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ON THE BIOGENESIS OF FLAVONE *O*-GLYCOSIDES AND *C*-GLYCOSIDES IN THE LEMNACEAE[1]

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Abstract—^{14}C-Labeled apigenin, luteolin, orientin, isoorientin, and isovitexin were obtained either directly or by hydrolyzing the glycosides obtained from *Spirodela* and *Lemna* species which had been cultured on media containing phenylalanine-1-^{14}C. The ^{14}C-labeled flavones were introduced into the growth media for *S. polyrhiza*, *S. oligorhiza* and *L. minor* plants. When the extracts from these plants were chromatographed and the two-dimensional chromatograms examined autoradiographically, radioactivity was detected in *O*-glycosylated and *O*-methylated flavones. However, radioactivity was not detected in flavones containing new *C*-glycosyl linkages. The data suggest that plants in the Lemnaceae *O*-substitute flavones but do not *C*-glycosylate them. Moreover, evidence was obtained that flavones containing a 4′-monohydroxy B-ring can be oxidized to flavones with a 3′,4′-dihydroxy B-ring. None of the ^{14}C-flavones was incorporated into any of the anthocyanins known to occur in the Lemnaceae. Isoorientin 3′-methyl ether and an *O*-β-D-glucoside of chrysoeriol were detected for the first time as constituents of *L. minor*.

INTRODUCTION

IT HAS been suggested that both *O*- and *C*-glycosylation of flavonoids probably occur after the synthesis of the flavonoid nucleus.[4] However, these proposals are supported by limited evidence, especially with respect to the stage at which *C*-glycosylation of flavonoids occurs. The evidence that *O*-glycosylation of flavonoids is a late or terminal process includes reports of the enzymatic *O*-glycosylation of quercetin to the rutinoside, rutin[5] and a number of genetic investigations which demonstrated the conversion of flavonoid mono-*O*-glycosides to di- and triglycosides.[6] Other genetic evidence indicated that *O*-methylation of flavonoids is also a biogenetically late process.[6]

We report here results which indicate that in the Family Lemnaceae flavones can be *O*-glycosylated and *O*-methylated but not *C*-glycosylated. In addition, autoradiographic

[1] (*a*) A preliminary account of this work was previously reported: J. W. WALLACE and R. E. ALSTON, *Plant Cell Physiol.* **1**, 699 (1966).
(*b*) Also additional comments on these investigations can be found in: R. E. ALSTON in *Recent Advances in Phytochemistry* (edited by T. J. MABRY, R. E. ALSTON and V. C. RUNECKLES), pp. 305–327, Appleton-Century-Crofts, New York, 1968.
(*c*) This study represents a portion of a dissertation presented in 1967 by the senior author to the Graduate School of the University of Texas at Austin in partial fulfilment for the degree of Doctor of Philosophy.

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[4] For example, see: (*a*) V. K. BHATIA and T. R. SESHADRI, *Current Sci.* **36**, 111 (1967).
(*b*) H. GRISEBACH in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), pp. 279–308, Academic Press, London (1965).
(*c*) W. B. WHALLEY in *The Chemistry of Natural Phenolic Compounds* (edited by W. D. OLLIS), pp. 20–58, Pergamon Press, London (1961).

[5] G. A. BARBER, *Biochem.* **1**, 463 (1962); *Arch. Biochem. Biophys.* **7**, 204 (1962).

[6] For a discussion of these results see J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, pp. 262–265, 271, Academic Press, London (1967).

results showed that 4'-hydroxyflavones can be oxidized to 3',4'-dioxygenated flavones and indicated that neither apigenin nor luteolin was converted to anthocyanins.

The present biogenetic investigations utilized four species of the Lemnaceae, *Spirodela polyrhiza*, *S. oligorhiza*, *Lemna minor* and *L. obscura*, because they were known to synthesize a large number of *O*- and *C*-glycosides containing the apigenin and luteolin oxidation patterns.[7] Moreover, these species appeared to be good experimental organisms because they reproduce asexually in a few days in axenic cultures. Prior to the present investigation, Van Dyke had established that *L. perpusilla* incorporated phenylalanine-1-^{14}C into all the flavonoids synthesized by that species.[8]

RESULTS AND DISCUSSION

Isolation of ^{14}C-*Luteolin*[9] *and* ^{14}C-*Apigenin*

Supplies of ^{14}C-luteolin and ^{14}C-apigenin, which were required for later biogenetic studies, were obtained from extracts of *Spirodela polyrhiza* plants which had been cultured on a medium containing phenylalanine-1-^{14}C. *S. polyrhiza* synthesizes a large number of flavonoids including apigenin (I), apigenin 7-*O*-β-D-glucoside (II), vitexin (III), luteolin (VII), luteolin 7-*O*-β-D-glucoside (VIII), orientin (X) and derivatives of cyanidin. Radioactivity was detected autoradiographically in all of these flavonoids when the species was cultured on a modified Hutner's medium containing phenylalanine-1-^{14}C (Fig. 1). *S. polyrhiza* produces only small amounts of apigenin and luteolin; therefore the following procedure was used to obtain sufficient amounts of these aglycones labeled with ^{14}C. The plants, which had been grown on a medium containing phenylalanine-1-^{14}C, were air-dried and then extracted with methanol containing aqueous HCl. The extract was chromatographed (two-dimensionally) and the region of the chromatogram containing the overlapping apigenin 7-*O*-glucoside (II) and vitexin (III) spots was eluted. The mixture of II and III was subsequently hydrolyzed with β-glucosidase to yield ^{14}C-apigenin and ^{14}C-vitexin. The latter two compounds were distinctly separated by paper chromatography. By a similar procedure ^{14}C-luteolin and ^{14}C-orientin were obtained from a mixture of ^{14}C-luteolin 7-*O*-glucoside and ^{14}C-orientin. ^{14}C-Isoorientin (XI) was obtained directly from the two-dimensional paper chromatogram of the extract of *Lemna obscura* plants which had been cultured in the presence of phenylalanine-1-^{14}C. In the same manner, ^{14}C-isovitexin was obtained from *S. oligorhiza*.

In addition to obtaining ^{14}C-labeled flavones from the phenylalanine-1-^{14}C experiments, the following important observation was made. *S. polyrhiza* incorporated a common precursor, phenylalanine-1-^{14}C, into apigenin 7-*O*-glucoside (II) and vitexin (III) to approximately the same extent (Table 1). Therefore, one would expect any common precursor biogenetically more advanced than phenylalanine to also be incorporated equally into II and III. Similar results were obtained for the incorporation of phenylalanine-1-^{14}C into luteolin 7-*O*-glucoside (VIII) and orientin (X) (Table 1), which are produced in almost equal amounts by this species.

[7] J. W. MCCLURE and R. E. ALSTON, *Am. J. Botany* **53**, 849 (1966).

[8] G. VAN DYKE, M.A. Thesis, The University of Texas at Austin (1965).

[9] Based on the known mode of phenylalanine-1-^{14}C incorporation into flavonoids[10], all the C^{14}-labeled flavones used in the present investigations are probably labeled at position C-4; however this was not confirmed.

[10] For a recent review of the biogenesis of flavonoids, see H. GRISEBACH in *Recent Advances in Phytochemistry* (edited by T. J. MABRY, R. E. ALSTON and V. C. RUNECKLES) pp. 379–404, Appleton-Century-Crofts, New York (1968).

TABLE 1. SPECIFIC ACTIVITY DATA* FOR ^{14}C-LABELED FLAVONOIDS[9] SYNTHESIZED IN CULTURED *Spirodela polyrhiza* PHENYLALANINE-1-^{14}C

Flavonoid	Specific activity (mc/mM)		
	Experiment No.		
	1	2	3
^{14}C-apigenin 7-*O*-glucoside† (II)	2·5	3·0	2·7
^{14}C-vitexin (III)	2·0	2·5	2·2
^{14}C-luteolin 7-*O*-glucoside† (VIII)	1·6	2·1	1·9
^{14}C-orientin (IX)	1·5	2·2	1·9

* A Packard Liquid Scintillation Counter (Model 314 EX) was used to determine the activity data. For each determination, a solution of the flavonoid in 0·5 ml of methanol was mixed in a Packard polyethylene counting vial with 15 ml of Packard dioxane base scintillation liquid. The molecular extinction coefficients (ϵ) were determined (in MeOH) at the long wavelength absorption maximum for each flavonoid on the purest samples available: apigenin (λ_{max} 336 nm, ϵ $2{\cdot}034 \times 10^4$), luteolin (λ_{max} 350 nm, ϵ $2{\cdot}034 \times 10^4$), vitexin (λ_{max} 335 nm, ϵ $1{\cdot}75 \times 10^4$) and orientin (λ_{max} 351 nm, ϵ $1{\cdot}885 \times 10^4$).

† The specific activity data recorded for these compounds were determined on the aglycones.

Incorporation of ^{14}C-*Apigenin and* ^{14}C-*Luteolin into the Flavone* O-*Glucosides of* S. polyrhiza

Because *S. polyrhiza* synthesizes the related series of *O*- and *C*-glycosides, apigenin 7-*O*-glucoside/vitexin and luteolin 7-*O*-glucoside/orientin, this species was used to test for the relative incorporation of ^{14}C-apigenin and ^{14}C-luteolin into their respective *O*- and *C*-glycosides.

When *S. polyrhiza* was cultured on a medium containing ^{14}C-apigenin, radioactivity was incorporated[11] into apigenin 7-*O*-glucoside and luteolin 7-*O*-glucoside (Fig. 2A). Significantly, no radioactivity was detected in vitexin or orientin. These results provide evidence on three biogenetic processes, namely B-ring oxidation, *O*-glycosylation and *C*-glycosylation. The data establish that the 4′-hydroxyflavones can be further oxidized in this plant to 3′,4′-dihydroxyflavones; however, it was not determined whether it was apigenin or its 7-*O*-glucoside which was oxidized.

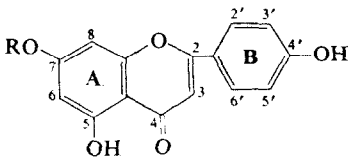
The observation that apigenin was *O*-glucosylated is in agreement with the hypothesis[4] that *O*-glycosylation is a terminal process in flavonoid biogenesis. On the other hand, the absence of incorporation of the ^{14}C-apigenin into vitexin suggests that flavones are not *C*-glycosylated and, therefore, that *C*-glycosylation is an early step in flavonoid biosynthesis.

In agreement with the above results, *S. polyrhiza* also incorporated[11] ^{14}C-luteolin into luteolin 7-*O*-glucoside but not into orientin (Fig. 2B). Furthermore radioactivity was not

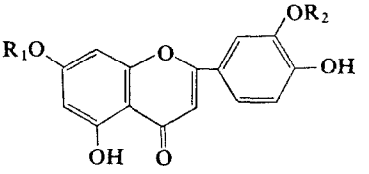
[11] All the incorporation results reported in this paper, unless otherwise noted, are based on autoradiographic examination of the two-dimensional paper chromatograms obtained from the acidic methanol extracts of the plant material.

detected in apigenin 7-*O*-glucoside; thus the reduction of the B-ring of flavones does not appear to occur in this species.

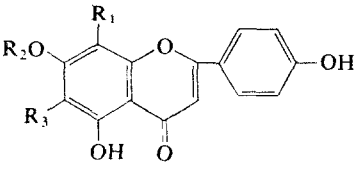
The autoradiograms from both the ^{14}C-apigenin and ^{14}C-luteolin experiments showed that radioactivity was not present in the various cyanidin glycosides synthesized by *S. polyrhiza* in accord with the previous results[10] that flavonols are not precursors of anthocyanins.



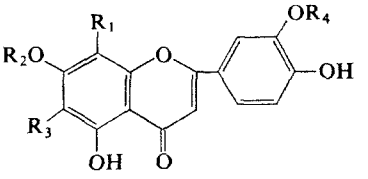
(I) R = H (apigenin)
(II) R = β-D-glucosyl



(VII) $R_1 = R_2 = H$ (luteolin)
(VIII) R_1 = β-D-glucosyl
$R_2 = H$
(IX) $R_1 = H$
$R_2 = CH_3$ (chrysoeriol)



(III) R_1 = *C*-glucosyl
$R_2 = R_3 = H$ (vitexin)
(IV) $R_1 = R_2 = H$
R_3 = *C*-glucosyl (isovitexin)
(V) $R_1 = R_2 = H$
R_3 = *O*-glycosyl-(*C*-glucosyl)
(VI) R_1 and R_3 = *C*-glucosyl
$R_2 = H$ (vicenin)



(X) R_1 = *C*-glucosyl
$R_2 = R_3 = R_4 = H$ (orientin)
(XI) $R_1 = R_2 = R_4 = H$
R_3 = *C*-glucosyl (isoorientin)
(XII) R_1 and R_3 = *C*-glycosyl
$R_2 = H$ (lucenin)
(XIII) $R_1 = R_2 = H$
$R_3 = H$
$R_4 = CH_3$ (isoorientin 3′-methyl ether)
(XIV) R_1 = *C*-glucosyl
R_2 = β-D-glucosyl
$R_3 = R_4 = H$ (lutonarin)

Lack of Incorporation of ^{14}C-*Apigenin and* ^{14}C-*Luteolin into the* 6- *and* 8-*mono- and* 6,8-*di-C-Glycosylflavones of* L. minor

Lemna minor was selected for additional experiments to test for the possible incorporation of ^{14}C-apigenin and ^{14}C-luteolin into *C*-glycosylflavones because this species had been previously reported[7] to synthesize not only vitexin (III) and orientin (X) but also isovitexin (IV), V (an *O*-glycoside of isovitexin which was referred to previously[7] as isosaponarin; however, the published u.v. data[7] are in accord with structure V), vicenin (VI), isoorientin (XI), lucenin (XII) and the 7-*O*-glucoside of orientin, lutonarin (XIV). In addition, in the course of the present investigation, it was found that this species also produced isoorientin 3′-methyl ether (XII) and an *O*-β-D-glucoside of chrysoeriol (IX).

When *L. minor* was cultured on a medium containing either ^{14}C-apigenin (Fig. 3A) or ^{14}C-luteolin (Fig. 3B) radioactivity was incorporated into an *O*-glucoside subsequently

identified as a chrysoeriol *O*-β-D-glucoside. In the ^{14}C-apigenin experiment some radioactivity appeared in the region where apigenin 7-*O*-glucoside would be expected to occur; however, insufficient material was available to establish conclusively that *L. minor* does synthesize this flavone glucoside; the intensity of this spot (as viewed under ultraviolet light), albeit always weak, was considerably enhanced when ^{14}C-apigenin was present in the growth medium for *L. minor*. Most of the ^{14}C-apigenin was further oxidized, *O*-methylated and *O*-glucosylated to yield an *O*-β-D-glucoside of chrysoeriol.

Notably, none of the ^{14}C-apigenin or ^{14}C-luteolin was incorporated into any of the nine C-glycosylflavones (III–VI, XI–XIV) produced by *L. minor*. These results complement those obtained with *Spirodela polyrhiza* and further support the hypothesis that *C*-glycosylation is an early flavonoid biogenetic process.

Lack of Incorporation of ^{14}C-*Orientin,* ^{14}C-*Isoorientin and* ^{14}C-*Isovitexin into the* 6,8-*di*-C-*Glycosides of* L. minor

Lemna minor was also selected for experiments to test for the possible conversion of the 6- and 8-mono-*C*-glucosylflavones into the 6,-8*C*-glycosylflavones since the species produces not only orientin (X), isoorientin (XI), vitexin (III), isovitexin (IV) but also vicenin (VI) and lucenin (XII).

When ^{14}C-orientin (Fig. 4A) was present in the growth medium for *L. minor*, radioactivity was subsequently detected[11] in orientin but not in lucenin thus suggesting that orientin is not the precursor of the lucenin (XII) which occurs in *L. minor*.[12] Similarly, when either ^{14}C-isoorientin or ^{14}C-isovitexin was present in the growth medium of *L. minor* radioactivity was not detected in the lucenin (XII) or the vicenin (VI) (Fig. 4B and 4C), which are produced by this species.[12] In the experiment with ^{14}C-isoorientin some of the material was methylated to yield ^{14}C-isoorientin 3′-methyl ether (Fig. 4B), while in the experiment with ^{14}C-isovitexin some of the material was converted to V, an *O*-glycoside of isovitexin, as well as to some compounds (K and L in Fig. 4C) which appear on the basis of their chromatographic behavior to contain additional *O*-glycosyl groups.

The results from the ^{14}C-orientin, ^{14}C-isoorientin and ^{14}C-isovitexin experiments indicate that none of these mono-*C*-glycosides[13] serve as precursors for the vicenin or lucenin compounds of *L. minor*[12] further supporting the hypothesis that *C*-glycosylation is an early flavonoid biogenetic step. The conversion of isoorientin to its 3′-methyl ether and isovitexin to an *O*-glycoside shows that *C*-glycosylflavones can be *O*-methylated and *O*-glycosylated.

Identification of Isoorientin 3′-*Methyl Ether and an* O-β-D-*Glucoside of Chrysoeriol from* L. minor

In the course of the present biogenetic investigations two flavones, isoorientin 3′-methyl ether and an *O*-β-D-glucoside of chrysoeriol, were detected in *L. minor* for the first time. We report here the evidence for their identifications.

Chrysoeriol O-β-D-*glucoside.* A dark absorbing spot near the orientin spot (see Fig. 4) was observed when the two-dimensional paper chromatogram of an extract from *L. minor* was viewed in u.v. light; it appeared bright yellow in the u.v. when treated with ammonia. The above data were essentially identical with those previously observed for luteolin

[12] There are a number of lucenins and vicenins, all of which are based on structures XII and VI, respectively but differing in their *C*-glycosyl moieties. For a recent review, see reference 1b.

[13] Additional preliminary experiments with ^{14}C-vitexin III indicate that this mono-*C*-glycoside also does not serve as a precursor for the di-*C*-glycosyl flavones.

7-*O*-glucoside.[14] There was not sufficient material to obtain a sample of the flavonoid glycoside completely free of orientin; therefore, the mixture was hydrolyzed with β-glucosidase to yield a readily separable mixture of an aglycone, different from luteolin, and orientin. The chromatographic behavior and u.v. spectra of this flavone aglycone corresponded precisely to those observed for chrysoeriol (IX)[14]; therefore, the original glycoside (based on its hydrolysis with β-glucosidase) is an *O*-β-D-glucoside of chrysoeriol.

Isoorientin 3′-methyl ether (XIII). This compound was first observed to be a constituent of *Lemna minor* upon examination of the autoradiogram which was obtained from the experiment in which ^{14}C-isoorientin was present in the growth medium of *L. minor*. The R_f-values for the compound (see Fig. 4C) were slightly greater than those observed for isoorientin in both TBA and HOAc, a result which suggested that the compound was an *O*-methyl ether. The compound was subsequently isolated from *L. minor* plants not cultured in the presence of ^{14}C-isoorientin in sufficient quantity for u.v. spectral analyses. The u.v. spectra in methanol (λ_{max} 344, 267 and 255 nm), methanol plus sodium methoxide (λ_{max} 408, 326 and 272 nm), methanol plus sodium acetate (λ_{max} 386 and 272 nm) and acidic $AlCl_3$ (λ_{max} 382, 257, 294, 273, and 263 nm) were essentially identical with thos previously observed for isoorientin.[14] However, the compound had spectra different from isoorientin in the presence of anhydrous $AlCl_3$ and sodium acetate plus boric acid; with these two reagents shifts resulting from the presence of an *ortho* dihydroxyl group were not observed.[15]

The autoradiographic results, which showed that the compound was readily formed from isoorientin in *L. minor*, combined with the u.v. spectral data, which indicated that the substance had free hydroxyl groups at the 5,7- and 4′-positions but not at the 3′, and the compounds' chromatographic behavior, which was in accord with an O-methyl ether of isoorientin, indicated that the flavone is isoorientin 3′-methyl ether (XIII).

CONCLUSIONS

The experiments in which either ^{14}C-apigenin, ^{14}C-luteolin, ^{14}C-orientin, ^{14}C-isoorientin and ^{14}C-isovitexin were present in the growth media of either *Spirodela polyrhiza* or *Lemna minor* plants gave the following results (based on autoradiographic data): (1) Apigenin was *O*-glucosylated and/or oxidized to a 3′,4′-dioxygenated flavone, but was not *C*-glycosylated; (2) Luteolin was *O*-methylated and *O*-glucosylated but was not *C*-glycosylated; (3) Orientin was not *C*-glycosylated; (4) Isoorientin was *O*-methylated but not *C*-glycosylated; (5) Isovitexin was *O*-glycosylated; and (6) None of the flavones was converted to anthocyanins.

These observations provide support for the hypothesis that *O*-glycosylation and *O*-methylation are late or terminal flavonoid biogenetic steps and suggest that *C*-glycosylation is an early biogenetic process.

EXPERIMENTAL

Isolation of ^{14}C-Flavonoids

^{14}C-Apigenin, ^{14}C-luteolin, ^{14}C-vitexin and ^{14}C-orientin were obtained by the following procedure. *Spirodela polyrhiza* (clone 7003)[16] plants were cultured in 20–30 125 ml Erlenmeyer flasks stoppered with

[14] See the following reference for a general tabulation of chromatographic and spectral data for flavonoids: T. J. MABRY, K. R. MARKHAM and M. B. THOMAS, *The Systematic Identification of Flavonoids*, Springer-Verlag, New York (1969).

[15] K. R. MARKHAM and T. J. MABRY, *Phytochem.*, in press, 1969.

[16] All clone numbers refer to the University of Texas at Austin collection of Lemnaceae species initiated by Dr. J. W. McClure (see J. W. MCCLURE, Ph.D. dissertation, The University of Texas at Austin, 1964).

cotton plugs. Each flask contained approximately 18 ml of solution (1 part Hutner's medium,[17] 2 parts H_2O) containing 0·18 g sucrose and $1·5 \times 10^{-6}$ moles of phenylalanine-1-^{14}C (sp. act. 12 mc/mM, Nuclear Chicago); the solution was autoclaved at 15 psi for 15 min prior to inoculation with about 10 plants (using a platinum loop which had been flame sterilized) from the stock clone. After 10–12 days under continuous illumination (3000 ft-c), the plants from each flask were harvested, rinsed in cold tap water to remove external ^{14}C-containing compounds, and then air-dried overnight. The dry plants from each flask were extracted overnight with 30 ml of methanol containing 0·3% conc. HCl. The extract was decanted from the plant material and the solvent was removed *in vacuo*. The residue from each flask was dissolved in about 0·5 ml of methanol and chromatographed two dimensionally on Whatman No. 3MM filter paper. The chromatograms were developed first (in the long, 57 cm, direction) in TBA (*t*-butyl alcohol: acetic acid: water; 3:1:1) and then HOAc (15% aqueous acetic acid).

Two areas, one containing orientin and luteolin 7-*O*-glucoside and one containing vitexin and apigenin 7-*O*-glucoside, were cut out from each chromatogram. The pieces of paper were extracted in 125 ml Erlenmeyer flasks with excess reagent-grade methanol three times (each time, overnight with shaking). The appropriate extracts were combined and the solvent removed *in vacuo*. The residue, from plants from 20 to 30 flasks, was mixed with 15 ml of water containing a few mg of β-glucosidase (Sigma Chemical Co.). The solution was allowed to stand overnight, after which time the solvent was removed *in vacuo*; the residue was rechromatographed two-dimensionally. The aglycone, either ^{14}C-apigenin or ^{14}C-luteolin, was distinctly separated from the corresponding *C*-glycosylflavone, either ^{14}C-vitexin or ^{14}C-orientin. All four ^{14}C-flavones, ^{14}C-apigenin, ^{14}C-luteolin, ^{14}C-orientin and ^{14}C-vitexin, were obtained from the appropriate chromatograms by extraction (as described above) with spectral grade methanol.

Isolation of ^{14}C-*Isovitexin and* ^{14}C-*Isoorientin*

^{14}C-Isovitexin and ^{14}C-isoorientin were obtained from *Spirodela oligorhiza* (clone 70) and *Lemna obscura* (clone 97), respectively, by the procedure described above; however, in these experiments, it was not necessary to treat the extracts from the original chromatograms with β-glucosidase. Both ^{14}C-isovitexin and ^{14}C-isoorientin were re-chromatographed for final purification.

Culturing of Plants in Media Containing ^{14}C-*Flavones*

All the experiments in which either ^{14}C-apigenin, ^{14}C-luteolin, ^{14}C-orientin, ^{14}C-isoorientin or ^{14}C-isovitexin was present in the growth media of either *S. polyrhiza* or *L. minor* plants were carried out by the following procedure. The appropriate ^{14}C-flavone ($1·5 \times 10^{-6}$ moles) in 12 ml of H_2O was added through a millipore filter (0·45 μ dia.) to a 125 ml Erlenmeyer flask containing 0·18 g of sucrose dissolved in 6 ml of Hutner's solution. The flask was inoculated (by the procedure described above) with about 20–30 plants from the stock clone. The remaining aspects of the experiments were the same as those used for obtaining a chromatogram of the plant extract when phenylalanine-1-^{14}C was present in the growth medium (see above).

Autoradiographic Procedures

All autoradiograms were prepared by placing Kodak Industrial X-Ray Film (Type KK, 36 × 43 cm) in direct contact (front and back) with each chromatogram. The film-chromatogram "sandwiches" were stored in the dark for one day (Fig. 1), 1 week (Figs. 2A, 2B, 3A, 4A, 4B and 5B), 1 month (Figs. 3B and 5C) and 3 months (Fig. 5A). Other exposure times were also employed for each experiment; all the autoradiograms including those selected for this report, are recorded elsewhere.[17] The film was processed in Kodak X-Ray Developer and Fixer. The autoradiograms were photographed with Kodak Royal Pan Film and the negatives were printed on Kodabromide F-5 enlarging paper.

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[17] J. W. WALLACE, Ph.D. dissertation, The University of Texas at Austin, 1967.