Tracer Studies on the Incorporation of [2-^{14}C]-DL-Mevalonate into Chlorophylls *a* and *b*, α-Chaconine, and α-Solanine of Potato Sprouts

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Chlorophyll and glycoalkaloids are synthesized in different parts of the potato plant including leaves, tubers, and sprouts. Although light stimulates the biosynthesis of both constituents, the question of whether the two biosynthetic pathways are under the same genetic control has not been resolved. This study investigated the dynamics of incorporation of labeled [2-^{14}C]-DL-mavalonate into chlorophyll *a*, chlorophyll *b*, and the glycoalkaloids α-chaconine and α-solanine in potato sprouts after 7 and 14 days of storage in the light and in the dark. No chlorophyll synthesis occurred in the dark. Fractionation of the "glycoalkaloid" extract followed by high-performance liquid chromatography produced four peaks. The fractions were collected and analyzed for radioactivity. About 80% of the radioactivity resided in fraction 1, the composition of which is unknown. Two of the fractions, with 1−14% of the original label, were α-chaconine and α-solanine. The radioactivity derived from mevalonate largely resides in unidentified compound(s) eluting as a single peak on the HPLC column before the peaks associated with the glycoalkaloids. The specific radioactivity of α-chaconine and α-solanine increased ∼2-fold in going from 7 to 14 days of exposure in the light and in the dark. These and additional observations point to the near identity of the dynamics of biosynthesis of the two glycoalkaloids. These data also implicate a non-mevalonate pathway for the synthesis of both chlorophylls and the glycoalkaloids and are consistent with independent genetic control of the concurrent formation of the two classes of compounds during greening of potatoes.

Keywords: *α-Chaconine; biosynthesis; chlorophyll; glycoalkaloid; HPLC; α-solanine; mevalonate; potato sprouts; tracer studies*

INTRODUCTION

Potatoes synthesize phenolic compounds such as chlorogenic acid, the glycoalkaloids α-chaconine and α-solanine, and inhibitors of trypsin, chymotrypsin, and carboxypeptidase A (*1*, *2*). These compounds exhibit pharmacological activity in animals and humans and have been postulated to act individually or jointly to protect the plant against phytopathogens and insects (*3*). Glycoalkaloids are produced in all parts of the potato plant including leaves, tubers, and sprouts (*4*, *5*). Exposure of potatoes to light after harvest leads to surface greening due to stimulation of chlorophyll biosynthesis and to concurrent increase in glycoalkaloid content (*6*−*9*). Previously, Dao and Friedman (*2*) showed that exposure of potatoes to light stimulates synthesis of chlorophyll, chlorogenic acid, and glycoalkaloids but not protease inhibitors. Experiments were also carried out to investigate whether these different classes of compounds are under the same genetic control. On the basis of the observation that partial immersion of cut potato tubers in water for 5 days resulted in formation of only 2.5 mg of chlorophyll/100 g of fresh peel weight compared to 6.7 mg for the sample placed in aluminum foil, whereas the glycoalkaloid contents of the two samples were identical, it was suggested that chlorophyll and glycoalkaloid biosynthesis are not linked and thus appear to be under separate genetic control. This conclusion is consistent with the observation of Conner (*10*), who also concluded that the syntheses of chlorophyll and glycoalkaloids are independent events. However, on the basis of detailed tracer studies on the incorporation of labeled CO_2, formate, glycine, pyruvate, acetate, mevalonate, and serine into solanidine (the aglycon of both α-chaconine and α-solanine) and chloroplasts isolated from greening potatoes, Ramaswamy et al. (*11*) concluded that chlorophyll and solanine syntheses are intimately related. Although chlorophyll synthesis preceded the synthesis of glycoalkaloids in cold-stored potatoes, the formation of both appeared to be related because the products of CO_2 fixation were also concurrently incorporated into the solanidine structure.

To obtain additional information about the dynamics of these biosynthetic events, this study examined the distribution of radioactivity among chlorophylls *a* and *b*, the individual glycoalkaloids α-chaconine and α-solanine, and other components of potato sprouts exposed to [2-^{14}C]-DL-mevalonate in the dark and in the light. Figure 1 shows the structures of the compounds discussed in this study.

MATERIALS AND METHODS

Materials. May-Queen potatoes were obtained from a supermarket in Himeji City, Japan. To allow sprouting, they were stored in the dark for 6 months at 20 °C. The sprouts

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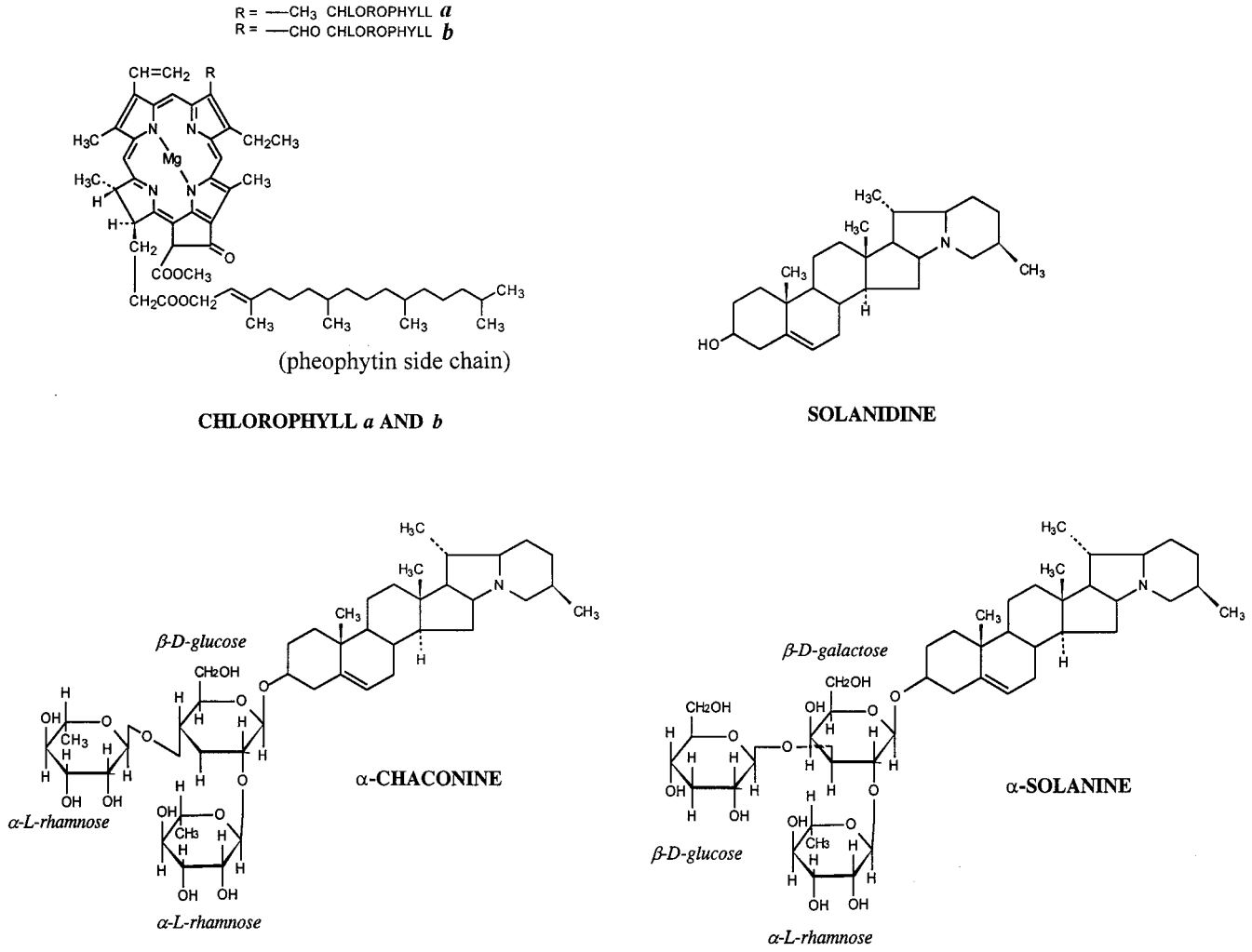

Figure 1. Structures of chlorophyll *a*, chlorophyll *b*, solanidine, α-chaconine, and α-solanine.

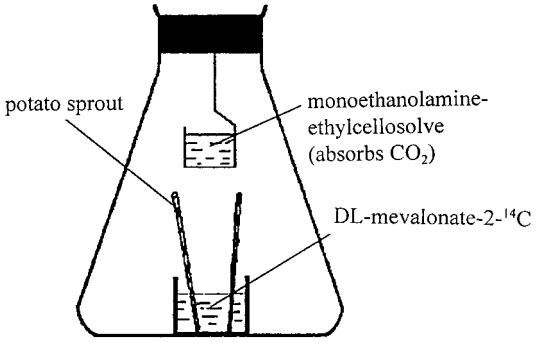

Figure 2. Design of tracer study.

(~10 cm long and weighing ~1 g) were used for the tracer experiment described below.

[2-^{14}C]-DL-Mevalonic acid sodium salt was obtained from New England Nuclear Corp., Boston, MA. DEAE-Sepharose CL-6B and Sepharose CL-6B were purchased from Pharmacia, Uppsala, Sweden. Wako Pure Chemical, Osaka, Japan, supplied all other compounds.

Tracer Experiments. The tracer experiments were carried out in a tightly closed 500-mL Erlenmeyer flask shown in Figure 2. Phosphate buffer (3 mL, 0.1 M) containing 6 μCi of [2-^{14}C]-DL-mevalonic acid was added to each flask, followed by two potato sprout segments. A small vial containing 2 mL of a mixture of monoethanolamine and ethylcellosolve (3:1 v/v) was then placed into each flask to trap the CO_2 produced. The flasks were then kept in an incubator at 15 °C for 7 or 14 days in the dark or under illumination with 15000 lx. At the end of each period, the radioactivity of $^{14}CO_2$ formed was counted by liquid scintillation counting in a scintillator (Aloka Liquid Scintillation System, LSC-3500) containing 6 g of 2,5-di(phenyloxazole) (PPO) and 0.5 g of 1,4-bi(2-phenyloxazolebenzene) (POP) in 667 mL of toluene plus 333 mL of Triton X-100 (*12*).

Radioactivity Measurements. After a rinse with distilled H_2O, the shoots were macerated in a glass mortar with 10 mL of 80% acetone and then centrifuged at 13000 rpm for 10 min at 1 °C. The pellet was re-extracted three times with 10 mL of 80% acetone and centrifuged each time. The combined acetone extracts were adjusted with 80% acetone to 50 mL. This solution (2 mL) was used for measurement of the radioactivity of fraction 1 of Figure 3. The acetone-insoluble residue, which contained no glycoalkalids, was dried at 40 °C and its radioactivity determined (Table 2).

Chlorophyll Content of Sprouts. The chlorophyll content of the sprouts was determined spectrophotometrically at 642.5 and 665 nm. Chlorophyll *a* (1 mg) and chlorophyll *b* (1 mg), isolated from spinach leaves according to the method of Omato and Murata (*13*), were added to the remaining acetone extract. Dioxan (7 mL) and H_2O (up to 20 mL) were then added separately dropwise until a green precipitate began to appear. The solution was placed in a freezer at −20 °C for 30 min. The green precipitate (chlorophyll−dioxan complex) was collected by centrifugation at 10000 rpm for 5 min. It was then dissolved in 30 mL of ethanol, and the solution was centrifuged. The filtrate was evaporated at 30 °C under reduced pressure. The crude residue was then dissolved in 3 mL of acetone. This solution (0.5 mL) was used to measure radioactivity as shown in fraction 2 of Figure 3.

The DEAE-Sepharose CL-6B column was washed with distilled water and then converted to an acetate by suspending

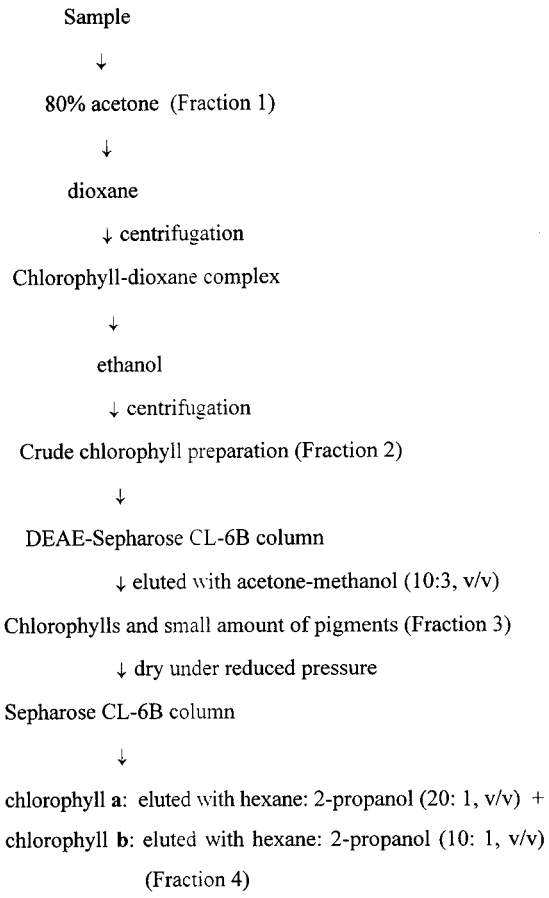

Figure 3. Scheme showing extraction and fractionation of chlorophyll *a* and chlorophyll *b* from potato sprouts by column chromatogrpahy on DEAE-Sepharose CL-6B and Sepharose CL-6B.

it in 1 M sodium acetate buffer (pH 7.0) for 3 min. It was then washed with H_2O to remove excess salt, then with acetone, and finally suspended in acetone. The acetone extract containing the crude chlorophyll was applied to the column (2.5 cm × 1.0 cm i.d.). Pigments (carotenoid, pheophytin, and plastoquinone) and lipids were eluted with acetone. The eluant flowed through the column at the rate of 1.5 mL/min. After the yellow compounds were removed from the column, the eluant was changed to acetone/methanol (10:3, v/v) to elute chlorophyll *a* and chlorophyll *b*. The volume of the eluant was adjusted to 100 mL with the same solvent. This solution (3 mL) was used for determination of radioactivity, as shown in fraction 3 of Figure 3.

The remaining solvent (90 mL) was evaporated at 30 °C under reduced pressure, and the residue was dissolved in 3 mL of hexane/2-propanol (20:1, v/v). This solution was subjected to chromatography on the Sepharose CL-6B column (11.5 cm × 2.5 cm i.d.). The column packing was washed with H_2O and then converted to the triethylammonium form by suspending it in 1 M triethylamine−HCl (pH 7.0) with NaOH. It was then washed with H_2O followed by acetone for complete removal of H_2O. It was further washed by filtration and resuspension successively with acetone/hexane (2:1, v/v), acetone/hexane (1:2, v/v), hexane/2-propanol (10:1, v/v), and finally hexane/2-propanol (2:1, v/v). This suspension was then stored until needed.

To elute chlorophyll *a*, the column was developed with 40 mL of hexane/2-propanol (20:1, v/v) at a flow rate of 8.0 mL/min. Chlorophyll *b* was eluted with 40 mL of hexane/2-propanol (10:1, v/v). The two eluants were combined, and the solvents were evaporated at 30 °C under reduced pressure. The residue was dissolved in 2 mL of acetone. This solution (0.5 mL) was transferred to a scintillation vial to measure radioactivity (see fraction 4 of Figure 3).

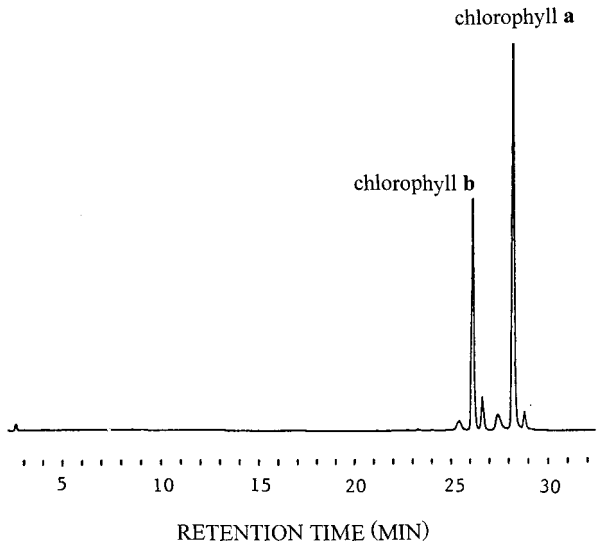

Figure 4. HPLC chromatogram of chlorophyll *a* and chlorophyll *b*.

HPLC Separation of Chlorophyll *a* and Chlorophyll *b*. HPLC was carried out with a Hitachi model 635 instrument equipped with a sampling valve of a 50-μL loop sample. The column was a Lichrosorb RP-18 (ODS, Merck), 4.0 mm × 2.5 cm; the detector was UV−visible, set at 660 nm; the chart speed was 5 mm/min. Solvents were (A) 80% methyl alcohol and (B) ethyl acetate (A/B linear gradient for 35 min). The flow rate was 1 mL/min. Figure 4 shows retention times of chlorophylls *a* and *b* on the HPLC column. A 50-μL acetone extract containing chlorophylls *a* and *b* obtained from the Sepharose column was injected into the instrument. Each component corresponding to chlorophyll *a* or *b* was collected, and their radioactivities were determined (Table 2).

Glycoalkaloid Analysis. The glycoalkaloids were extracted with chloroform/methanol (2:1, v/v) and analyzed as previously described (*14*−*16*). HPLC analysis was carried out with the aid of a Hitachi liquid chromatograph model 655A-11 with an autosampler model 655-40. Two stainless steel chromatographic columns [25 cm × 4.0 mm (i.d.)] connected in series were packed with Nucleosil NH_2 (particle diameter = 10 μM, Nagel). Glycoalkaloids were eluted with tetrahydrofuran/acetonitrile/0.025 M KH_2PO_4 (50:30:20, v/v/v) at the flow rate of 1 mL/min. The UV detector (Hitachi, model-655A UV monitor) was set at 208 nm. The separation of individual peaks of the "glycoalkaloid " extract is shown in Figure 5.

Statistics. All experiments were carried out in duplicate with two *separate* sprouts of about equal weight and length. Therefore, the treatments have been replicated. It is valid to use the standard error (SE) based on $n = 2$. With $n = 2$, the duplicate determinations can be computed from the mean standard error.

RESULTS AND DISCUSSION

Biosynthesis of Chlorophylls *a* and *b*. Two independent pathways exist in plants for the biosynthesis of isopentenyl pyrosphate (IPP), the common C_5 precursor of isoprenoids: the well-known mevalonate pathway and a recently proposed non-mevalonate biochemical pathway that proceeds via 1-deoxy-D-xylulose-5-phosphate, the so-called DOXP pathway (*17*, *18*). In plants and bacteria (*19*), the chloroplast-bound isoprenoids, which include the phytyl side chain of chlorophyll, seem to be formed via the DOXP pathway, whereas sterols, which accumulate in the cytosolic compartments of the plant cells, are created via the acetate−mevalonate pathway.

A major step in the biosynthesis of the chlorophyll ring involves condensation of glycine with succinyl

Table 1. Chlorophyll Content of Potato Sprouts (Micrograms per Gram of Fresh Weight)[a]

	storage time				
	0 days (initial)	light		dark	
		7 days	14 days	7 days	14 days
chlorophyll *a*	0	13.7 ± 1.14	29.7 ± 1.84	0	0
chlorophyll *b*	0	10.7 ± 0.58	14.7 ± 0.56	0	0
total		24.4 ± 1.72	44.4 ± 2.40	0	0

[a] Values are averages for two different sprouts ± SE.

Table 2. Radioactivity from [2-^{14}C]-DL-Mevalonic Acid Incorporated into Chlorophyll of Potato Sprouts (Disintegrations per Minute per Gram of Fresh Weight)

	storage time			
	light		dark	
	7 days	14 days	7 days	14 days
chlorophyll *a*	0	20	0	0
chlorophyll *b*	0	10	0	0

coenzyme A to form δ-aminolevulinate. Two molecules of this intermediate then condense to form the pyrrole derivative porphobilinogen. The latter then condenses to form the chlorin part of chlorophyll. The biosynthesis of the chlorophyll ring therefore does not seem to involve mevalonate as a precursor (*20*).

Table 1 shows that potato sprouts stored in the light produce chlorophyll, whereas those stored in the dark do not. The time study shows that under light, the amount of chlorophyll *a* produced doubled from 13.7 to 29.7 μg/g of fresh weight for the two time periods, 7 and 14 days, respectively. For chlorophyll *b*, the increase was only ~50%, from 10.7 to 14.7 μg/g, respectively.

Table 2 shows that (a) for the light-exposed sprouts, neither chlorophyll *a* nor *b* incorporated any radioactivity after 7 days of exposure to the labeled mevalonic acid, whereas, after 14 days, the radioactivity count for chlorophyll *a* was 20 dpm/g of fresh weight and for chlorophyll *b*, 10 dpm/g; and (b) for the sprouts stored in the dark, the corresponding values for chlorophyll *a* after 7 and 14 days were 0 dpm/g of fresh weight for both time periods.

Biosynthesis of Glycoalkaloids. The biosynthesis of cholesterol proceeds via the following steps: acetate (C_2) → mevalonate (C_6) → isopentenyl pyrophosphate (C_5) → squalene (C_{30}) → cholesterol (C_{27}) (*21*). Guseva and colleagues (*22*, *23*) showed that both labeled acetate and mevalonate are incorporated into the aglycon part of α-chaconine and α-solanine in potato leaves, seeds, and sprouts. These observations suggested that cholesterol is an intermediate in the biosynthesis of both potato glycoalkaloids. They observed the following: (a) On a molar basis, the amount of mevalonate utilized by leaves of *Solanum aviculare* was more than twice that of acetate; (b) the difference in the incorporation between mevalonate and acetate was less for potato seedlings than for the leaves; and (c) the percent uptake of mevalonate by the glycoalkaloids was much less than is the case for the biosynthesis of cholesterol in animals. In related studies, Eltayeb and Roddick (*24*) found that tomato fruits incorporated mevalonic acid [2-^{14}C]lactone into tomatidine, the aglycon of α-tomatine. The cited observations suggest that cholesterol is a precursor of both solanidine and tomatidine.

Glycosyltransferase enzymes then catalyze the glycosylation of the solanidine aglycon to form the final glycosides. For commercial potatoes, these are the

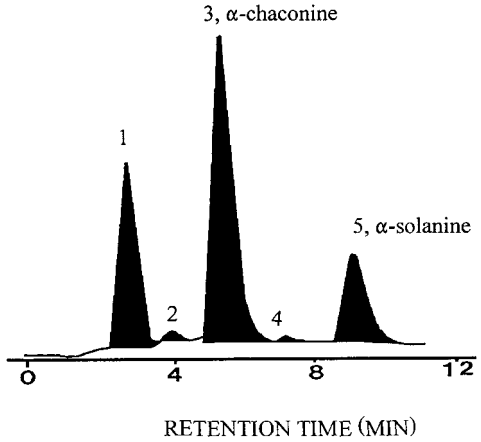

Figure 5. HPLC chromatogram of a potato sprout extract showing elution times of α-chaconine and α-solanine and three unknown peaks. Each peak was collected and analyzed for radioactivity.

trisaccharides α-chaconine and α-solanine, which share the common aglycon solanidine. Related studies (*25*−*28*) showed that cloning and anti-sense suppression of the gene encoding the enzyme which glucosylates solanidine to the monosaccharide γ-chaconine resulted in a decrease in the glycoalkaloid content of transgenic potato plants harboring the suppressed gene. The expected accumulation of solanidine was not apparent. Evidently, solanidine induces a biofeedback mechanism that shuts off the synthesis of the trisaccharides α-chaconine and α-solanine, possibly by inhibiting a specific enzyme active in the biosynthetic pathway. This discovery makes it possible to create low-glycoalkaloid potatoes with improved compositional and nutritional qualities. Two other potato enzymes were also cloned in the course of these studies, an epoxide hydrolase (*29*) and a transaldolase (*30*).

Light, heat, and mechanical injury such as bruising or slicing stimulate glycoalkaloid synthesis. Agents or treatments that inhibit sprouting, such as γ-radiation, suppress glycoalkaloid production in potatoes. With the cited considerations as a background, we will now examine the relevance of our findings to the biosynthesis of chlorophyll and glycoalkaloids.

Radioactivity of Glycoalkaloids. The HPLC chromatogram of glycoalkaloids of sprout extract contained five peaks (Figure 5). Peaks 3 and 5 were identified as α-chaconine and α-solanine by comparison with known compounds. However, the other three peaks could not be identified. Each of the five peaks was repeatedly eluted and collected from the HPLC columns. The radioactivity of each peak was then determined. The data are shown in Tables 3−6. Tables 3 and 4 show the mevalonate-derived radioactivity of the various fractions, Table 5 shows that of the two glycoalkaloids, and Table 6 shows that of the CO_2 and the insoluble residue. The glycoalkaloid contents of the sprouts stored in the light and dark are given in Table 7.

These results show that for both light- and dark-stored sprouts, the highest activity was present in peak 1, the composition of which is unknown. The amount ranged from 70 to 94% of the total. The radioactivity of peak 1 derived from the light-stored sprouts was ~2.6−3.4 times that of the corresponding peak from the dark-stored sprouts for the two time periods shown. The radioactivity associated with α-chaconine was quite low compared to that of peak 1. It ranged from 1.9 to 14.5%

Table 3. Radioactivity from [2-^{14}C]-DL-Mevalonic Acid Incorporated into Four Fractions of Potato Sprouts (Disintegrations per Minute per Gram of Fresh Weight)[a]

	storage time			
	light		dark	
fraction	7 days	14 days	7 days	14 days
1	1405060 ± 70250	2060150 ± 12770	1187890 ± 67710	1570190 ± 31400
2	50170 ± 3260	42650 ± 1360	32720 ± 2380	37900 ± 2000
3	41230 ± 3380	14170 ± 1500	16340 ± 780	13460 ± 1020
4	17970 ± 1650	8150 ± 850	11350 ± 990	3130 ± 240

[a] See Figure 1. Listed values are averages for two separate sprouts ± SE.

Table 4. Radioactivity from [2-^{14}C]-DL-Mevalonic Acid Incorporated into Individual Glycoalkaloids Separated from Potato Sprouts (Disintegrations per Minute per Gram of Fresh Weight)[a]

	storage time			
	light		dark	
	7 days	14 days	7 days	14 days
fraction P_1	3550 ± 600 (83.8)[b]	28210 ± 1230 (94.4)	1350 ± 100 (69.8)	8370 ± 800 (87.6)
fraction P_2	110 ± 20 (2.7)	590 ± 40 (2.0)	150 ± 20 (7.9)	270 ± 30 (2.9)
α-chaconine	290 ± 30 (6.9)	570 ± 80 (1.9)	280 ± 40 (14.4)	570 ± 110 (6.0)
fraction P_3	100 ± 10 (2.3)	260 ± 30 (0.9)	30 ± 6 (1.7)	140 ± 20 (1.5)
α-solanine	190 ± 20 (4.3)	250 ± 30 (0.8)	120 ± 20 (6.2)	210 ± 50 (2.0)

[a] Values are averages for two different sprouts ± SE. [b] Values in parentheses are percent of total radioactivity.

Table 5. Specific Radioactivity from [2-^{14}C]-DL-Mevalonic Acid Incorporated into α-Chaconine and α-Solanine from Potato Sprouts (Disintegrations per Minute per Milligram)[a]

	storage time			
	light		dark	
	7 days	14 days	7 days	14 days
α-chaconine	236.0 ± 6.1	527 ± 58.1	226.3 ± 21.3	498.6 ± 71.1
α-solanine	118.1 ± 12.4	177.4 ± 0.1	69.7 ± 9.6	136.9 ± 30.0

[a] Values are averages for two different sprouts ± SE.

Table 6. Radioactivity Incorporated into Carbon Dioxide and Insoluble Residue of Potato Sprouts from [2-^{14}C]-DL-Mevalonic Acid (Disintegrations per Minute per Gram of Fresh Weight)[a]

	storage time			
	light		dark	
	7 days	14 days	7 days	14 days
carbon dioxide	40800 ± 6600	29950 ± 2430	18000 ± 3270	107900 ± 6360
insoluble residue	81580 ± 8560	140800 ± 16470	184000 ± 33480	176330 ± 22570

[a] Values are averages for two different sprouts ± SE.

Table 7. α-Chaconine and α-Solanine Contents of Potato Sprouts Synthesized in the Dark and in the Light (Micrograms per Gram of Fresh Weight)[a]

	storage time				
	0 days (initial)	light		dark	
		7 days	14 days	7 days	14 days
α-chaconine	1093.3 ± 42.4	1226.7 ± 104.7	1075.0 ± 67.9	1230.9 ± 94.8	1132.0 ± 106.5
α-solanine	1300.2 ± 104.6	1629.8 ± 289.9	1409.0 ± 168.5	1710.4 ± 120.2	1514.0 ± 130.5

[a] Values are averages for two different sprouts ± SE.

of the total under the various conditions listed in Table 3. The data also show that the amount of radioactivity present in α-solanine was much less than the listed values for α-chaconine. On the other hand, comparison of specific activities for the two glycoalkaloids listed in Table 5 shows that for the 7-day sprouts, the activities of α-chaconine were the same for light- and dark-stored samples and that the activity of α-chaconine was ~2−3 times that of α-solanine. For the light-stored samples, the specific activity increased 2-fold from 7 to 14 days for α-chaconine and 1.5 times for α-solanine. For the dark-stored samples, there was a 2-fold increase for α-chaconine and α-solanine. On a weight basis the respective amounts of α-chaconine to α-solanine present in the dark- or light-treated sprouts did not differ significantly from each other (Table 7).

These results demonstrate that light stimulates the biosynthesis of α-solanine slightly more than it does that of α-chaconine. They also imply that the biosynthesis of the two glycoalkaloids may not proceed by the putatively common intermediate solanidine. The rate of incorporation of radioactivity per milligram of each glycoalkaloid (the specific activity) should be identical for both compounds if they are both derived from cholesterol via solanidine. As this is not the case, it is possible that mechanisms other than those mentioned earlier may be involved in the biosynthesis of the two potato glycoalkaloids.

Because the glycoalkaloids decrease in overall content from day 7 to day 14, there may be some metabolism and one may be formed as a result of the metabolism of the other. It is also possible that there is a “pool” effect;

the two glycoalkaloids are each formed in a different plant cell or location and the specific radioactivity is affected by endogenous solanidine. If no accumulation of glycoalkaloids has taken place, any incorporation of labeled mevalonate into the glycoalkaloids can only be the result of turnover. It therefore appears to be unlikely that a large amount of mevalonate would be incorported into the glycoalkaloid pool during the 14-day experiment even if glycoalkaloid synthesis did proceed via the mevalonate pathway.

The low incorporation of radioactivity into the glycoalkaloids could be consistent with a mevalonate-independent pathway of isoprenoid biosynthesis mentioned earlier. Elucidation of the biosynthetic intermediates associated with peak 1 of Figure 3, where most of the radioactivity derived from the labeled mevalonate resides, may help in efforts to find out whether a mevalonate pathway, a non-mevalonate pathway, or both pathways govern glycoalkaloid and/or chlorophyll biosynthesis.

Our results support the view that light-induced formations of chlorophyll and glycoalkaloid are independent biosynthetic events, in view of the apparent lack of parallel trends in the incorporation of mevalonate-derived radioactivity as well as in the differing rates of formation of the two classes of plant constituents under similar light and storage conditions.

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