that flavones cannot be readily reduced to flavanones (of naringenin). In fact, the low incorporation of apigenin into the *C*-glycosides probably indicates a turnover of the flavone to produce a 4-hydroxyphenylpropane moiety which is then incorporated. A direct enzymatic conversion of apigenin into the *C*-glycosylflavones would be expected to give higher activity.

Radioactivity from [^{14}C]-vitexin was mainly detected in endogenous vitexin demonstrating that it was not readily oxidized to orientin. Based on the previous rationale, the slight incorporation of [^{14}C]-vitexin into other *S. polyrhiza* flavonoids (Tables 1 and 2), was probably due to turnover. However, it is interesting that apigenin-7-*O*-glucoside contains a higher degree of incorporation than do luteolin-7-*O*-glucoside or orientin. This implies that when vitexin undergoes turnover the

Table 2. Incorporation of various radioactive precursor molecules into flavones and C-glycosylflavones of *Spirodela polyrhiza*

Product	%*I** from ^{14}C-labeled precursors fed						
	Phenylalanine	Tyrosine	DOPA	Naringenin	Apigenin	Vitexin	Luteolin
Apigenin-7-*O*-glucoside	20	1·1	0·45	20	32	0·46	0·28
Vitexin	17	0·94	0·42	18	0·16	15	0·14
Luteolin-7-*O*-glucoside	14	0·95	0·41	3·1	3·8	0·093	17
Orientin	12	0·84	0·37	3·5	0·082	0·087	0·48

* $\%I$ = % incorporation into a given molecule = (sp. act. prod.)/(sp. act. precur.) × 100.

phenylpropanoid moiety may not be further hydroxylated (to the 3′,4′-pattern) but is incorporated directly into naringenin, etc.

[^{14}C]-Luteolin (5,7,3′,4′-tetrahydroxyflavone) was incorporated as expected to a high degree into luteolin-7-*O*-glucoside; however, it does not appear to be incorporated directly into the other compounds examined (Tables 1 and 2).

Analysis of the nutrient solution after the feeding experiments were terminated demonstrated in all cases that the above reactions occurred *in vivo*. None of the above "turnover products" were detected in the nutrient solution.

That neither of the hydroxylated amino acids were incorporated into the flavonoid pool to the same degree as phenylalanine or naringenin implies that the B-ring oxidation pattern was not determined at the amino acid level and probably not at the cinnamic acid stage during the biosynthesis of flavones or *C*-glycosylflavones. If oxidation does occur at the cinnamic acid level, one would expect a multienzyme complex which accepted a phenylalanine type precursor in preference to tyrosine or DOPA. Data to support the existence of multienzyme complexes for flavonoid biogenesis has been reviewed by Stafford [19]. In contrast, the relatively high incorporation of the flavanone, naringenin, into apigenin-7-*O*-glucoside, vitexin, luteolin-7-*O*-glucoside and orientin, suggests that 4′-hydroxyflavanones are precursors to 3′,4′-dihydroxyflavonoids of higher oxidation state. The observation that apigenin is incorporated into luteolin-7-*O*-glucoside shows that oxidation can occur at the flavone level but does not indicate whether apigenin or its 7-*O*-glucoside is the precursor of luteolin-7-*O*-glucoside. Based on published data the oxidation probably occurs at the flavone aglycone stage and not at the glycoside level [3]. However, apigenin was not readily reduced to the flavanone stage. If this had occurred the *C*-glycosylflavones would have been expected to contain appreciable label via the main flavonoid biosynthetic pathway. In contrast the *C*-glycosylflavone, vitexin, was not incorporated to any appreciable amount into orientin, demonstrating that the B-ring oxidation pattern of *C*-glycosylflavones probably occurs prior to the *C*-glycosylflavone level. Based on flavonoid-*O*-glycoside biosynthetic data the oxidation pattern of *C*-glycosylflavones is probably determined at the flavanone and not at the *C*-glycosylflavanone level [3, 4].

EXPERIMENTAL

Culturing plant materials. Spirodela polyrhiza L. (clone 7003) [10] was axenically cultured in 1/3 strength Hutner's mineral nutrient solution [15] which contained 1% sucrose [9]. Sterile techniques were used in subculturing duckweeds: the containers being autoclaved for 20 min before use. Approx 12–18 plants were inoculated per 18 ml medium: the plants grew and asexually reproduced for 10 days (except for the naringenin expt which lasted for 3 days) at 26° under continuous $3{\cdot}2 \times 10^4$ lx fluorescent–incandescent illumination. After growth the plants were harvested, air dried, and extracted with MeOH.

Radioactive precursors. The ^{14}C-labeled amino acids, [1-^{14}C]-phenylalanine (12 mCi/mM), [1-^{14}C]-tyrosine (5·8 mCi/mM), and [1-^{14}C]-dihydroxyphenylalanine (DOPA, 2·36 mCi/mM), were obtained from Nuclear Chicago Corp. [2-^{14}C]-Naringenin (2 mCi/mM, a flavanone) was synthesized according to Wallace and Grisebach [7]. The ^{14}C-labeled flavones, [^{14}C]-apigenin (2·4 mCi/mM), [^{14}C]-vitexin (2·03 mCi/mM) and [^{14}C]-luteolin (1·63 mCi/mM) were obtained according to Wallace *et al.* [9].

Feeding precursors. The following techniques were used for administrating the ^{14}C-labeled compounds. The radioactive amino acids (1·5 μmol) were added to the nutrient solution before autoclaving [5]: for the radioactive flavonoids the compounds (1·5 μmol) were dissolved in 12 ml hot H_2O and filtered (0·45 μm Millipore filter) into the autoclaved solution containing 6 ml of undiluted Hutner's nutrient soln and 0·18 g sucrose. Each expt was performed twice. Nutrient soln was examined chromatographically and autoradiographically at the conclusion of each expt [9].

Specific activity determination. Methanolic duckweed extract was chromatographed in TBA and 15% HOAc according to Mabry *et al.* [16]. The *C*-*O*-glycosidic areas were cut from the chromatograms, eluted with MeOH, and solvent removed. Residue was taken up in H_2O and allowed to react with β-glucosidase (Sigma) for 24 hr and rechromatographed. This separated the *C*-glycosylflavone from the aglycone; the compounds were then quantitated for counting [9] using 15 ml of BBOT scintillation fluid [17]. The samples were each counted 3× for 10 min periods. The average sp. act. are presented in Table 1.

REFERENCES

1. Grisebach, H. (1965) *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., ed.). Academic Press, London.
2. Harborne, J. (1967) *Comparative Biochemistry of the Flavonoids*, Academic Press, London.
3. Halbrock, K. and Grisebach, H. (1975) *The Flavonoids* (Mabry, T. J. and Harborne, J. B., eds.). Chapman and Hall.
4. Grisebach, H. (1975) *Recent Advances in Phytochemistry* (Runeckles, V. and Conn, E., eds.), Vol. 9. Academic Press, New York (in press).
5. Van Dyke, G. D. (1966) M.A. Thesis, University of Texas at Austin.
6. Grisebach, H. and Barz, W. (1967) *Z. Naturforsch.* **22b**, 746.
7. Wallace, J. W. and Grisebach, H. (1973) *Biochem. Biophys. Acta* **304**, 837.

8. Wallace, J. W. and Alston, R. E. (1966) *Plant Cell Physiol.* **7,** 699.
9. Wallace, J. W., Mabry, T. J. and Alston, R. E. (1969) *Phytochemistry* **8,** 93.
10. McClure, J. W. and Alston, R. E. (1966) *Am. J. Botany* **53,** 849.
11. Hess, D. (1964) *Planta* **60,** 568.
12. Hess, D. (1965) *Z. Planzenphysiol.* **53,** 1.
13. Hess, D. (1966) *Z. Planzenphysiol.* **55,** 374.
14. Patschke, L. and Grisebach, H. (1965) *Z. Naturforsch.* **20,** 1039.
15. Hunter, S. H. (1953) *Growth and Differentiation in Plants*, p. 417 (Loomis, W. E., ed.). Iowa State College Press, Ames.
16. Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids*, Springer-Verlag, New York.
17. Snyder, F. (1968) *Recent Advances in Tracer Methodology*, p. 81 (Rothchild, R., ed.), Vol. 4. Plenum Press, New York.
18. Geissman, T. A. (1967) *Biogenesis of Natural Products* (Bernfeld, P., ed.). Pergamon Press, Oxford.
19. Stafford, H. A. (1974) *Recent Advances in Phytochemistry* (Runeckles, V. and Conn, E., eds.). Vol. 9. Academic Press, New York.
20. Patschke, L., Hess, D. and Grisebach, H. (1964) *Z. Naturforsch.* **19,** 1114.
21. Halbrock, K., Wong, L., Schill, L. and Grisebach, H. (1970) *Phytochemistry* **9,** 949.
22. Grambow, H. J. and Grisebach, H. (1971) *Phytochemistry* **10,** 789.
23. Alston, R. E. (1968) *Recent Advances in Phytochemistry* (Mabry, T. J., Alston, R. E. and Runeckles, V. C., eds.), Vol. 1. Appleton-Century-Crofts, New York.