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BIOSYNTHESIS OF THE COUMARINS: SCOPOLETIN FORMATION IN TOBACCO TISSUE CULTURES

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Abstract—Feeding experiments of tobacco tissue cultures with (U)-^{14}C-phenylalanine, 2-^{14}C-cinnamic acid, 2-^{14}C-glucosidoferulic acid and methyl-^{14}C-methionine were carried out over periods from 3 min to 10 hr. The use of short feedings enabled us to study the kinetic aspect of the biosynthesis and to demonstrate the following main pathway: phenylalanine → *free* cinnamic acid → *free* p-coumaric acid → *free* caffeic acid → *free* ferulic acid → scopoletin → scopolin. Turn-over rates of free forms were shown to be much higher than those of the corresponding bound forms.

INTRODUCTION

THE BIOSYNTHESIS of coumarin (I) and some 7-oxygenated coumarins (II, III, IV) has been previously described.[1,2] Evidence has been obtained that the pathway involves phenylalanine, cinnamic acid and glycosides of hydroxycinnamic acids as intermediates. The biosynthesis of scopoletin (VI) has been studied very little. However, it has been noticed that in tobacco leaves scopolin (VII) becomes labelled after a 24 hr feeding of radioactive ferulic acid[3-6] as well as of radioactive glucosidoferulic acid.[4-6]

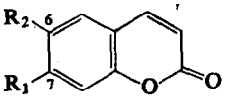

FIG. 1. COUMARINS CITED IN THE TEXT.

(I) Coumarin	$R_1 = H$	$R_2 = H$
(II) Umbelliferone	$R_1 = OH$	$R_2 = H$
(III) Skimmin	$R_1 = O$Glucose	$R_2 = H$
(IV) Herniarin	$R_1 = OCH_3$	$R_2 = H$
(V) Esculetin	$R_1 = OH$	$R_2 = OH$
(VI) Scopoletin	$R_1 = OH$	$R_2 = OCH_3$
(VII) Scopolin	$R_1 = O$Glucose	$R_2 = OCH_3$

The present paper reports the results of studying the biosynthesis of scopoletin in tobacco tissue cultures. The following objectives were considered: (1) determination of the biosynthetic pathway; (2) determination of the turn-over rates of scopoletin, scopolin and all related compounds; and (3) determination of the individual reactions and the relative importance of each of them *in vivo*.

[1] S. A. BROWN, G. H. N. TOWERS and D. CHEN, *Phytochem.* 3, 469 (1964).
[2] D. J. AUSTIN and M. B. MEYERS, *Phytochem.* 4, 245 (1965).
[3] V. C. RUNECKLES, *Can. J. Biochem. Physiol.* 41, 2259 (1963).
[4] W. STECK, *Can. J. Biochem.* 45, 889 (1967).
[5] W. STECK, *Can. J. Biochem.* 45, 1995 (1967).
[6] W. STECK, *Phytochem.* 7, 1711 (1968).

RESULTS AND DISCUSSION

FORMATION OF LABELLED SCOPOLETIN AND SCOPOLIN FROM PHENYLALANINE (U)-^{14}C: RELATIONSHIP BETWEEN THE TWO COUMARINS

In *Hydrangea macrophylla*, umbelliferone (7-hydroxycoumarin) seems to be formed from cinnamic acid as the glycoside and it is believed that the traces of free umbelliferone recovered represent an artefact of extraction.[1,2] In tobacco leaves[3-6] a similar picture concerning the free and bound forms of scopoletin has been observed: the monoglycoside scopolin is the main form while the aglycone scopoletin is hardly detectable. In tobacco tissue cultures, free scopoletin occurs however in significant and measurable amounts,[7-8] and therefore the use of this material enabled us to study the biosynthetic relationship between aglycone and glycoside. How can the large difference in their concentration be explained in terms of biosynthetic pathways? Is scopolin first synthesized and scopoletin then formed by

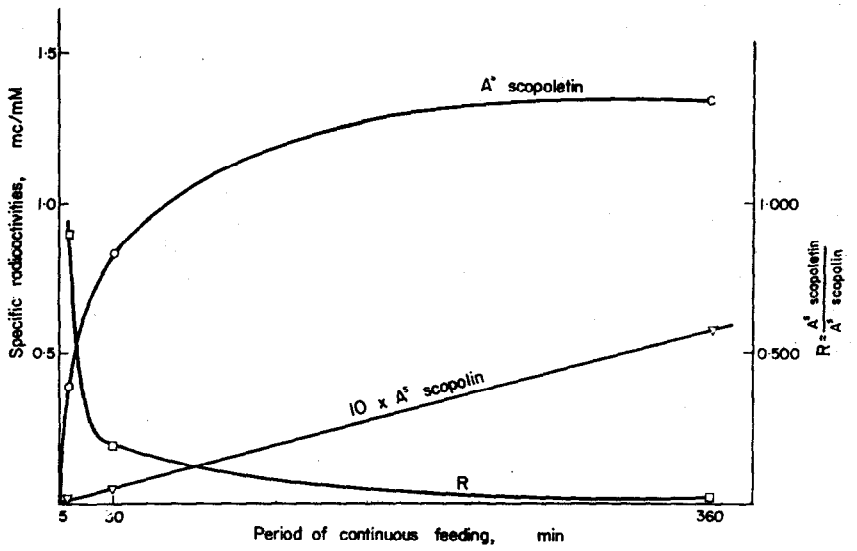

FIG. 2. RATE OF LABELLING OF SCOPOLETIN AND SCOPOLIN FROM (U)-^{14}C-PHENYLALANINE.

hydrolysis, as is the case of skimmin (III) and umbelliferone (II)? Or is scopolin a storage form produced from scopoletin? Or is there no biosynthetic relationship between the two?

To answer these questions we carried out a kinetic study of labelling of both compounds from a common radioactive precursor. ^{14}C-Labelled phenylalanine was fed, since Reid[9] had shown this aminoacid to be a much better precursor of scopoletin in tobacco plants than phenylacetic acid, acetic acid or CO_2. The specific radioactivities of free and bound scopoletin were followed over periods of *continuous feeding* from 3 min to 10 hr. Figure 2 shows the curves obtained with three such parallel experiments in which fresh weights of tissue and amounts of precursor fed were the same. Scopoletin becomes labelled very fast and the shape of its specific activity curve is characteristic of a compound of high turnover. By contrast, there is a slow and almost constant increase of the label in the glycoside, scopolin. The lower

[7] B. FRITIG, L. HIRTH and G. OURISSON, *Compt. Rend.* **263D**, 838 (1966).
[8] F. SKOOG and G. MONTALDI, *Proc. Natl. Acad. Sci. U.S.* **47**, 36 (1961).
[9] W. W. REID, *Chem. Ind.* 1439 (1958).

specific activities of scopolin cannot be explained by an abnormal isotopic dilution by various pools of unlabelled glycoside present in the cells. If this were the case, and supposing that the turnover of scopolin at its sites of biosynthesis is the same as that of scopoletin, the curves obtained should be parallel; the ratio of specific activities should be almost constant with time. As shown in Fig. 2, there is a strong decrease of this ratio; the *shorter* the time of feeding, the *larger* the difference of specific activities. These results clearly indicate that scopoletin, present in only small amounts, has a high turnover rate while its glycoside, present in large amounts, has a low turnover rate.

This finding enables us to answer the above questions: the main pathway leading to scopoletin does *not* involve scopolin as an intermediate; scopolin is probably a storage form produced from scopoletin, though it might equally well arise through an independent pathway from glucosidoferulic acid.

Comparison of the curves of specific activity gives more information (Fig. 2); the flow of radioactivity out of free scopoletin is much higher than that going into scopolin. This means that most of the aglycone is metabolized to something else than the glycoside. What is the fate of this substance? *In vitro* experiments have shown that scopoletin is one of the best substrates for oxidation in presence of peroxidases and H_2O_2.[10] It probably undergoes polymerization via free radicals. Since tobacco tissue cultures show high peroxidase activity, the important utilization of scopoletin could be accounted for by a similar oxidation *in vivo*, though no evidence of such a catabolism has yet been reported.

CINNAMIC AND HYDROXYCINNAMIC ACIDS PRESENT IN TOBACCO TISSUE CULTURES: THEIR ROLE IN SCOPOLETIN AND SCOPOLIN BIOSYNTHESIS

The biosynthesis of coumarins from phenylalanine involves hydroxylation of the aromatic ring and cyclization. The formation of umbelliferone from phenylalanine was shown to proceed via cinnamic acid, *p*-coumaric acid and glucosido *p*-coumaric acid.[1,2] In the case of scopoletin the picture is complicated by the presence of an additional methoxyl group in position 6. Since there is no evidence of direct methoxylations in plants, two more steps are probably required: hydroxylation and *O*-methylation, so that caffeic and ferulic acids might be intermediates. Thus, tobacco tissue cultures were investigated for their cinnamic acid content. After either a long or a short phenylalanine feeding none of the activity of the cinnamic compounds was found in the insoluble derivatives previously described for wheat.[11] The compounds identified in the methanolic extract are listed in Table 1. Only a

TABLE 1. CINNAMIC AND HYDROXYCINNAMIC ACID DERIVATIVES IDENTIFIED IN TOBACCO TISSUE CULTURES

	Cinnamic	*p*-Coumaric	Caffeic	Ferulic
Free form	+	+	+	+
Glucose ester	+	+	−	++
Quinic acid ester	+	+	+++	+
Glycoside		−	−	++

Key: +, in small amounts, detection by use of radioactive tracers; ++, detection by use of both fluorescence in u.v. light and radioactive tracers; +++, present in large amounts; −, not detectable.

[10] G. W. SCHAEFFER, J. G. BUTA and F. SHARPE, *Physiol. Plantarum.* **20**, 342 (1967).
[11] S. Z. EL-BASYOUNI and A. C. NEISH, *Phytochem.* **5**, 683 (1966).

few were detected without use of radioactive tracers, i.e. chlorogenic acid, the most abundant, its glucose ester and the glycoside of ferulic acid. In addition to these three bound forms, another ester was detected which represents the most abundant form of ferulic acid; it has not yet been clearly identified, but according to Thies[12] it may be the shikimic acid ester. No bound forms of *o*-coumaric and *m*-coumaric acids were found, even using carrier compounds.

In order to compare the rate of labelling of scopoletin and scopolin with that of the cinnamic derivatives, a 3 mn pulse feeding of phenylalanine was carried out using 15 g of fresh material and 100 μc of highly labelled precursor (200 mc/mM). The specific activities obtained in free scopoletin and in scopolin were compared to those found in *p*-coumaric, caffeic and ferulic acids after alkaline and acidic hydrolysis of the methanolic extract; these later values each represent the average specific activity of a mixture of forms. As can be seen from Table 2, the specific activities of the three hydroxycinnamic acids are *lower* than that of scopoletin. Since the precursors of scopoletin must have a higher turnover rate than scopoletin itself, this result seems to exclude the cinnamic compounds as intermediates. In fact the change in order of specific activity can readily be explained in terms of their respec-

TABLE 2. SPECIFIC RADIOACTIVITIES OF HYDROXYCINNAMIC ACIDS* AND COUMARINS AFTER A 3 min ^{14}C-PHENYLALANINE FEEDING†

	Compounds				
	p-Coumaric acid	Caffeic acid	Ferulic acid	Scopoletin	Scopolin
Specific radioactivity (dpm/μM $\times 10^{-3}$)	60	16	10	93	0·090

* The specific radioactivity of *p*-coumaric, caffeic and ferulic acids have been measured after alkaline and acidic hydrolysis of the methanolic extract.

† 15 g of fresh material and 100 μc of (U)-^{14}C-phenylalanine (200 mc/mM) were used in this experiment.

tive pools: it provides good evidence that the pathway leading to scopoletin involves only some of the forms identified above. To select the possible intermediates, we investigated the kinetics of their labelling from ^{14}C-phenylalanine in a manner similar to that described above for scopoletin and scopolin. Similar results were obtained: very fast labelling of the free acids with levelling of their specific activity after 30 min of feeding; but the increase in the label of the bound forms is more progressive and equilibrium is still not reached even after 6 hr of continuous feeding. In our material, the classification of the substances by decreasing rate of turnover is: free hydroxycinnamic acids (10) > scopoletin (1) > bound hydroxycinnamic acids ($\frac{1}{20}$ to $\frac{1}{2}$) > scopolin ($\frac{1}{200}$). The figures in parentheses show the relative specific activities measured after a 40 min supply of ^{14}C-phenylalanine. Therefore, *on kinetic grounds*, scopoletin very plausibly is formed via the *free* phenolic acids while scopolin might arise from either free scopoletin or bound hydroxycinnamic acids like glucosidoferulic acid.

STUDY OF SOME STEPS IN SCOPOLETIN BIOSYNTHESIS

In addition to an adequate rate of labelling from a common precursor, there is another requirement for possible intermediates in a biosynthetic pathway: their efficient conversion into one another and into the final product. This aspect will now be considered.

[12] W. THIES, Dissertation, Göttingen, Germany (1965).

The role of cinnamic acid in scopoletin biosynthesis. Thies[12] has suggested that in tobacco tissue cultures the pathway leading to scopoletin did not involve cinnamic acid as an intermediate. He did not find any trace of this compound or any radiocativity corresponding to it after feeding labelled phenylalanine. In our material, a weak radioactivity could be detected only by use of carrier cinnamic acid and phenylalanine of very high specific activity (200 mc/mM). But an intermediate role for cinnamic acid still appeared to be unlikely, since it follows phenylalanine closely in the pathway to hydroxycinnamic acids and would therefore be expected to be highly labelled. Moreover, tyrosine is not converted into *p*-coumaric acid and the following compounds in our material.[13] To test the possibility of a very high turnover, we tried to inhibit the hydroxylation of cinnamic to *p*-coumaric acid. This was done under two different experimental conditions (Table 3): an *in vivo* feeding in nitrogen (Experiment 2) or the incubation of a supernatant obtained from a cell-free extract by centrifuging at 100,000 *g*. This latter fraction contains soluble enzymes and does not contain cinnamic acid hydroxylase which is a particle-bound enzyme. In both cases there is an accumulation of radioactivity in cinnamic acid, when compared with the label found in a normal aerobic feeding (Experiment 1). This clearly indicates that an important flow of radioactivity goes through cinnamic acid and that this compound has a very high turnover.

TABLE 3. PHENYLALANINE AMMONIA-LYASE ACTIVITY IN TOBACCO TISSUE CULTURES: RESULTS FROM (U)-^{14}C-PHENYLALANINE FEEDINGS (200 mc/mM)

Experiment	Experimental conditions	Radioactivities (dis/min) found per 10 g of fresh material in: Total cinnamic acid	Radioactivities (dis/min) found per 10 g of fresh material in: Total *p*-coumaric acid
1	Tobacco tissue cultures, normal aerobic conditions	2500	200,000
2	Tobacco tissue cultures, anaerobic conditions	600,000	15,000
3	Cell free extract (pH = 8·8), anaerobic conditions	100,000	0

When 2-^{14}C-cinnamic acid is fed to tobacco cultures more than 50 per cent of it is metabolized after 4 hr and more than 40 per cent of the metabolized activity is found in free and bound *p*-coumaric, caffeic and ferulic acids (about 20 per cent in chlorogenic acid alone). Thus, as expected, cinnamic acid appears to be a very efficient and specific precursor of the hydroxycinnamic derivatives. The label obtained in scopoletin and scopolin is indicated in Table 4; cinnamic acid is efficiently incorporated into both. The dilution value into scopoletin is very low, about 30 times lower than that of phenylalanine. Moreover an analogous difference of specific activity between scopoletin and scopolin is observed, in the same way as from phenylalanine as precursor.

The Two Possible Pathways in Scopoletin Biosynthesis

The two successive hydroxylations which convert cinnamic acid into caffeic acid have already been described[14–16] and it has been shown that they occur with the free acids as well

[13] B. FRITIG, L. HIRTH and G. OURISSON, *Compt. Rend.* **263D**, 860 (1966).
[14] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 537 (1959).
[15] P. M. NAIR and L. V. VINING, *Phytochem.* **4**, 161 (1965).
[16] D. W. RUSSEL and E. E. CONN, *Arch. Biochem. Biophys.* **122**, 256 (1967).

TABLE 4. COMPARISON OF CINNAMIC, FERULIC AND GLUCOSIDOFERULIC ACIDS FEEDINGS

Compound fed	Amount (μM) fed per 10 g of fresh material	Radioactivity		Period of feeding (hr)	% Incorporation (and dilution values) into				
		Fed (μc)	Recovered (%)		Ferulic acid			Scopoletin	
					Free	Glycoside	Esters	Free	Glycoside
2-^{14}C Cinnamic acid	0·25	0·5	44	4	1·2	1·5	7·1	1·4 (20)	2·6 (1000)
2-^{14}C Ferulic acid	0·20	1·4	7	6	—	3·3	4·4	0·33 (155)	1·3 (2200)
2-^{14}C Glucosido ferulic acid	0·25	5·0	55	6	0·70	—	9·5	0·50 (160)	2·8 (2600)

as with the bound forms. After caffeic acid two possible pathways leading to scopoletin have to be considered (Fig. 3). In the first, methylation would be the last step, so that esculetin (or 6,7-dihydroxycoumarin) would be involved as an intermediate. Such a pathway is quite reasonable since methyltransferases converting esculetin into scopoletin are known[17] and since esculetin has been detected in tobacco plants.[18] In the second hypothesis, cyclization would be the last step and ferulic acid would then be an obligatory intermediate.

The identification of esculetin is complicated by the spontaneous cyclization of the *cis* isomer of caffeic acid to esculetin; therefore esculetin must be separated from caffeic acid in the first step of the analytical procedure. Doing so, we were unsuccessful in identifying esculetin in our material: it could not be detected, neither by its strong blue fluorescence in u.v. light nor by any label from cinnamic acid or phenylalanine feedings. To exclude the possibility of a very high turnover, trapping experiments were carried out in which unlabelled esculetin and very highly labelled phenylalanine were fed together. The results were also negative: no radioactivity was found in the carrier esculetin recovered and comparison with

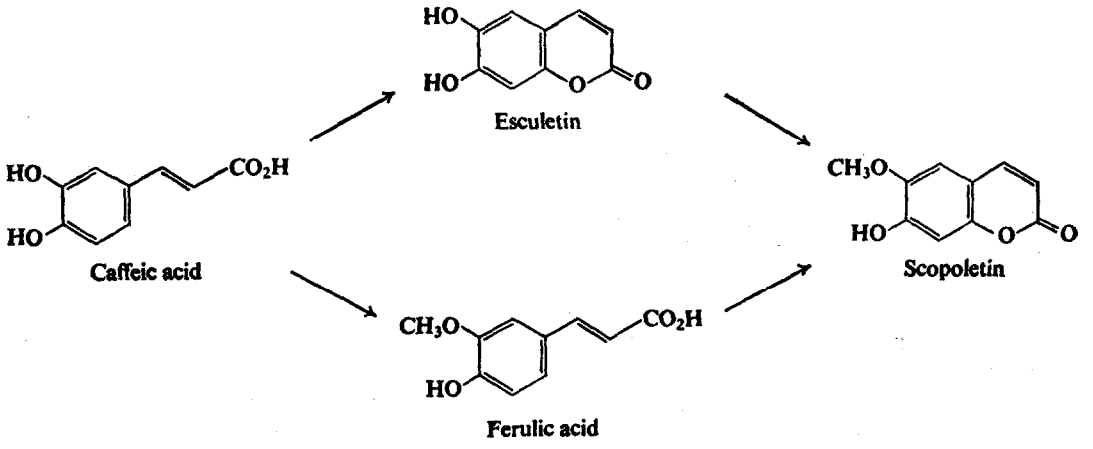

FIG. 3. THE POSSIBLE BRANCHING AT THE LEVEL OF CAFFEIC ACID.

a normal ^{14}C-phenylalanine feeding did not indicate any significant dilution into scopoletin or scopolin through the unlabelled esculetin. Therefore esculetin does not seem to be involved in the pathway.

To test the alternative pathway, 2-^{14}C-ferulic acid was fed and the results are given in Table 4. There is a significant incorporation of ferulic acid into scopoletin and scopolin; however, this incorporation is much lower than expected. Cinnamic acid, for instance, appears as a much better precursor though ferulic acid is theoretically the nearest intermediate to scopoletin. But it must be pointed out that only 7 per cent of the precursor was recovered and that a high radioactivity seemed to be attached to the cell walls: ferulic acid probably underwent polymerization in the presence of peroxidase or peroxidase-like enzymes during penetration and only a small amount of it was left for scopoletin biosynthesis. However, the results obtained in the ferulic acid feedings were unsatisfactory and more evidence was necessary to demonstrate a main pathway through this compound.

[17] C. YANG, M. D. BRAYMER, P. L. PETRAKIS, M. R. SHETLER and S. H. WENDER, *Arch. Biochem. Biophys.* **75**, 538 (1958).
[18] L. J. DIETERMANN, C. M. YANT, Y. NAGAGAWA and S. H. WENDER, *J. Org. Chem.* **24**, 1134 (1958).

By feeding tobacco leaves with labelled glucosidoferulic acid, Steck[4,5] showed that this compound was an efficient precursor of scopolin, but when we supply tobacco tissue cultures with 2-^{14}C-glucosidoferulic acid, scopolin is *not* the most highly labelled compound (Table 4). The distribution of radioactivity in ferulic acid bound forms and in the coumarins is similar to that obtained from free ferulic acid, but there is a better recovery of the precursor. This shows that scopolin biosynthesis does not involve glucosidoferulic acid as a main intermediate: scopolin arises by glucosidation of scopoletin. Previous observations[3-5,19] can be explained by the label found in free ferulic acid and its esters (Table 4): it is obvious that glucosidoferulic acid undergoes cleavage of its glycosidic group, all the interconvertible forms of ferulic acid become labelled, and over a long feeding period most of the radioactivity will be recovered in the compound of lowest turnover, i.e. in scopolin.

Ferulic Acid as an Intermediate

Various experiments provided indirect evidence for this role: we summarize below the results obtained.

Comparison of Methyl-^{14}C-methionine and (U)-^{14}C-phenylalanine feedings. In a ^{14}-C phenylalanine feeding, the label is introduced much before the branching indicated in Fig. 3. But if ^{14}C-methyl methionine is supplied, the label can be introduced at two different levels: after the branching (methylation of esculetin) in the case of a pathway through esculetin, or at the level of the branching (methylation of caffeic acid) in the case of a pathway through ferulic acid. It is obvious that the latter pathway involves an additional dilution of radioactivity by the endogenous amounts of ferulic acid and this should decrease the ratio between dilution values into scopoletin and ferulic acid. The results are given in Table 5: the ratios obtained in Experiments 2 ($\frac{780}{1100} \simeq 0{\cdot}71$) and 3 ($\frac{49}{61} \simeq 0{\cdot}80$) are of the same order of magnitude. A direct methylation of esculetin appears therefore unlikely since it should have resulted in a relatively higher label of scopoletin to ferulic acid in Experiment 3 than in Experiment 2.

In vitro *methylations.* The supernatant (pH 7·4) obtained from a cell-free extract by centrifuging at 100,000 *g* showed an *O*-methylase activity. With this enzymatic fraction and in presence of ATP and ^{14}C-methyl methionine, radioactive ferulic acid and feruloylquinic acid were obtained from caffeic acid and chlorogenic acid, respectively, as substrates. But esculetin was not a substrate: no labelled scopoletin was produced. Thus caffeic acid, present in our material, is a substrate for this enzymatic methylation system while esculetin, not detectable in our tissue cultures, is not a substrate. Of course this negative result is not a direct proof but only an indication that methylation might not be the last step in scopoletin biosynthesis.

Chase experiments. In vivo parallel incubations with ^{14}C-methyl methionine were carried out under nitrogen: as expected, ferulic acid was the only labelled phenolic acid, and scopoletin and scopolin remained unlabelled because an oxidation step is necessary for the cyclization into coumarins. A chase experiment consisting of an aerobic incubation with non-radioactive methionine was compared with several controls and showed a decrease in the labelling of ferulic acid at the same time as a strong increase in that of scopoletin and scopolin. As shown by the controls, this increase cannot be explained only by the presence in the cells of residual radioactive methionine, since in such a case there would have been almost no decrease in the label of ferulic acid.

[19] D. J. AUSTIN and M. B. MEYERS, *Chem. Commun.* 125 (1966).

TABLE 5. COMPARISON OF (U)-^{14}C-PHENYLALANINE AND METHYL-^{14}C-METHIONINE FEEDINGS

Experiment	Amounts* of compound fed	Radioactivity fed (μc)	Period of feeding (hr)	Dilution values into		
				Total ferulic acid	Scopoletin	Scopolin
1	60 μg of labelled phenylalanine	40	4	1200	1000	34,000
2	750 μg of non-labelled methionine +60 μg of labelled phenylalanine	40	4	1100	780	29,000
3	60 μg of non-labelled phenylalanine +750 μg of labelled methionine	50	4	61	49	2400

* 30 g fr. wt. of material were used in each experiment.

Isotopic dilution experiments. A 2 mM solution of non-labelled ferulic acid containing radioactive phenylalanine was supplied during 40 min. Comparison with a normal radioactive phenylalanine feeding showed that the label of the free phenolic acids was the same or even higher while a 90-fold dilution into scopoletin occurred. This is a very strong experimental support for a pathway through ferulic acid since a similar experiment with esculetin did not result in any significant dilution into scopoletin.

The Cyclization

How can ferulic acid be converted into scopoletin? At least two steps seem to be necessary: a *trans–cis* isomerization at the level of a phenolic acid, and an oxidative step. In our material isomerization is probably not a pure photochemical process:[20,21] there is no significant change in the label of any phenylpropanoid when feeding experiments with phenylalanine are carried out in darkness. Concerning the oxidative step, 2-hydroxyferulic acid is a logical intermediate following ferulic acid in the pathway. Its identification is complicated by the spontaneous cyclization of the *cis*-isomer into scopoletin and by its spontaneous production from scopoletin by alkaline hydrolysis. All our attempts to find it in tobacco tissue cultures were unsuccessful. Even trapping experiments with simultaneous feeding of non-labelled 2-hydroxyferulic acid and radioactive phenylalanine, cinnamic acid, ferulic acid or methionine did not result in any significant label in the carrier recovered or in the glucose ester formed from it by tobacco tissue cultures. The cyclization of this compound might be so fast that a very high turnover is to be expected. Another reasonable possibility is that ferulic acid itself is cyclized into scopoletin via radicals formed in the presence of peroxidase-like enzymes, and this seems in agreement with the isotopic dilution experiment described above.

CONCLUSION

The proposed main pathway leading from phenylalanine to scopoletin and scopolin is indicated in Fig. 4. One particular problem arose from the presence of a 6-methoxy-7-hydroxy substitution: at which level does methylation occur? That ferulic acid is involved as intermediate in the pathway shows that methylation precedes cyclization, and this is in good agreement with the pathway demonstrated in the case of the biosynthesis of herniarin (7-methoxycoumarin).[22] The possible presence and role of esculetin in our material cannot be excluded, but we could not find any experimental support for a pathway involving this compound as an intermediate.

Important results concerning the relationships between free and bound forms were found, especially by studying the kinetic aspects of the biosynthesis: the free forms have a much higher turnover rate than their respective bound forms. This result, obtained first from phenylalanine feedings, was confirmed with cinnamic acid, ferulic acid and methionine as precursors. It shows important differences with the pathway demonstrated for umbelliferone[1,2] and with that proposed for scopolin in tobacco leaves.[4–6]

The use of tobacco tissue cultures as a material played a decisive role in these findings. These cultures can be divided into equivalent fragments and this makes parallel or chase experiments easy to carry out. It is also possible to obtain good anaerobic conditions by incubating under nitrogen. Moreover, since these tissue cultures are undifferentiated cells and

[20] F. A. HASKINS and H. J. GORZ, *Biochem. Biophys. Res. Commun.* **6**, 298 (1961).
[21] K. G. EDWARDS and J. R. STOCKER, *Phytochem.* **6**, 655 (1967).
[22] S. A. BROWN, *Phytochem.* **2**, 137 (1962).

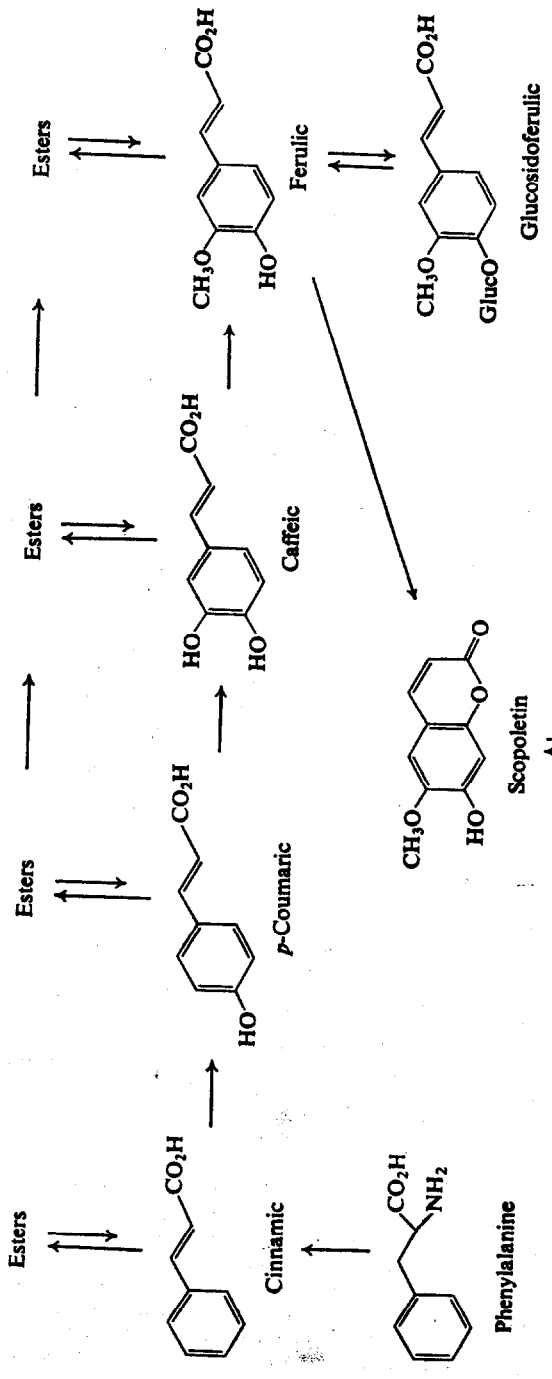

FIG. 4. THE PROPOSED MAIN PATHWAY LEADING TO SCOPOLETIN AND SCOPOLIN IN TOBACCO TISSUE CULTURES.

have no organs, there is no problem of mechanical transport of precursors, as in whole plants; the problem of transport across a cell wall alone remains. Consequently, incorporation times can be kept very short and thus the main pathway is easier to find: the lower the feeding periods, the larger the differences of specific radioactivity between the compounds of high and low turnover rates.

EXPERIMENTAL

Cultivation of Plant Material

The tobacco tissue cultures used in these experiments were obtained by successive transfers from tobacco tissue previously isolated from *Nicotiana tabacum* by Morel. They are grown on a basic mineral medium containing saccharose, microelements and agar. These tissue cultures are "habituated", i.e. are growing without kinetin and auxin.

Feeding Experiments

Aerobic feeding experiments were carried out with either an agar medium or a liquid medium. In the former case cultures were transferred onto Petri dishes containing the normal growth medium and the radioactive precursor was applied on the surface of cultures as drops of a concentrated buffered solution. In the second case an Erlenmeyer flask was used, containing growth medium without agar, cultures and radioactive precursor. Then the flask was put on a rotary shaker for incubation. This latter procedure was used in the case of short feedings (less than 4 hr), whereas the former was preferred in the case of longer periods of feeding.

For anaerobic feeding a round-bottom flask was used, containing liquid growth medium. After introduction of the cultures, air was removed by successive connexion to a vacuum pump and to N_2. Then the solution of precursor was injected through a rubber cap into the flask by means of a sterile syringe, and a N_2 flow was maintained during the metabolic period. Good anaerobic conditions were obtained.

Isolation and Quantitative Determination of Compounds

After freeze-drying of the cultures, extraction was accomplished with methanol. For isolation of both free and bound phenolic acids classical paper chromatography was performed.[3,4,12,14,23,24]

Scopoletin and scopolin were isolated by means of successive TLC (with silica-gel as adsorbent and 20% CH_3OH in $CHCl_3$ as solvent) and paper chromatography [with C_6H_6–AcOH–H_2O (6:7:3) for the former and *n*-BuOH–HoAc–H_2O, (4:1:5) for the second].

For the knowledge of specific activities, measurements of both concentrations and radioactivities were necessary. Phenolic acids were determined by double counting as described later. We measured the concentrations of ethanolic solutions of scopoletin by either spectrophotometry of spectrophotofluorimetry.[18] Scopolin was determined as its hydrolysis product.

Measurements of Radioactivity

Countings were performed with either a gas-flow apparatus (for higher amounts of compound) or a scintillation spectrometer (for lower amounts, because of the strong fluorescence of the compounds studied). Only radioactivities over 1000 dis/min (^{14}C) were regarded as significant.

Prior to counting, the radiochemical purity was checked by means of recrystallization to constant specific activity and of functional radiochromatography. In this latter case, coumarins and phenolic acids were chromatographed in various systems after chemical transformations like isomerization of the cinnamic double bond, alkaline and acidic hydrolysis of bound forms, acetylation of phenolic groups, methylations of carboxylic acid and phenolic groups. Chromatograms were scanned with a Berthold radioactive scanner and it was checked that before and after any chemical transformation the radioactive peak was superimposed with the compound detected in u.v. light.

Quantitative Determination of the Phenolic Acids: U.v. and Fluorescence Measurements, Double Counting

Great difficulties were encountered with the spectrophotometric determinations. First, a shoulder at 275 nm in the u.v. spectrum of each cinnamic compound was observed after paper chromatography, probably because of the formation of a complex with paper. Blanks did not show this shoulder and an elution of the paper with any solvent prior to the chromatography did not help. The importance of the shoulder increased with the ratio: surface of spot on paper/amount of compound. Second, *trans–cis* and *cis–trans* isomerizations of the cinnamic derivatives took place, especially on dry chromatograms and under u.v. light. Even after separation of the isomers by means of chromatography with an aqueous solvent both spots again are mixtures of

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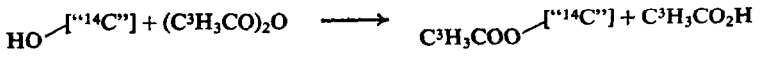
isomers. Since it has been shown that their ϵ are different[25] it is obvious that the spectrophotometric determination cannot be very accurate. In the case of caffeic acid a third complication derives from the spontaneous cyclization of the *cis* isomer into esculetin that has quite different spectral properties. Thus we used the following procedure: the ^{14}C-labelled phenolic acid was acetylated with ^{3}H-labelled acetic anhydride of known specific activity. The reaction scheme is indicated below:



This method has been successfully used as the "double isotope assay" in steroid hormones measurements.[26] In the case of *p*-coumaric, caffeic and ferulic acids we were able to demonstrate the absence of any isotopic effect and the quantitative acetylation of both *trans* and *cis* isomers.[27] Double counting of ^{14}C and ^{3}H leads to the knowledge of the specific activity which is proportional to the ratio $^{14}C/^{3}H$. During purification of the doubly labelled acetate, carrier substances may be added without changing the results. The sensitivity of the determination can be increased by increasing the specific activity of tritiated Ac_2O: thus we were able to measure the specific activity of free *p*-coumaric acid and this, as far as we know, has never been reported. Determinations of bound forms were carried out after separation and hydrolysis, as described below.

The phenolic acid to determine is acetylated at room temperature with a mixture containing 10 μl of pyridine and 20 μl of a solution of 10% tritiated Ac_2O in benzene. Reagents in excess are then evaporated under vacuum and 0·5–1·0 mg of non-labelled acetate of the phenolic acid is added as carrier. A first paper chromatography with C_6H_6–AcOH–H_2O (6:7:3) as solvent is carried out and the doubly labelled acetate (detected under u.v. light) eluted with acetone. After evaporation of acetone, a solution of CH_2N_2 in ether is added in order to methylate the carboxylic acid function. The compound obtained is then chromatographed on silica-gel HF (giving a yellow-greenish fluorescent background under u.v. light) with 50% EtOAc in cyclohexane when most of the tritiated contamination is removed. The dark absorbing band is eluted with ether. A final purification of the methyl ester and acetate of phenolic acid is carried out by means of a second paper chromatography with the benzene solvent. After elution with ether, double counting is performed with a liquid scintillation spectrometer.

When the amount of phenolic acid to be determined was less than 50 μg, scopoletin (500 μg) was added as protecting agent and carrier before acetylation; the tritiated acetate of scopoletin was very easily removed during the TLC.

Bound forms (esters or glycosides) were isolated and purified separately, hydrolysed into the corresponding free form which was then subjected to acetylation. When they were present in very low amounts, scopoletin was added as a carrier already before hydrolysis.

Commercial tritiated Ac_2O was diluted with non-labelled anhydride in order to obtain a ^{3}H–^{14}C counting ratio of about 10. This represents the best ratio for an accurate double-counting. The exact specific activity of the tritiated anyhdride was determined by applying the above-described procedure to a known amount of known radioactivity of a ^{14}C-labelled phenolic acid.

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