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BIOSYNTHESIS OF TRITERPENOID SAPOGENOLS IN SOYBEAN AND ALFALFA SEEDLINGS

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Abstract—By incubation of germinating soybeans with mevalonate-[2-^{14}C] (MVA), radioactivity was incorporated into four sapogenols which were identified by TLC. Unequivocal evidence for the identity of three of the four sapogenols was provided by co-crystallization to constant specific radioactivity. The partition of incorporated radioactivity into lipid- and water-soluble fractions and the pattern of radioactivity of individual sapogenols varied with the mode of administering labeled substrates to soybean seedlings, such as incubation of germinating soybeans with MVA-[2-^{14}C], immersion of roots into MVA-[2-^{14}C] or foliar application of squalene-[^{14}C]. When alfalfa seedlings were incubated with MVA-[2-^{14}C], about two-thirds of the radioactivity incorporated into the sapogenols was associated with medicagenic acid.

INTRODUCTION

Considerable knowledge has accumulated on the chemical structure and physiological properties of saponins in soybean seeds and alfalfa plants, two important food sources for farm animals [1–3]. These saponins are glycosides which yield, on hydrolysis, sugars and aglycones. The aglycones present in soybean saponins are the five soyasapogenols A, B, C, D and E, which are pentacyclic triterpenoids substituted with OH groups differing from each other in the number of oxygen functions. A report on the structure elucidation of soybean saponins was recently provided by Kitagawa *et al.* [4]. Alfalfa saponins contain, in addition to soyasapogenols, medicagenic acid as the dominant aglycone. Medicagenic acid contains two hydroxyl and two carboxyl groups. It is responsible for adverse effects of alfalfa saponins on animals, such as the growth inhibition of chicks.

Very little information is available on saponin biosynthesis; Arigoni [5] has shown that when germinating soybeans were grown on a substrate containing ^{14}C-labeled acetic acid, the specific activity was equally distributed between the soybean sapogenins and the sterols, and was also found in squalene. The biosynthesis of sapogenols from acetate-[2-^{14}C] was investigated in germinating seeds of alfalfa by Nowacki *et al.* [6].

The purpose of the present work was to study the biosynthesis of sapogenins in soybean and alfalfa seedlings by feeding labeled precursors such as MVA-[2-^{14}C]. The identity of the labeled sapogenols isolated from plants was established by co-crystallization with authentic carrier material to constant specific radioactivity. The influence of the following factors on distribution of radioactivity among the individual sapogenols formed and between saponins and other plant fractions was investigated: plant species (soybeans and alfalfa), nature of the labeled precursor (mevalonic acid and squalene) and route of administration (leaves and roots).

RESULTS AND DISCUSSION

Incubation of germinated soybeans with MVA-[2-^{14}C]

Two incubations of germinating soybeans with MVA-[2-^{14}C], lasting five and ten days, respectively, were carried out. Our extraction procedure for labeled fractions from plant material is given in the Experimental. The amounts of radioactivity supplied and the extent of incorporation into various fractions, as well as the distribution of radioactivity among individual sapogenols are shown in Table 1.

Considerable radioactivity was incorporated into the lipid-(*A'*) and water-soluble fractions (*A*). The presence of labeled sitosterol and squalene in the lipid-soluble fraction was evident from comparisons with authentic compounds by TLC. The water-soluble fraction, containing the saponins, was hydrolysed, and the unsaponifiable material (*B*) was subjected to TLC revealing labeled sapogenols A, B, C and E, and sterols. A zone near the solvent front contained *ca* 70% of the radioactivity. When fraction *B* was boiled with ethanolic KOH and extracted with Et_2O–$CHCl_3$ (1:1), the unsaponifiable material (*C*) no longer contained that zone. The distribution of radioactivity among individual sapogenols in fraction *C* is given in Table 1.

A greater portion of radioactivity was associated with sapogenol B than with the sum of sapogenols A, C and E. Neither the distribution of radioactivity between the water-(*A*) and lipid-(*A'*) soluble fractions nor its distribution among individual sapogenols were significantly influenced by extension of the incubation period from five to ten days.

Labeled sapogenols A, B and C were eluted from TLC plates and identified by co-crystallization with authentic carrier material to constant specific radioactivity. Independent evidence for the identity of sapogenols A, B, C and E was provided by MS and NMR spectra [7].

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Table 1. Incorporation of radioactivity from MVA-[2-^{14}C] and squalene-[^{14}C], administered to soybean and alfalfa plants, into various plant fractions and distribution of radioactivity among individual sapogenols

Experiment No.	1	2	3	4	5
Species	Soybeans	Soybeans	Soybeans	Soybeans	Alfalfa
Precursor	MVA-[2-^{14}C]	MVA-[2-^{14}C]	MVA-[2-^{14}C]	Squalene-[^{14}C]	MVA-[2-^{14}C]
Mode of application	Germinating seeds	Germinating seeds	Root application	Foliar application	Germinating seeds
Duration (days)	5	10	14	14	10
Radioactivity applied (cpm × 10^{-6})	13.13	14.81	4.1	2.15	19.7
Incorporation into sapogenols (cpm × 10^{-5})	1.05	1.77	0.615	0.215	2.36
Per cent of administered radioactivity incorporated					
Water-soluble fraction (*A*)	35.5	36.9	25.9	20	22.2
Lipid-soluble fraction (*A'*)	28	22	7.3	13	3.9
Sapogenols	0.8	1.2	1.5	1.0	0.8
Radioactivity in individual sapogenols in per cent of that incorporated into total sapogenols					
Sapogenol A	10.8	11.7	10.9	8.9	8.3
Sapogenol B	57.9	54.9	39.5	43.4	10.9
Sapogenol C	16.6	18.7	27.1	27.0	9.6
Sapogenol E	14.6	14.5	22.2	20.0	6.8
Medicagenic acid	—	—	—	—	64.2

Immersion of roots of soybean seedlings in MVA-[2-^{14}C] *solution*

The roots of 14-day-old soybean seedlings were kept for an additional 14 days in a solution of MVA-[2-^{14}C]. A much lower percentage (7.3%) of the administered radioactivity was incorporated into the lipid-soluble fraction (*A'*) than after the incubation of germinating soybeans with MVA-[2-^{14}C] (average 25%) (Table 1). The percentage of radioactivity incorporated into the water-soluble fraction (*A*) was also lower in this experiment than in the incubation of germinating soybeans, but the percentage of radioactivity recovered in the sapogenols was higher (1.5% vs 1.0%). Comparing the distribution of radioactivity among the individual sapogenols, after root application the per cent incorporation into sapogenol B declined somewhat, while that of sapogenols E and C increased relative to the incubation of seedlings in MVA-[2-^{14}C].

Distribution of radioactivity in different parts of soybean plants treated with MVA-[2-^{14}C]

(a) Seeds of soybeans were incubated for ten days with MVA-[2-^{14}C] solution and the distribution of radioactivity in the different parts of the seedlings was examined by three independent procedures. (1) Radioautograms of the whole plants revealed intensive darkening by the cotyledons and less, but distinct darkening by the other parts of the plants. (2) Samples of cotyledons, leaves, stems and roots combusted separately in an oxidizer manifested higher labeling of the cotyledons than the other parts of the plants (Table 2). (3) Individual parts of the treated plants were extracted and worked up as described for preparing radiochromatograms of sapogenols. The radioactivity associated with the sapogenols in each part, expressed in per cent of the radioactivity of the sapogenols in the whole plant, gave the following results: cotyledons 70%, leaves 10.4%, roots 10.1% and stems 6.2%. Thus, all three methods showed that the radioactivity accumulated mainly in the cotyledons, the part of the plants to which MVA-[2-^{14}C] was originally applied, but smaller amounts of radioactivity were also found in other parts of the plants, such as leaves, stems and roots, indicating the translocation of labeled substances in the plant.

(b) When mevalonate was administered through the roots, most of it was incorporated into leaves and stems and only a small portion of the radioactivity remained associated with the roots. This indicates that either the radioactive substrate supplied to the roots or radioactive metabolites produced in the roots are transported to the other parts of the plant. Quantitative results obtained by combustion of various plant parts in the oxidizer are presented in Table 2.

Administration of squalene-[^{14}C] *to soya plants*

Because squalene-[^{14}C] is insoluble in water, it was applied to plant leaves as a solution in silicone oil–ether (1:99). In order to avoid leaf damage by the solvents, their amount and therefore that of squalene-[^{14}C] applied had to be limited. Fourteen-day-old seedlings were treated with squalene and then kept growing for two more weeks. The fraction of applied radioactivity incorporated into sapogenols was about the same after foliar application of

Table 2. Radioactivity in various parts of soya plants treated with MVA-[2-^{14}C]

Treatment	Incubation of seedlings for 10 days	Immersion of roots for 14 days
Radioactivity in whole plant (cpm)	2.18×10^4	1.59×10^4
Per cent of radioactivity in the whole plant found in various parts		
Cotyledons	72.5	19.9
Leaves	8.0	37.9
Stems	9.5	35.0
Roots	9.8	6.5

squalene-[^{14}C] as after treating germinating seeds with MVA-[2-^{14}C] (Table 1). The distribution pattern of radioactivity in individual sapogenols after foliar application of squalene-[^{14}C] resembled that after root application of MVA (Table 1).

Radioautograms of plants treated with squalene-[^{14}C] showed that the radioactivity was mainly incorporated into the leaves treated with the squalene solution, but minor radioactivity was also recognizable in other parts of the plants, such as stems and roots. The migration of radioactive substances through the phloem was also visible in the radioautogram. Since squalene is not water-soluble, the radioactivity may have been transported by water-soluble saponins and other glycosides, an assumption supported by the detection of radioactive sapogenols after alkaline hydrolysis (fraction *C*) of the extract of squalene-[^{14}C]-treated plants.

Incubation of germinating alfalfa seeds with MVA-[2-^{14}C] *for ten days*

A much lower percentage (3.9 %) of the applied radioactivity was incorporated into the lipid fraction (*A'*) of alfalfa plants than into the corresponding fraction of soybean seedlings (average 25 %) (Table 1). The per cent incorporation into the water-soluble fraction (*A*) of alfalfa seedlings was somewhat lower (22.2 %) than in the case of soybean seedlings (average 36.2 %). The distribution of radioactivity in sapogenols isolated from alfalfa seedlings is noteworthy: 64.2 % of the radioactivity incorporated into the sapogenols was associated with medicagenic acid, whereas no indication for the presence of medicagenic acid in the respective extracts of soybean plants could be found. Medicagenic acid was isolated after alkaline hydrolysis from the aqueous fraction *C'* by an additional step (*D*) in the procedure (see Experimental). It was identified by scanning the radiochromatogram developed with solvent system 5 and comparison with an authentic reference compound. The predominance of medicagenic acid among the labeled sapogenols produced in alfalfa seedlings under these conditions agrees with the results of Nowacki *et al.* [6] who applied acetate-[^{14}C] to alfalfa seedlings.

EXPERIMENTAL

Seeds. Soybeans (Lincoln variety). Alfalfa seeds (high saponin synthetic—a composite of high saponin lines out of Du Puits, Ladak, Lahontan, Ranger, Uinta and Vernal—provided by Dr. Marion W. Pederson, U.S. Department of Agriculture, Western Region Crops Research Laboratory, Utah State University, Logan, Utah, U.S.A.). Radioactive materials: DL-mevalonic acid-[2-^{14}C] DBED salt (42 μCi/mg) was purchased from Radiochemical Centre, Amersham, England. Squalene-[^{14}C] was prepared biosynthetically by germination of seeds of *Pisum sativum* according to the procedure of ref. [8].

Administration of MVA-[2-^{14}C] *to germinating soybean and alfalfa seeds.* Sterilized soybeans or alfalfa seeds were germinated in a covered Petri dish in the presence of a measured amount of MVA-[2-^{14}C] and an amount of water limited by adequate aeration of the seeds. The experiments were carried out at a controlled temperature, 26° during the day and 24° at night, for the first 3 days in the dark and for the following 2 to 7 days under illumination with white fluorescent light (Cool White, Photon fluence rate (Ph AR) 400–700 μ Einstein m^{-2} sec^{-1}. The Petri dish was opened 5 days after beginning the incubation to allow expansion of the seedlings.

Administration of MVA-[2-^{14}C] *to soybean seedlings through their roots.* Whereas in the previous experiments seeds were incubated from the beginning of germination with MVA-[2-^{14}C], in this experiment the roots of 14-day-old soybean seedlings were placed in a soln of MVA-[2-^{14}C] for an additional 14 days. In order to enable transport of metabolites through the xylem, the main root was cut off. The roots were kept in vials containing the mevalonate, dissolved in a minimal amount of H_2O. The requisite humidity was retained by a cotton plug which also supported the growing plants.

Administration of squalene-[^{14}C] *to soya plants.* Carried out according to the technique of ref. [9] for applying water-insoluble precursors to plants. Squalene-[^{14}C] was dissolved in a 1 % soln of Dow-Corning Silicone Oil 200 in Et_2O and applied to the leaves with a dropper. Non-incorporated squalene-[^{14}C] was removed by washing with CH_2Cl_2.

Separation of labeled plant fractions and isolation of labeled sapogenols. Plant material was homogenized with 80 % EtOH, filtered and the residue washed with hot EtOH and hot Me_2CO. Filtration gave a residue which was discarded and a filtrate which was concd to leave an aq. residue which was partitioned with H_2O–Me_2CO–$CHCl_3$ (2:3:4). The organic soln (*A'*) contained squalene, sterols and pigments. The aq. soln (*A*) was hydrolysed by boiling for 10 hr with 2 N H_2SO_4, then extracted with Et_2O–$CHCl_3$ (1:1) to yield the aq. soln (*B'*) and an organic soln (*B*). The latter was evapd and the residue boiled with 6 % KOH in EtOH for 1 hr, then extracted with $CHCl_3$–Et_2O (1:1). The organic phase (*C*) contained sapogenols and sterols. The aq. soln (*C'*) was acidified with 2 N HCl and extracted with Et_2O to yield in the organic soln (*D*) medicagenic acid. The last step in the procedure (*D*) was carried out only with plants containing medicagenic acid. Radiochromatography was conducted on Si gel plastic TLC sheets (20 × 20 cm, 0.25 mm) (60F_{254}, Merck). Solvent system 1, C_6H_6–EtOAc (97:3), was used for the separation of squalene, β-amyrin and sitosterol. Solvent systems 2–5 served for the separation of sapogenols: solvent 2: C_6H_6–EtOH (9:1); solvent 3: C_6H_6–EtOH (19:1); solvent 4: C_6H_6–EtOH (49:1); solvent 5: Me_2CO–$(Me_2CH)_2O$ (2:5) + 1 drop HOAc/70 ml. Solvent system 5 was particularly suited to the isolation of medicagenic acid. An efficient separation of sapogenols with solvent system 3 or 4, which was also used for preparative purposes, was achieved by continuous ascending development for 4.5 and 20 hr, respectively by allowing the top edge of the plate to protrude from the lid of the chamber [10]. The individual sapogenols were located by spraying with conc H_2SO_4 and briefly heating the plates in an oven at 120°. The following colours were observed: sapogenol A—violet, sapogenol B—brown, sapogenol C—brown, sapogenol E—blue, medicagenic acid—yellow.

Radioactivity measurements. The distribution of radioactivity on TLC was determined with a radiochromatogram scanner. Alternatively, zones corresponding to the appropriate standards were scraped from the plates and the separated compounds were eluted from the Si gel with MeOH–$CHCl_3$ (1:1). Radioactivity was measured by scintillation counting. Aliquots of the radioactive solns were evapd to dryness in counting vials and 5 ml scintillating fluid was added. The latter contained 5 g 2,5-diphenyloxazole and 200 mg 1,4-bis (2-(4-methyl-5-phenyl-oxazol-2-yl) benzene) in 666 ml toluene and 334 ml Triton X-100. The counting efficiency was 85 %. The location of radioactivity in whole plants was detected by autoradiography with Kodak No-Screen Medical X-ray film. The radioactivity of plant parts was determined by combustion in a Tri-Carb Oxidizer, Packard Model 306. For the oxidizer, Permafluor (Packard) was used as scintillation fluid.

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