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OBSERVATIONS ON SCOPOLETIN AND SCOPOLIN METABOLISM

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Abstract—The specific activities of scopoletin and scopolin synthesized by tobacco callus from uniformly labeled phenylalanine reached a maximum at 18 hr and then decreased. The formation of scopolin was not influenced by the presence or absence of 2 mg/l. IAA in the incubation medium in 15-hr experiments. In the range 21 to 165 mg/l. phenylalanine, the specific activities of the scopoletin and scopolin produced as well as their concentrations were constant; at higher phenylalanine concentrations, their specific activities decreased. The specific activity of scopoletin was higher than that of scopolin in all experiments. On incubation of tobacco callus with labeled scopolin, little of the activity was incorporated into true lignin; most of the activity was associated with the protein and cell-wall fractions. When pea-stem segments were incubated with scopoletin, they formed an insoluble material which, when oxidized with nitrobenzene, produced little extra vanillin.

INTRODUCTION

SCOPOLETIN and its glycoside scopolin is found in many plants.[1–9] It accumulates in diseased plants,[10–12] boron-deficient plants[13, 14] and 2,4-D-treated plants.[15, 16] The occurrence of scopoletin in tobacco callus has been established by several investigations.[7, 17, 18] Tryon[19] reported that two strains of tobacco callus of similar origin that differed markedly in their ability to form buds also differed in their scopoletin content; the bud-forming strain contained twenty times as high a concentration of scopoletin as the non-differentiating strain. Scopoletin inhibits indoleacetic acid (IAA) degradation;[20, 21] however, IAA and kinetin influence the concentrations of scopoletin and scopolin found in tobacco callus.[7, 22]

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Despite the common occurrence of scopoletin in plants, the metabolism of this compound has received comparatively little attention. Dewey and Stepka[23] showed that during the post-harvest conversion of ^{14}C-labeled nicotine to nornicotine, the methoxy group of scopoletin became radioactive. Steck[24] showed that tobacco leaves form scopoletin from phenylalanine by way of glucosidoferulic acid and scopolin, and that scopoletin is readily converted to scopolin.[25] The work of Fritig *et al.*[26, 27] also indicates that scopoletin is the precursor to scopolin.

This paper presents some data on the conversion of phenylalanine to scopoletin and scopolin by tobacco callus and on the metabolism of these two compounds by tobacco callus and pea-stem segments.

RESULTS AND DISCUSSION

Phenylalanine is readily converted to scopoletin and scopolin.[27, 28] When tobacco callus was incubated 36 hr with a constant amount of uniformly labeled precursor, the specific activities of both scopoletin and scopolin reached maxima at 18 hr while the concentration

TABLE 1. PRODUCTION OF SCOPOLETIN AND SCOPOLIN IN TOBACCO CALLUS FROM PHENYLALANINE*

Duration (hr)	Specific activity (cpm/μg) Scopolin†	Scopoletin‡	Radioactivity in tissue (%)
12	1,150	3,970	96
18	2,170	14,100	95
24	1,250	7,940	93
36	1,460	6,100	91

* 0·01 mc of 8·53 mc/mM uniformly labeled phenylalanine in 5 ml medium with 1 g callus.
† 103 μg scopolin/g fresh wt. callus.
‡ 1 μg scopoletin/g fresh wt. callus.

of both compounds remained constant (Table 1), indicating that they are metabolites. In agreement with the results of Fritig *et al.*[26] the specific activity of scopoletin was higher than that of its glucoside for the entire period studied.

To test the effect of IAA on scopolin formation, tobacco callus was incubated in medium supplemented with IAA, phenylalanine, or glutamic and aspartic acids or a combination of these compounds (Table 2). IAA, neither alone nor in combination with glutamic and aspartic acids, had any detectable effect on the specific activity of the scopolin produced. Thus it appears that the effect of IAA on scopolin accumulation, and probably also on scopoletin accumulation, is indirect. In this experiment, however, increasing the phenylalanine concentration in the medium increased the total and specific activity of scopolin about threefold.

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The effect of increasing the phenylalanine concentration in the presence of a constant amount of labeled precursor is given in Table 3. In the range 21–165 mg/l. phenylalanine the specific activities of scopoletin and scopolin were essentially constant; only when higher concentrations were used did they decrease. Contrary to the results of Skoog and Montaldi[22] that phenylalanine promoted scopoletin produced in experiments lasting 2 months, in this 15-hr experiment the levels of these two coumarins were essentially the same over the entire

TABLE 2. SCOPOLIN PRODUCTION IN TOBACCO CALLUS ON VARIOUS MEDIA*

Supplement	% Activity in tissues	Scopolin specific activity† (cpm/μg)
Control (none)	93	1370
IAA (2 mg/l.)	92	1250
Phenylalanine (41 mg/l.)	79	3650
IAA and phenylalanine	81	3890
Glutamic acid (65 mg/l.) and aspartic acid (51 mg/l.)	94	1440
IAA, glutamic and aspartic acids	93	1460
Phenylalanine, glutamic and aspartic acids	81	4170
IAA, phenylalanine, glutamic and aspartic acids	80	3600

* 550 mg fresh wt. callus was incubated in 5 ml medium containing 0·001 mc of uniformly labeled phenylalanine (215 mc/mM) for 15 hr.

† Average scopolin content 17 μg/g fresh wt.

TABLE 3. EFFECT OF PHENYLALANINE CONCENTRATION ON SCOPOLETIN AND SCOPOLIN FORMATION IN TOBACCO CALLUS*

Phenylalanine in medium (mg/l.)	Label in tissue (%)	Specific activity (cpm/μg)	
		Scopoletin†	Scopolin‡
2,643	37·5	685	38
1,321	30·5	1,470	52
661	35·2	2,870	89
330	52·5	5,470	122
165	58·2	19,800	412
83	73·8	16,500	445
41	83·1	18,400	334
21	87·8	21,000	341

* 540 mg fresh wt. callus was incubated in 5 ml medium containing 0·25 μc uniformly labeled phenylalanine (215 mc/mM) for 15 hr.

† Average scopoletin content 0·7 μg/g fresh wt.

‡ Average scopolin content 54 μg/g fresh wt.

concentration range. Since most of the externally supplied phenylalanine was taken up by the tissue at the lower concentrations, these results indicate that only a small fraction was converted to scopoletin and scopolin, and that most of the phenylalanine was converted to other compounds. Probably the phenylalanine in the medium suppressed the formation of this amino acid by the callus. It thus appears that scopoletin accumulation in diseased, mineral-deficient or otherwise abnormal plants may be not so much the result of conversion of bound scopoletin to the free form but that it is a direct result of interference with the normal pathways of phenylalanine utilization.

Scopolin metabolism was studied by incubating tobacco callus with ^{14}C-labeled scopolin for 24 hr (Tables 4 and 5). Approximately 40 per cent of the label found in the tissue was extracted with ethanol and benzene, about 2 per cent was released by pronase digestion, 33 per cent was released by nitrobenzene oxidation and about 5 per cent remained in the insoluble residue. The fact that the yield of lignin-derived aldehydes on nitrobenzene oxidation was less than 0·5 per cent of the dry extracted weight of the tissue shows the low lignin content of the callus. Similar results were obtained when tobacco callus was incubated with labeled scopoletin. While over one-third of the total label taken up by the tissue was associated with the pronase-resistant fraction, very little radioactivity was found in vanillin on nitrobenzene oxidation of the tobacco callus lignin. As much activity was found in

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN TOBACCO CALLUS INCUBATED WITH ^{14}C-SCOPOLIN*

	(cpm)	% of Total
Ethanol extract	48,200	39
Benzene extract	9,640	8
Pronase digest	14,480	12
"Lignin" fraction (nitrobenzene oxidation)	41,000	33
Residue	6,025	5

* 0·5 g tobacco callus was incubated 15 hr in 5 ml medium containing 20 μg scopolin-U-^{14}C (124,000 cpm).

TABLE 5. DISTRIBUTION OF RADIOACTIVITY IN THE CELL-WALL FRACTION OF TOBACCO CALLUS INCUBATED WITH ^{14}C SCOPOLIN AFTER NITROBENZENE OXIDATION*

	Amount (per g fresh wt.)	
	(μg)	(cpm)
Vanillin	40	482
p-Hydroxybenzaldehyde	16	4,550
Syringaldehyde	18	435
Unknowns (water-soluble)	—	41,000

* For conditions of incubation see Table 4.

syringaldehyde as in vanillin. More activity was found in *p*-hydroxybenzaldehyde than in vanillin even though the callus, on nitrobenzene oxidation, gave rise to more of the latter; the *p*-hydroxybenzaldehyde probably originates from protein tyrosine rather than from true lignin.[29] Since the conversion of scopolin to *p*-hydroxybenzaldehyde (or tyrosine) involves the loss of a methoxy group, the data suggests that scopolin can serve as a methyl donor.

The efficacy of scopoletin and scopolin as lignin precursors was also studied by incubating these two compounds and some known lignin precursors separately with pea-stem sections (Table 6). Scopoletin as well as ferulic acid and *p*-hydroxycinnamic acid promoted the formation of an insoluble lignin-like materials; scopolin did not. The apparent phenol content of the material formed from scopoletin was even higher than that formed from ferulic acid; on nitrobenzene oxidation of the pronase-treated sections, the scopoletin-treated segments

[29] J. E. STONE, M. J. BLUNDELL and K. G. TANNER, *Can. J. Chem.* **29**, 734 (1951).

produced no more vanillin and *p*-hydroxybenzaldehyde than did the controls; the *p*-hydroxycinnamic acid and ferulic acid-incubated sections, however, produced increased amounts of vanillin and *p*-hydroxybenzaldehyde, as expected. That is, while the pea sections produced an insoluble polymer from scopoletin, this material is at best a very unusual lignin.

TABLE 6. LIGNIN CONTENT OF PEA SECTIONS INCUBATED WITH POTENTIAL LIGNIN PRECURSORS

Precursor†	Amount (per g fresh wt.)			
	Extracted dry wt. (mg)	Phenolics (units)*	Vanillin (μg)	*p*-hydroxybenzaldehyde (μg)
Control	10·5	113	65	6
p-Hydroxycinnamic acid	15·6	232	64	130
Scopolin	9·7	110	60	6
Scopoletin	14·3	675	63	10
Ferulic acid	11·7	530	178	70

* Arbitrary units; determined by Gierer's method.[33]
† 1 mM.

The relatively efficient incorporation of scopolin into the protein fraction and into unidentified compounds shows that scopolin has a much greater role in the metabolism of these compounds than it has in lignin biosynthesis.

EXPERIMENTAL

The callus, originally derived from stem segments of *Nicotiana tabacum* L., Wisconsin 38, was maintained on the medium used by Loewenberg.[30]

For the biosynthesis of scopoletin and scopolin from phenylalanine, four to eight tobacco callus slices, weighing about 1 g, were incubated in 5 ml liquid medium with uniformly labeled precursor in the dark at 24° with gentle shaking. The tissue was then harvested, weighed, and ground in a chilled homogenizer with ice-cold 80% EtOH. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant removed. The tissue was extracted twice more with hot 80% EtOH and the alcohol extracts combined and concentrated. The residue was dissolved in a small volume of 75% EtOH and chromatogrammed on Whatman No. 3 paper with acetone/NH_3 (2 N) 85/15 v/v; the scopoletin and scopolin bands were then separately chromatogrammed with HCOOH/NH_4HCOO/water, 1/7/200 v/wt/v. The scopolin and scopoletin bands were then eluted, and brought to a volume of 1 ml each. The radioactivity was measured with a gas flow counter at infinite thinness; scopoletin and scopolin were determined fluorometrically.[7] Emulsin was used to convert scopoletin to scopolin. The scopolin concentrations and specific activities stated in this paper are based entirely on the scopoletin moiety of the glycoside: that is if one unit of labeled scopoletin were converted entirely to scopolin the amounts and specific activities of precursor and product as reported here would remain identical.

The labeled scopolin and scopoletin used were biosynthesized as above: to remove possible contaminants, they were also chromatographed with *n*-butanol/acetic acid/water, 4/1/2 (vol.).

The conversion of scopolin to lignin was tested by incubating 600–700 mg fresh wt. tobacco callus in 5 ml liquid medium containing 20 μg scopolin (124,000 cpm) for 24 hr. The callus was extracted in a Soxhlet with EtOH and EtOH/C_6H_6 (1/1 v/v) and finally rinsed 4 × with EtOH. The dried material was incubated 24 hr in 0·1 mg/ml Pronase (45,000 units/g) in 0·03 M Na-phosphate, pH 7·4, and 5% ethanol. After the residue had been thoroughly washed with water and ethanol, its lignin content was determined by using a modification of the method of Stone and Blundell.[31]

To test the production of lignin from potential precursors by pea-stem sections a modification of Bland's procedure[32] was used. Peas were grown in vermiculite at 18° under continuous fluorescent light for 11 days; at this time the plants were about 15 cm tall. Two 5-cm sections were cut from the center of the uppermost

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internode 4–6 cm long. The sections were floated on distilled water for 2 hr and randomized. Groups of 100–150 sections were then incubated with gentle shaking in 10 ml 10 mM KH_2PO_4 containing 0·03% H_2O_2 and 1 mM precursor for 18 hr at 22° in the dark before they were extracted, digested with Pronase and the lignin determined as described above.

The free hydroxyl groups in the nitrobenzene oxidized samples were determined by the method of Gierer.[33]

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