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Studies on the Ginseng Plants(II)*

Radioactive Squalene-H^3 Feeding Experiments

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人蔘植物에 관한 연구(Ⅱ)*

同位元素化合物 Squalene-H^3을 투여한 실험

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美國人蔘(五加科, *Panax quinquefolium* L.) 2年生 및 4年生 植物에 심지法을 使用하여 squalene-H^3를 投與했을때 美國人蔘 사포닌(panaquilins)과 그 非糖體에 H^3-incorporation 可能性 與否를 검토 하였다. squalene-H^3 섭취율은 매우 낮은 값을 보였으며 (40~86 %), squalene-H^3는 또한 panaquilin의 非糖體 部分에는 incorporation 되지는 않았으나 糖體에는 squalene 代謝物質이 incorporation 된 것으로 思料 되었다.

Introduction

In the previous paper[1], the analytical procedures of American and Korean ginseng plant saponins and sapogenins were described. In American ginseng plants, 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 G-2, A, (c) and (d) (unknown genin saponins) were also reported to be present. Ginsenosides Rd, Re_2 and Re_3 for the Korean ginseng saponins were rearranged according to our two-dimensional thin-layer chromatographic patterns in comparison with the American ginseng plant saponins panaquilins.

With such results tracer experiments were attempted to biosynthesize American ginseng saponins and sapogenins for further future pharmacological and chemical studies. The radioactive materials sodium acetate-U-C^{14} [2] and squalene-H^3 were administered to American ginseng plants by the wick method[3]. Squalene-H^3 used in this study was prepared through pea seeds with mevalonic acid-5H^3 [4,5]. Sodium acetate-U-C^{14} was a good precursor for the panaquilins and their sapogenins[2]. 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.

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Materials and Methods

A. 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 m*l*, 3.5 Ci/mM, Lot No. VR 1086) used as precursor was purchased from Schwartz Bio Research, Orangeburg, N.Y. Non-radioactive MVA was purchased from Sigma Chemical Co., St. Louis, Missouri.

2. Feeding of $5H^3$-MVA to peas[4,5]

The tritiated MVA (1 mCi/0.5 m*l*) was diluted with distilled water (10 m*l*). To a 5-m*l* aliquot, non-radioactive MVA (18 mg) was added and neutralized with a 0.1N sodium bicarbonate solution. The aliquot was then diluted to 500 μCi/500 m*l*, 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 m*l* of distilled water were added. After 24 hrs., the treated peas were removed, washed. The activity of radioactive material remaining was 232 μCi.

3. Extraction

The washed peas were immediately homogenized in a Waring blendor with acetone (1 *l*) 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 solutions (2 *l*, 170 μCi) were evaporated, dissolved in 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 extracted three times with ether. The ether layers were dried over anhydrous sodium sulfate, filtered, 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 (13 m*l*) and eluted with 30~100% ether-petroleum ether on an alumina column (100 g, neutral, grade 1) (Fig. 1). Fractions 10~15 (60 m*l*) were collected and found to contain tritiated squalene (123 mg, 58 μCi or sp. act. 0.47 μCi/mg).

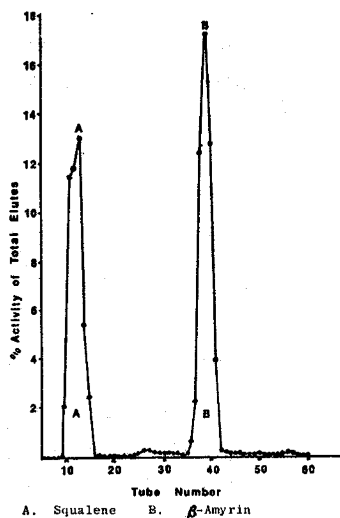

Fig. 1. Isolation of Radioactive Squalene from Pea Extracts.

Tritiated squalene (1.03 μCi) was mixed with non-radioactive squalene (261 mg) and dissolved in acetone (5 m*l*). 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).

B. 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 80 g, respectively. Squalene-H^3 (60 mg, 25.7 μCi) was added to distilled water (10 m*l*) containing approximately 1% Tween 80, and then mixed vigorously for 30 min. before feeding on an electric vibrator (Super Mixer, No. 1290, Labline Instruments, Inc., Melose Park, Illinois). A 0.5-m*l* aliquot contained approximately 3 mg or 1.23 μCi of squalene. Tritiated squalene su-

spensions were fed by the wick method[3] to 5 two-year-old intact plants (0.64 μCi/0.25 ml) and 5 stem cuttings (0.64 μCi/0.25 ml), and to 5 four-year-old intact plants (1.28 μCi/0.5 ml) and 5 stem cuttings (1.28 μCi/0.5 ml). 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 (0.2 ml) 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 59.6 %, 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 %.

C. Radioisotope Measurements

1. Isotope Standards

Toluene-H^3 (3.00x10^6±1% dpm/g) was purchased from the Packard Instrument Co., Inc., Downers Grove, Illinois.

2. Scintillation Fluids

PPO (2,5-diphenyloxazole, scintillation grade) and thixotropic gel powder (Cab-O-Sil, for liquid scintillation counting systems) were purchased from the Packard Instruments Inc., Co.; 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 (10.5 g), POPOP (0.45 g), naphthalene (150 g), Cab-O-Sil (72 g), 1.5 l of dioxane and then diluted with distilled water to 1.8 l. b. *Dioxane Solution*; PPO (0.7 g), POPOP (0.05 g) and dioxane (100 ml). c. *Toluene Solution*; PPO (0.4 g), POPOP (5 mg) and toluene (100 ml).

3. Detection and Measurement of Radioactivity

Measurements of radioactivity were 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 15 ml 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 (0.01～0.05 ml) in scintillation fluid (10 or 15 ml). Toluene-H^3 (approximately 0.01 ml, 70～100 mg) was weighed exactly and added to the methyl red containing scintillation fluid[6,10].

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, ethanol or water was used to check the background (10～30 dpm).

D. Isolation and Detection of H^3-Labeled Panaquilins and Panaxadiol

The tritiated panaquilins and panaxadiol analysis used in this study was identical to that reported[1].

Results and Discussion

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.), but 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 %).

1. Extracts

The amounts of extracts obtained from the plants

Table I. Extracts from Two- and Four-year-old September Collected Ginseng Plants and Cuttings: Squalene-H^3 Experiments.

Plant Material	Dry Wt. (g)	Extract (%)*					
		Ether	Chloroform	Methanol-1	Residue	Methanol-2	Total
Two-year-old							
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	0.6	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
Four-year-old							
Plants							
Leaf	13.4	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

* 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 (%).

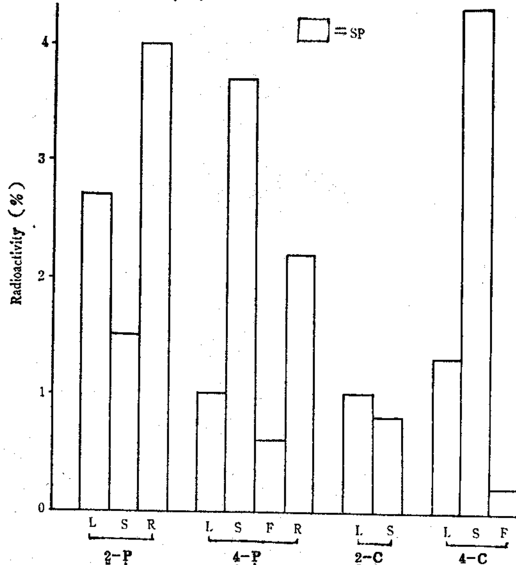

Fig. 2. American Ginseng Methanol-2 H^3-Radioactivity Distribution (%).

* Abbreviations: L-leaf; S-stem; F-fruit; R-root; Sp-September collection; 2-P: Two-year-old plants; 4-P: Four-year-old plants; C-cuttings.

** Radioactivity (%): (Radioactivity in methanol-2 (μCi)/Radioactivity uptake (μCi))$\times$100.

and cuttings (Table I) 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 (8.9 %) than that in chloroform (0.6 %) and methanol-1 (2.4 %) (Table II). 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-year-old cuttings (1.8 %) (Fig. 2). In two-year-old plants, the percentage of squalene-H^3 incorporation into methanol-2 was higher (4.0 %) in the roots than the 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 observations 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 (0.02 mμCi/mg) and roots (0.02 mμCi/mg). This is probably related to the fact that the stem wick method was used to administer the radioactive compound. The extract activity was quite low. This may be due to low amounts (0.43 μCi/mg, 0.64 or 1.28 μCi/plant) of activity fed as well as low percent-

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

Plant Meterial	H^3-Activity (%)			
	Ether	Chloroform	Methanol-1	Total
Two-year-old				
Plants				
Leaf	27.3	0.6	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
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

* H^3-Activity: (Total radioactivity in extract/Total radioactivity uptake)×100. NA-not available. September collection.

age uptake by plants (40~86 %).

2. Hydrolysis of Radioactive Methanol-2

a. Radioactivity of Hydrolysates. Aliquots of radioactive methanol-2 extracts (0.55~1.55 g, activity 0.014~0.096 mμCi/mg) (Table III) were hydrolyzed. The ether extracts of hydrolyzed products weighed 0.14~0.28 g 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).

b. Radioactivity of Panaxadiol. Panaxadiol from the ether-soluble extracts of hydrolysates obtained by methanol elution of silica gel bands contained 0.013~0.216 mμCi/mg (20~42 mg) (Table IV). This activity was lower than that of the original ether extracts of hydrolysates with the exception of four-year-old

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

Plant Material*	Methanol-2**		Hydrolysates***	
	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 methanol-1 with cold methanol (5° C).
*** Hydrolysates: Ether extracts of hydrolysates of methanol-2 with 30 % hydrochloric acid and methanol (1 : 4).

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

Plant Material*	Panaxadiol**		Panaxadiol Added***	Purified (1×)****	
	Amount (mg)	Activity (mμCi/mg)		Amount (mg)	Activity (mμCi/mg)
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.
*** Panaxadiol added: Non-radioactive panaxadiol addition to radioactive panaxadiol fraction
**** Purified (1×): First recrystallization of radioactive panaxadiol.

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 (10~14 mg) was added to the panaxadiol (20~42 mg), and by recrystallization purified (1x) panaxadiol was obtained (6~27 mg) (Table IV). The average radioactivity of diluted panaxadiol was 0.013 $m\mu Ci/mg$ in two-year-old and 0.087 $m\mu C/mg$ in four-year-old. After three additional recrystallizations, the radioactivity decreased to zero. This result suggests that squalene was not incorporated into the American ginseng sapogenin.

Conclusion

1. The radioactive compound squalene-H^3 prepared from peas with $5H^3$-mevalonic acid was administered to two- and four-year-old September plants and cuttings. The squalene-H^3 uptake was low (40~86 %).

2. Squalene-H^3 was not incorporated into panaquilin sapogenins. 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.

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