PLANT BIOCHEMISTRY OF GINSENG SAPONINS(II)

Radioactives(1) Squalene–H^3 Feeding Experiments

Abstract.

The radioactive compound squalene-H^3 prepared from peas (*Pisum sativum* L.) with 5H -mevalonic acid was administered to two- and four-year-old American ginseng (*Panax quinquefolium* L.) Plants and cuttings in September. The squalene-H^3 uptake was low (40 ~ 86%). Squalene-H^3 was not incorporated into the panaquilin sapogenin panaxadiol. This may be due to its poor solubility characteristics and plant absorption, or to the low specific activity. It is possible, but unknown, if any squalene was metabloized into the carbohydrate portion of the panaquilins.

Introduction

In the previous paper the analytical procedures of American and Korean ginseng plant saponins and sapogenins were described. In American ginseng plants (*Panax quinquefolium* L.), panaquilins B and C (panaxadiol-type saponins), panaquilin D (an oleanolic acid saponin), panaquilins E-1, E-2 and E-3 (both panaxadiol and panaxatriol-type saponins), panaquilin G-1 (a panaxatriol-type saponin), and panaquilins A, (c), (d) and G-2 (unknown genin saponins) were also reported to be present. Ginsenosides Rd, Re_2 and Re_3 for the Korean ginseng saponins have been rearranged according to our two-dimensional thin-layer chromatographic patterns in comparison with the American ginseng plant saponins panaquilins.

With such results, tracer experiments have been attempted to biosynthesize American ginseng saponins and sapogenins for further pharmacological and chemical studies. The radioactive materials sodium acetate-U-C^{14} and squalene-H^3 were administered to American ginseng plants by the wick method (2). Squalene-H^3 used in this study was prepared through pea seeds (*Pisum sativum* L.) with mevalonic acid-$5H^3$ (3,4). Sodium acetate-U-C^{14} was a good precursor for the panaquilins and their genins. But squalene-H^3 was not. This may be due to its poor solubility characteristics and plant absorption, or to the low specific activity. It is possible, but unknown, if any squalene was metabolized into the carbohydrate portion of the panaquilins.

Results and Discussions

The squalene-H^3 experiments were done with plants and cuttings studied only in September. The time required for squalene-H^3 uptake was great (10–12 hrs.) and the percentage uptake of squalene-H^3 was considerably low (two-year-old plants-40.4% and cuttings-81.3%; four-year-old plants-85.8% and cuttings-76.0%.

Table 1. Extracts from Two- and Four-year-old Ginseng Plants and Cuttings.*

a. Two-year-old

	Dry Wt. (g)	Extract (%)**					
		Ether	Chloroform	Methanol-1	Residue	Methanol-2	Total
Plants							
Leaf	1.3	3.9	0.8	44.6	1.5	43.1	49.3
Stem	0.5	2.2	0.4	15.6	2.3	13.3	18.2
Root	8.0	2.4	0.1	26.9	3.4	23.5	29.4
Average	3.3	2.8	0.4	29.0	2.4	26.6	32.2
Cuttings							
Leaf	2.5	2.4	1.6	52.8	1.2	51.6	56.8
Stem	0.7	2.9	4.3	25.7	1.4	24.3	32.9
Average	1.6	2.7	3.0	39.3	1.3	38.0	45.0
	b. Four-year-old						
Plants							
Leaf	13.8	3.3	1.7	47.0	25.0	22.0	52.0
Stem	8.1	1.2	0.1	24.4	7.0	17.4	25.7
Fruit	11.8	15.9	2.8	18.4	6.1	12.3	37.1
Root	50.1	0.6	0.4	45.6	5.3	40.3	46.6
Average	20.9	5.3	1.3	33.9	10.9	23.0	40.5
Cuttings							
Leaf	11.7	6.4	3.9	48.4	14.3	34.1	58.7
Stem	7.6	1.1	0.3	30.7	2.8	27.9	32.1
Fruit	6.1	17.4	1.2	24.9	9.8	15.1	43.5
Average	8.5	8.3	1.8	34.7	9.0	25.7	44.8

*September collections.
**Residue: Insoluble material of methanol-1 extracted with cold methanol (5°C); Methanol-2: Soluble extracts of methanol-1 extracted with cold methanol (5°C); Total = Ether (%) + chloroform (%) + methanol (%).

Table 2. H^3-Activity of Ether, Chloroform and Methanol-1 Extracts from American Ginseng Plants and Cuttings*.

a. *Two-year-old*

	H^3-Activity			
	Ether	Chloroform	Methanol-1	Total
Plants				
Leaf	27.3	0.9	2.7	30.9
Stem	13.3	0.5	1.6	15.4
Root	1.0	NA	5.0	6.0
Total	41.6	1.4	9.3	52.3
Cuttings				
Leaf	1.3	0.3	1.0	2.6
Stem	3.2	0.3	0.8	4.3
Total	4.5	0.6	1.8	6.9
b. *Four-year-old*				
Plants				
Leaf	NA	0.6	2.8	3.4
Stem	15.7	1.0	4.4	21.1
Fruit	0.6	0.1	1.0	1.7
Root	0.4	0.1	3.8	4.3
Total	16.7	1.8	12.0	30.5
Cuttings				
Leaf	NA	1.0	1.8	2.8
Stem	26.4	1.2	3.9	31.5
Fruit	0.2	0.01	0.2	0.4
Total	26.6	2.2	5.9	34.7

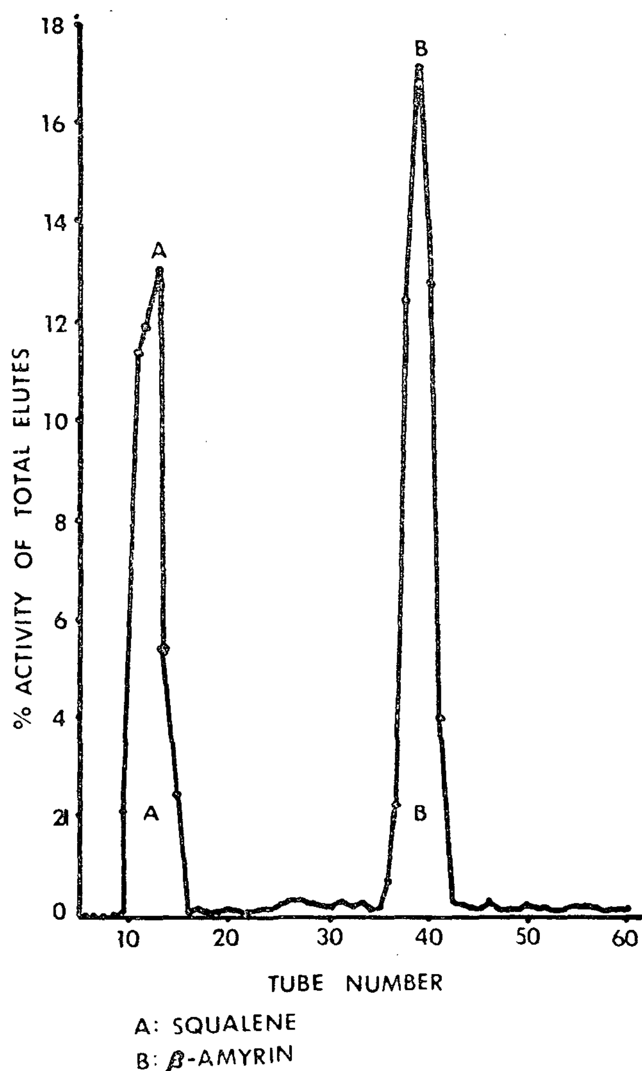
*September collections. H^3 -Activity; (Total activity in extract/Total activity uptake)x100. NA; Not available.

1. Extracts.

The amounts of extracts obtained from the plants and cuttings (Table 1) were similar to those obtained from the non-radioactive studies (1).

The average percentage of squalene-H^3 incorporation into ether extracts was significantly higher (8.94%) than that in chloroform (0.55%) and methanol-1 (2.42%) (Table 2). The average percentage of squalene-H^3 incorporation into methanol-2 extracts was higher in two-year-old plants (8.2%) than four-year-old plants (7.5%), but that in cuttings higher in four-year-old cuttings (5.8%) than two-

Figure 1. Isolation of Radioactive Squalene from Pea Extracts.



year-old cuttings (1.8%) (Fig.1). In two-year-old plants, the percentage of squalene-H^3 incorporation into methanol-2 was higher (4.0%) in the roots than leaves (2.7%) or stems (1.5%). In four-year-old plants, the percentage of squalene-H^3 incorporation was highest in stems (3.7%), and then followed by roots (2.2%), leaves (1.0%) and fruits (0.6%).

In cuttings, the percentage of squalene-H^3 incorporation in two-year-old leaves was higher (1.0 %) than that in two-year-old stems (0.8%), whereas the percentage of squalene-H^3 incorporation in four-year-old stems was higher (4.3%) than that in leaves (1.3%) and fruits (0.2%). These observation were similar to those observed in plants.

When extract activity was expressed as mμCi per mg dry weight it was consistantly higher in stems (0.22 mμCi/mg) than leaves (0.04 mμCi/mg), fruits (0..2 mμCi/mg) and roots (0.02 mμCi/mg). This is probably related to the fact that stem wick method was used to administered the radioactive compound. The extract activity was quite low. This may be due to low amounts (0.43 μCi/mg, 0.64 or 1.28μCi/plant) of activity fed as well as low percentage uptake by plants (40–86%).

2. Hydrolysis of Radioactive Methanol-2

a. Radioactivity of Hydrolysates.

Aliquots of radioactive methanol-2 extracts (0.55–1.55g, activity 0.014–1.096 mμCi-mg) (Table 3) were hydrolyzed. The ether extracts of hydrolyzed products weighed 0.14–0.28g and were radioactive 0.06–0.18 mμCi-mg.

The activity of hydrolysates was higher in the above-ground parts (0.18, 0.15 mμCi/mg) than that in the roots (0.06, 0.11 mμCi/mg, respectively). In cuttings, the hydrolysates from the above-ground parts contained 0.11 and 0.16 mμCi/mg. The average activity of hydrolysates was lower in two-year-old plants and cuttings (0.12 mμCi/mg) than four-year-old (0.14 mμCi/mg).

Table 3. Hydrolysates of Methanol-2: H^3 -Activity.

Plant	Methanol-1**		Hydrolysates***	
Material*	Aliquot (g)	Activity (mμCi/mg)	Amount (g)	Activity (mμCi/mg)
Two-year-old				
Above-ground	0.55	0.096	0.15	0.18
Root	1.01	0.958	0.20	1.06
Average	0.78	0.977	0.18	0.12
Above-ground (Cutting)	1.01	0.974	0.23	0.11
Four-year-old				
Above-ground	1.09	0.053	0.20	0.15
Root	1.37	0.014	0.14	0.11
Average	1.23	0.034	0.17	0.13
Above-ground (Cutting)	1.55	0.042	0.28	0.16

* September collection.

** Methanol-2: Soluble extracts of metanol-1 with cold methanol (5°C).

***Hydrolysates: Ether extracts of hydrolysates of methanol-2 with 30% hydrochloric acid and methanol (1:4).

Table 4. Dilution and Recrystallization of H^3-Panaxadiol.

Plant	Panaxadiol**		Panaxadiol	Purified (1x)****	
Material*	Amount	Activity	Added***	Amount	Activity
Two-year-old					
SpA	24	0.045	10	9	0.007
SpR	35	0.031	14	27	0.018
SpA(C)	31	0.015	10	10	0.013
Four-year-old					
SpA	20	0.216	10	10	0.071
SpR	26	0.043	11	17	0.014
SpA(C)	42	0.176	11	6	0.177

*Abbreviations; Sp: September collection; A: Above-ground parts; R: Root; C: Cuttings.
**Panaxadiol: Radioactive panaxadiol fractions obtained from preparative tlc bands; Amount (mg); Activity (mμCi/mg).
***Panaxadiol added: Non-radioactive panaxadiol addition to radioactive panaxadiol fraction (mg).
****Purified (1X): First recrystallization of radioactive. Panaxadiol; Amount (mg); Activity (mμCi/mg)

b. Radioactivity of Panaxadiol.

Panaxadiol from the ether-soluble extracts of hydrolysates obtained by methanol elution of silica gel bands contained 0.031–0.216 mμCi/mg (20–41 mg) (Table 4). This activity was lower than that of the original ether extracts of hydrolysates with the exception of four-year-old cuttings which remained essentially the same.

The panaxadiol obtained by preparative tlc was not perfectly recrystallized, probably due to the presence of impurities. Non-radioactive panaxadiol (10–14mg) was added to the panaxadiol (20–42mg), and by recrystallization purified (1x) panaxadiol was obtained (6–27mg) (Table 4). The radioactivity of diluted panaxadiol was 0.013 mμCi/mg (average) in two-year-old and 0.087 mμCi/mg in four-year-old. After three additional recrystallizataions, the radioactivity decreased to zero. This result suggests that squalene was not incorporated into American ginseng sapogenins.

Materials and Methods

Preparation of Squalene-H^3

1. Materials.

Burpee's Blue Bantham pea seeds (W. Atlee Burpee Co., Clinton, Iowa) were used for the biosynthesis of labeled squalene from mevalonic acid (MVA). The $5H^3$-MVA (1.0 mCi/0.5 ml, 3.5 Ci/mM, Lot No. VR 1086) used as precursor was pruchased from Schwartz Bio Research, Orangeburgh, N. Y. Non-radioactive MVA was purchased from Sigma Chemical Co., St. Louis, Missouri.

2. Feeding of 5H -MVA to peas (3, 4).

The tritiated MVA (1 mCi/0.5ml) was diluted with distilled water (10ml). To a 5-ml aliquot, non-radioactive MVA (18mg) was added and neutralized with a 0.1 N sodium bicarbonate solution. The aliquot was then diluted to 500 μCi/500 ml, and 460 g pea seeds mixed for 2 min. with 1% Arasan-75 (E. I. du Pont de Nemours and Co., Wilmington, Delaware). Approximately every 6 hours, 300 ml of distilled water were added. After 24 hours, the treated peas were removed, Rashed. The activity of material remaining was 232 μCi.

3. Extraction.

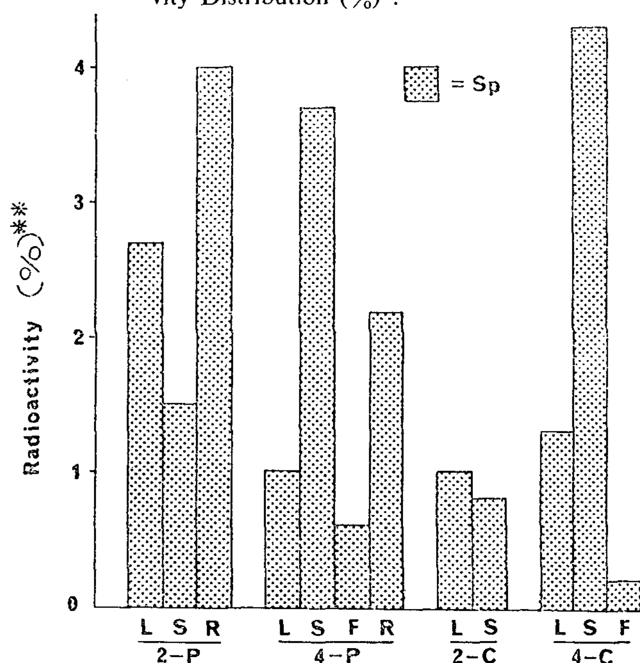
The washed peas were immediately homogenized in a Waring blendor with acetone (1 1.) and allowed to macerate for approximately 12 hrs. The suspension was filtered by aspiration through four layers of cheese cloth. The residue was then extracted with acetone in a Soxhlet overnight. The combined acetone solution (21., 170 μCi) were evaporated, dissolved in a distilled water, and extracted three times with ether. The combined ether layers were concentrated and refluxed for 1 hr. with 5% alcoholic potassium hydroxide to principally remove fatty acids and lipids. The reaction mixture was evaporated, the residue suspended in distilled water, and ex-

tracted three times with ether. The ether layers were dried over anhydrous sodium sulfate, and when evaporated formed a yellowish semi-solid residue (1.31 g, 115 μCi).

4. Isolation of Squalene.

The residue was dissolved in 30% ether-petroleum ether (13ml) and eluted with 30–100% ether-petroleum ether on an alumina column (100g, neutral grade 1) (Fig. 2). Fractions 10–15 (60ml) were collected and found to contain tritiated squalene (123mg, 58 μCi, sp. act. 0.47 μCi/mg).

Figure 2. American Ginseng Methanol-2 H-3 Radioactivity Distribution (%)*.



* Abbreviations: L-leaf; S-stem; F-fruit; R-root, sp-September collection; 2-P: two-year-old plants; 4P-: four-year-old plants; C-cuttings.
**Radioactivity (%): (Radioactivity in methanol-2 (μCi)/ Radioactivity uptake (μCi))x100.

Ttritiated squalene (1.03 μCi) was mixed with non-radioactive squalene (261mg) and dissolved in acetone (5ml). The resulting solution was saturated with hydrogen chloride at –5°C for 1 hr. Squalene hexachloride formed, and was recrystallized from acetone m.p. 107–111°C, sp. act. 6.3 mμCi/mg). Radioisotope Plant Feeding Experiments.

All tracer experiments were performed on plants grown in the field at the Fromm Brothers Farm, Hamburg, Wisconsin. The fresh weight of two- and four-year-old ginseng plants was approximately 3 and 80g, respectively.

Squalene-H^3 (60mg, 25.7 μCi) was added to distilled water (10ml) containing approximately 1% Tween 80, and then mixed vigorously for 30min. before feeding on an electric vibrator (Super Mixer, No. 1280, Labline Instruments, Inc., Melose Park, Illinois). A 0.5-ml aliquot contained approximately 3mg or 1.28 μCi of squalene. Tritiated squalene suspensions were fed by the wick method (2) to 5 two-year-old intact plants (0.64 μCi/0.25ml) and 5 stem cuttings (0.64 μCi/0.25ml), and to 5 four-year-old intact plants (1.28 μCi/0.5ml) and 5 stem cuttings (1.28 μCi/0.5ml). A plant absorbed the squalene solution (0.25 or 0.5 ml) in 10–12 hrs., and the vials were washed twice with distilled water (0.2ml) and the resulting solution was also taken up by the plant. The washing procedure was again repeated. The following day the thread and vials were removed from the plants and bamboo supports provided for plant. After 5 days or one week, the stem cuttings and intact plants were collected, respectively. The radioactivity remaining in the thread and vials for two-year-old intact plants was approximately 58.6 %, for two-year-old stem cuttings 18.7%, for four-year-old intact plants 14.2%, and for four-year-old stem cuttings 24.0%.

Radioisotope Measurements.

1. Isotope Standards.

Toluene-H^3 ($3.00 \times 10^6 \pm 1\%$ dpm/g) was purchased from the Packard Instrument Co., Inc., Downers Grove, Illinois.

PPO (2,5-diphenyloxazole, scintillation grade) and thixotropic gel powder (Cab-O-Sil, for liquid scintillation counting systems) were purchased from the Packard Instruments Co., Inc.; POPOP (2,2' -p-phenylene bis (5-phenyloxazole)) and naphthalene (scintillation grade) from Eastman Kodak Co., Rochester, N.Y.; dioxane (spectrophotometric grade) from Aldrich Chemical Co., Milwaukee, Wisconsin;

and toluene (analytical grade) from Mallinkrodt Chemical Co., St. Louis, Missouri.

The scintillation fluids used (6–9) were composed of; a. Cab-O-Sil-Dioxane-Water; PPO (10.5g), POPOP (0.45g), Naphthalene (150g) Cab-O-Sil (72g), 1.51. of dioxane and then diluted with distilled water to 1.81. b. Dioxane Solution; PPO (0.7g) POPOP (0.05g) and dioxane (100ml). c. Toluene Solution; PPO (0.4g), POPOP (5mg) and toluene (100ml).

3. Detection and Measurement of Radioactivity.

Measurement of radioactivity was done on a Beckman Model 100–C liquid scintillation counter (Beckman Instruments Inc., Fullerton, California) containing a Cs^{137} external standard.

Radioactive compounds were detected on thin-layer chromatograms by spraying a small side portion of each plate with a ceric sulfate solution (3% in 3N sulfuric acid). Materials absorbed to silica gel G were assayed quantitatively for radioactivity by their direct transfer to 15ml of scintillation fluid (Cab-O-Sil-Dioxane-Water).

4. Preparation of Quenched Radioactive Standard and Background.

The standard consisted of 0.01% methyl red methanolic solution (0.01–0.05ml) in scintillation fluid (10 or 15ml). Toluene-H^3 (approximately 0.01 ml, 70–100mg) was weighed exactly and added to the methyl red containing scintillation fluid (7,10).

The background samples were prepared by adding 0.01% methyl red methanolic solution (0.01–0.30 ml) to the scintillation fluid (11).

Disintegration per minute (dpm) was converted from counts per minute (cpm) using the external channels ratio method (11,12) and by counting quenched liquid scintillation samples. A blank containing the same amounts of scintillation fluid, methanol ethanal or water was used to check the background (10–30 dpm).

Isolation and Detection of H^3-labeled Panaquilins and Panaxadiol.

The tritiated panaquilins and panaxadiol analysis used in this study was identical to that has been reported (1).

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