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BIOSYNTHESIS OF *DIOSCOREA* SAPOGENINS FROM CHOLESTEROL*

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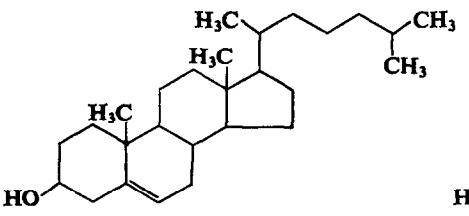
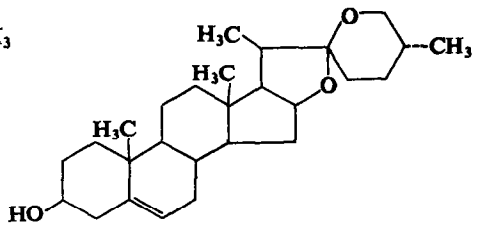
Abstract—Radioactive cholesterol was converted to the sapogenins diosgenin and kryptogenin by *Dioscorea spiculiflora* seedlings. The sapogenins were isolated by chromatography and their radiochemical purity established by dilution with carrier material and crystallization to constant specific activity.

INTRODUCTION

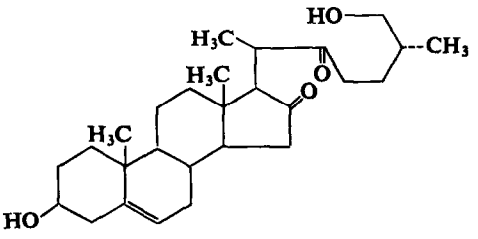
IN PREVIOUS work we found that mevalonic acid-2-^{14}C is incorporated into three sterols, including cholesterol, and four sapogenins by *Dioscorea spiculiflora* plants.[1] The very high specific activity of the cholesterol suggested that it may serve as a precursor of the sapogenins, especially since both are Δ^5-3β-sterols containing 27 carbon atoms. We wished to test this possibility by administering radioactive cholesterol to seedlings.



Cholesterol



Diosgenin



Kryptogenin

* Work conducted under a cooperative agreement with the California Institute of Technology, at the Division of Biology, Pasadena, California.

† A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

[1] R. D. BENNETT, E. HEFTMANN, W. H. PRESTON, JR. and J. R. HAUN, *Arch. Biochem. Biophys.* **103**, 74 (1963).

Adult *D. spiculiflora* plants contain the sterols β-sitosterol (24α-ethyl-Δ^5-cholesten-3β-ol), stigmasterol (24α-ethyl-$\Delta^{5,22}$-cholestadien-3β-ol), and cholesterol (Δ^5-cholesten-3β-ol), and the sapogenins diosgenin (25α-Δ^5-spirosten-3β-ol), yamogenin (25β-Δ^5-spirosten-3β-ol), gentrogenin (25α-Δ^5-spirosten-3β-ol-12-one), correllogenin (25β-Δ^5-spirosten-3β-ol-12-one), and kryptogenin (Δ^5-cholestene-3β, 26-diol-16, 22-dione). Preliminary chromatographic investigations showed that *D. spiculiflora* seeds contain the same three sterols but no detectable amounts of the sapogenins. The latter begin to appear about 2–3 weeks after germination, and their concentration increases slowly over the next 2 months as tubers begin to form. During this period cholesterol-4-^{14}C administered via the leaves is converted into sapogenins, as was demonstrated by purification of diosgenin and kryptogenin to constant specific radioactivity.

RESULTS

When radioactive cholesterol was applied to the leaf surfaces, uptake was so slow that most of it was destroyed by air oxidation. This difficulty was overcome by spraying the leaves with a petroleum ether solution of silicone oil, which was rapidly absorbed through the surfaces and carried considerable amounts of cholesterol with it.

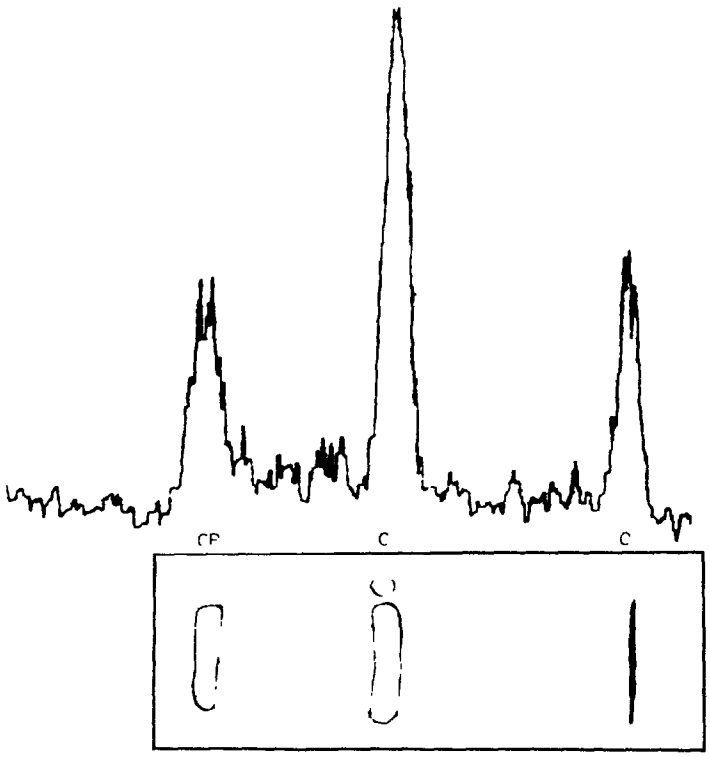


FIG. 1. RADIOCHROMATOGRAM OF LIPID EXTRACT OF *Dioscorea* SEEDLINGS, TREATED WITH CHOLESTEROL-4-^{14}C.

A Silica Gel G plate was developed first with hexane, dichloromethane (9:1) and then with cyclohexane:ethyl acetate (3:2) and scanned at 1·5 in/hr, using a time constant of 40 sec. and a slit width of 4 mm. *O*, origin; *C*, cholesterol; *CP*, cholesterol palmitate.

In preliminary experiments, seedlings so treated were extracted after varying time periods and the extracts separated into lipid and glycoside fractions. Figure 1 shows a typical scan of the radioactivity on a thin-layer chromatogram of a lipid fraction. The three major peaks correspond to unchanged cholesterol, fatty acid esters of cholesterol, and unidentified more polar material. Acid hydrolysis of the glycoside fractions gave the free genins, a radiochromatogram of which is shown in Fig. 2. Cholesterol and highly polar material account for most of the radioactivity, but a small peak can be observed corresponding to diosgenin. The nature of the radioactive material slightly less polar than gentrogenin could not be determined, although it gave the characteristic sapogenin fluorescence on spraying with sulfuric acid. Apparently gentrogenin is not present in seedlings at this stage.

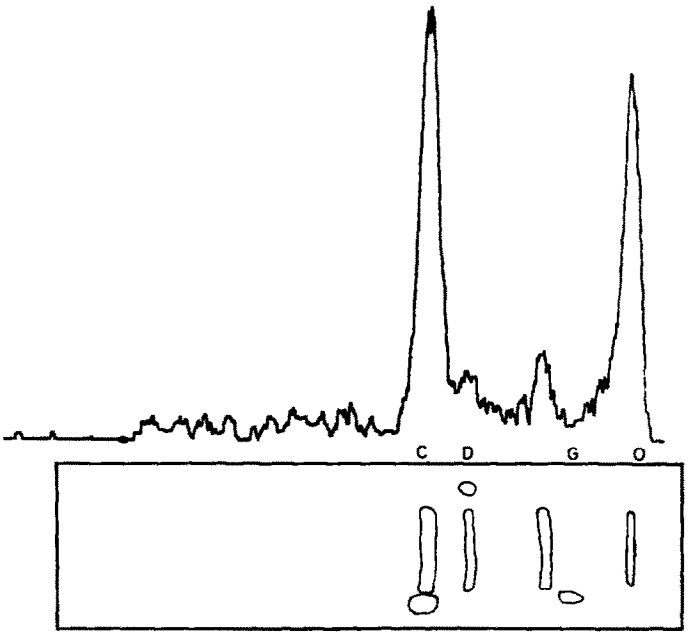


FIG. 2. RADIOCHROMATOGRAM OF AN AQUEOUS HCl HYDROLYZATE OF THE GLYCOSIDE FRACTION IN *Dioscorea* SEEDLINGS, TREATED WITH CHOLESTEROL-4-^{14}C.

A Silica Gel G plate was developed with dichloromethane:acetone (19:1)/water and scanned at 1·5 in/hr, using a time constant of 100 sec and a slit width of 4 mm. *O*, origin; *G*, gentrogenin; *D*, diosgenin; *C*, cholesterol.

The radioactive material corresponding in mobility to cholesterol palmitate was isolated by thin-layer chromatography (TLC), hydrolyzed, and acetylated. By continuous ascending TLC on Anasil B[2] it was found that no significant conversion of cholesterol to β-sitosterol or stigmasterol had occurred (Fig. 3). By similar methods this was also shown to be true of the cholesterol from the lipid fractions and the glycoside hydrolyzates.

In order to establish definitely the conversion of cholesterol to diosgenin, two seedlings were treated with cholesterol-4-^{14}C and extracted and fractionated separately after 19 (Plant A) and 36 days (Plant B). The radioactivity of the glycoside hydrolyzate of Plant A was about twice that of Plant B. The hydrolyzate was subjected to TLC and a sterol-sapogenin

R. D. BENNETT and E. HEFTMANN, *J. Chromatog.* **12**, 245 (1963).

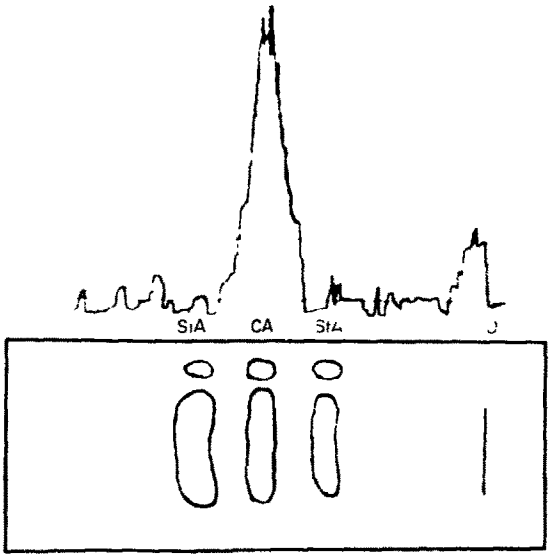


FIG. 3. RADIOCHROMATOGRAM OF STEROL ACETATES, PREPARED FROM THE STEROL ESTER FRACTION (SEE FIG. 1).

An Anasil B plate was developed continuously with hexane:ether (99:1) for 3 hr and scanned at 3 in/hr, using a time constant of 40 sec and a slit width of 4 mm. *O*, origin; *St A*, stigmasterol acetate; *CA*, cholesterol acetate; *St A*, β-sitosterol acetate

fraction was isolated and acetylated. Diosgenin acetate was then separated from the sterols and other sapogenins by TLC. The material from Plant A, having two-thirds of the radioactivity of that from Plant B, was combined with the latter, diluted with pure diosgenin acetate, and purified to constant specific activity (Table 1).

TABLE 1. PURIFICATION OF DIOSGENIN ACETATE TO CONSTANT SPECIFIC ACTIVITY *

Compound	Solvent used for crystallization	Cpm/μmole
Diosgenin Acetate		45·7 ± 1·9
	Methanol	40·6 ± 1·8
	Methanol	41·5 ± 1·8
	Ethanol	37·5 ± 1·7
	Ethanol	38·2 ± 1·7
	Hexane	38·2 ± 1·7
Diosgenin†	Acetone	37·8 ± 1·6
	Hexane-dichloromethane	38·8 ± 1·7

* 0·2-mg aliquots were plated from benzene solutions on copper planchets over an area of 2·8 cm² and counted in duplicate, using a Nuclear Chicago Model D-47 detector with micromil window mounted in a Model C110B sample changer to the 0·9 level of confidence.

† By treatment of diosgenin acetate with lithium aluminum hydride[1].

In the previous experiments little or no kryptogenin could be found by TLC in the glycoside hydrolyzates. Although this sapogenin remains at the origin in the solvent system used in Fig. 2 and thus coincides with a radioactive peak, in more polar systems it was evident that this activity was not associated with kryptogenin. Marker and Lopez[3] believed that kryptogenin is an artifact formed during hydrolysis of the saponin nolonin with ethanolic hydrochloric acid, while in our work we had used aqueous hydrochloric acid for hydrolysis. Accordingly, we used the former method to hydrolyze a glycoside fraction from seedlings treated with cholesterol-4-^{14}C. Figure 4 indicates that under these conditions radioactive kryptogenin was obtained. Our failure to isolate kryptogenin using aqueous acid hydrolysis is consistent with the recent observations of Blunden and Hardman[4] on other *Dioscorea* species.

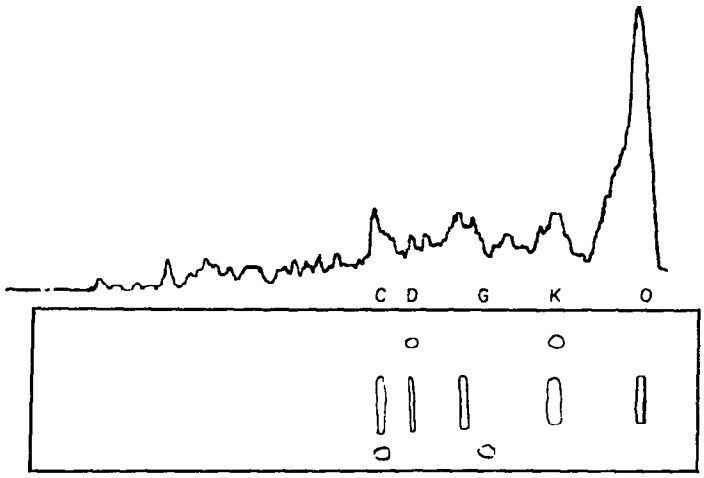


FIG. 4. RADIOCHROMATOGRAM OF AN ETHANOLIC HCl HYDROLYZATE OF THE GLYCOSIDE FRACTION IN *Dioscorea* SEEDLINGS, TREATED WITH CHOLESTEROL-4-^{14}C.

A Silica Gel G plate was developed first with dichloromethane: acetone (7:3)/water for 60 mm and then with a 97:3 mixture for 170 mm and scanned at 0·75 in/hr, using a time constant of 300 sec and a slit width of 4 mm. *O*, origin; *K*, kryptogenin; *G*, gentrogenin; *D*, diosgenin; *C*, cholesterol.

The kryptogenin was separated by TLC, rechromatographed, acetylated, and the kryptogenin acetate purified by TLC, diluted with carrier material, and crystallized to constant specific activity (Table 2). It was then hydrolyzed to kryptogenin, the specific activity of which was unchanged by further recrystallizations. As final proof of radiochemical purity, the kryptogenin was converted chemically, by the method of Mazur and co-workers,[5] to diosgenin, which, after separation by TLC and recrystallization, retained the radioactivity.

DISCUSSION

The chromatographic methods used for isolation of diosgenin separate it from all other steroids known to occur in *D. spiculiflora*, including the C-25 methyl isomer yamogenin

[3] R. E. MARKER and J. LOPEZ, *J. Am. Chem. Soc.* **69**, 2386 (1947).
[4] G. BLUNDEN and R. HARDMAN, *J. Chromatog.* **15**, 273 (1964).
[5] Y. MAZUR, N. DANIELI and F. SONDHEIMER, *J. Am. Chem. Soc.* **82**, 5889 (1960).

TABLE 2. PURIFICATION OF KRYPTOGENIN ACETATE TO CONSTANT SPECIFIC ACTIVITY*

Compound	Solvent used for crystallization	Cpm/μmole
Kryptogenin Acetate		10 10 ± 0·70
	Hexane-benzene	8·43 ± 0·62
	Hexane-benzene	8·29 ± 0·62
	Hexane-acetone	7·12 ± 0 52
	Hexane-acetone	6·94 ± 0·52
Kryptogenin†	Hexane-acetone	6 97 ± 0 52
	Ethanol	6 85 ± 0·52

* Conditions same as in Table 1, except that 1 0-mg aliquots were counted and a correction was made for differences in self absorption between krytpogenin and kryptogenin acetate.

† By hydrolysis of kryptogenin acetate with methanolic potassium bicarbonate.[6]

(Fig. 5). The sequence of purification steps used, including separation by TLC, acetylation, separation of diosgenin acetate by TLC, dilution with authentic diosgenin acetate and crystallization to constant specific activity in three solvents, conversion to diosgenin, and again crystallization to constant specific activity in two solvents, insures beyond reasonable doubt that the diosgenin obtained was radiochemically pure. The proof of the biosynthesis of kryptogenin from cholesterol involved the same steps and, in addition, chemical conversion

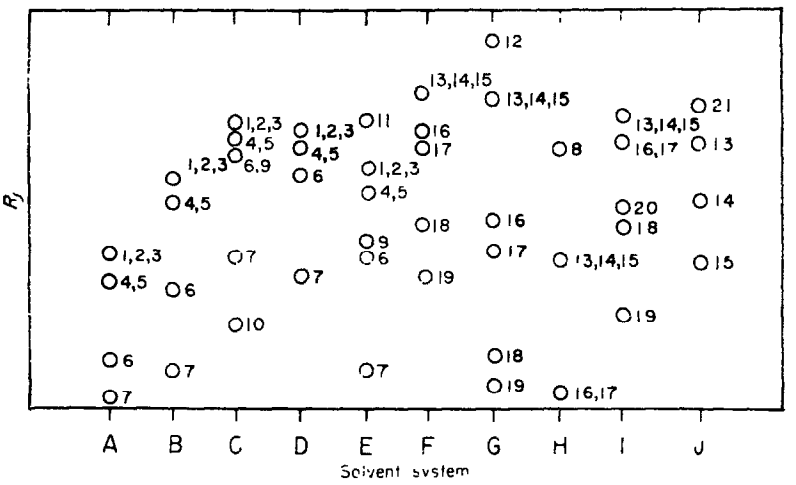


FIG. 5. SEPARATION OF STEROIDS BY TLC

For solvent systems *A–J* see text. Numbers refer to the following compounds: *1*, β-sitosterol; *2*, cholesterol; *3*, stigmasterol; *4*, diosgenin; *5*, yamogenin; *6*, gentrogenin; *7*, kryptogenin; *8*, cholesterol palmitate; *9*, pregnenolone; *10*, cortisol; *11*, lanosterol; *12*, squalene; *13*, β-sitosterol acetate; *14*, cholesterol acetate; *15*, stigmasterol acetate; *16*, diosgenin acetate; *17*, yamogenin acetate; *18*, gentrogenin acetate; *19*, kryptogenin diacetate; *20*, pregnenolone acetate *21*, lanosterol acetate.

[6] R. E. MARKER, R. B. WAGNER, P. R. ULSHAFER, E. L. WITTBECKER, D. P. J. GOLDSMITH and C. H. RUOF, *J. Am. Chem. Soc.* **69**, 2167 (1947).

of the former to diosgenin. This is a particularly rigorous test of purity, as the reduction of a ketone by sodium borohydride leads, in almost all cases, to an alcohol, causing a significant decrease in chromatographic mobility. In the case of kryptogenin, however, two keto groups and one hydroxyl group are replaced by the nonpolar spiroketal system, resulting in a great increase in mobility. Thus, it is highly unlikely that the kryptogenin could contain a radioactive impurity which, after such a reaction, would still be inseparable by chromatography.

The radioactivity of the diosgenin represents 1·1% of the radioactivity taken in by the plants, while the yield of kryptogenin is 0·8%. If the latter is an artifact in this case, as it appeared to be in *D. mexicana*,[3] the radioactivity of the parent saponin may have been even higher.

In assessing the significance of these rather low rates of incorporation of cholesterol into sapogenins, it is perhaps noteworthy that Tschesche and Lilienweiss,[7] in a somewhat analogous case, observed a 2·2% conversion of pregnenolone glucoside to digitoxigenin in *Digitalis lanata* leaves. The question of whether cholesterol lies on the major pathway of sapogenin biosynthesis cannot be definitely settled at this time.

It is evident from Figs. 1–4 that the major part of the radioactivity recovered by extraction of the plants is associated with cholesterol (free, esterified, and glycoside) and highly polar, unidentified material. Although a variety of extraction procedures was used in the course of this work, in every case from one-third to one-half of the radioactivity originally taken up by the plant could not be recovered in the extracts. This suggests that some transformations involving degradation of Ring A and loss of C-4 may have occurred.

The intermediary steps in the biosynthetic pathway leading from cholesterol to sapogenins remain to be elucidated. The role of kryptogenin, in particular, deserves further study. Marker and Lopez[3] found that Beth root yielded increasing amounts of diosgenin and decreasing amounts of kryptogenin during storage. They postulated a metabolic conversion of a sapogenin, nologenin, to diosgenin, while kryptogenin is an artifact formed during hydrolysis by dehydration of any remaining nologenin. Their findings as well as our own do not exclude the possibility that plants may also be able to synthesize kryptogenin and perhaps utilize it as a precursor of diosgenin and other sapogenins. The amount of kryptogenin present at any time may be quite low in this case.

EXPERIMENTAL

Methods

Thin-layer plates were 50 × 200 mm, with adsorbent layers 0·3 mm thick. Samples were applied as bands, 20–30 mm long, 15 mm from the bottom of the plate. The bands were centered, and reference compounds were cochromatographed as spots, 10 mm from the side edge of the plate. Chromatograms were scanned for radioactivity using a Strip Scanner I apparatus with TLC holder.* For preparative work, Rhodamine 6G was used to locate zones to be isolated, which were scraped off the plate and eluted in a chromatographic column with acetone. For analytical thin-layer chromatograms, 50% sulfuric acid was used as the spray reagent.[8]

Typical separations of the steroids of *D. spiculiflora*, as well as other biologically important steroids, in the solvent systems used in this work are shown in Fig. 5. The compositions of

* Atomic Accessories, Inc., Valley Stream, N.Y.

[7] R. Tschesche and G. Lilienweiss, *Z. Naturforsch.* **19b**, 265 (1964).

[8] R. D. Bennett and E. Heftmann, *J. Chromatog.* **9**, 353 (1962).

the systems are as follows: *A*, benzene:ethyl acetate (3:1); *B*, cyclohexane:ethyl acetate (3:2); *C*, dichloromethane:acetone (7:3); *D*, chloroform:methanol:water (188:12:1); *E*, dichloromethane:acetone (9:1); *F*, dichloromethane:ether (9:1); *G*, dichloromethane:ether (97:3); *H*, hexane:dichloromethane (1:1); *I*, cyclohexane:ethyl acetate (4:1); *J*, hexane:ether (99:1). System *J* was used only with Anasil B* plates, developed continuously. All other systems were used on Silica Gel G† plates. Systems *C*, *E*, and *F* were saturated with water.[9]

Aliquots of radioactive samples were counted on planchets at infinite thinness (except for material diluted with carrier) under a gas flow detector (see Table 1, legend, for details).

Administration of Cholesterol

Cholesterol-4-^{14}C, having a specific activity of 50 μc/mg, was purchased from New England Nuclear Corporation. This material showed only a single radioactive peak when subjected to TLC with System B and, after acetylation, on Anasil B with System J.

D. spiculiflora Hemsl. seeds‡ were placed on a wet piece of filter paper in a closed Petri dish and kept at 25°; the water was replenished as necessary. Most of the seeds germinated within 2–3 weeks. When the seedlings were about 2 cm long, they were planted in soil in pots and watered daily. Just before cholesterol was to be applied, the leaf was washed well with a 0·1% aqueous solution of Tween 20 and allowed to dry. A solution of cholesterol-4-^{14}C in 80% ethanol (20–50 μl), containing 0·05% Tween 20, was spread over the surface of the leaf. It was then sprayed with a petroleum ether solution of Silicone DC-200 (Chromatospray)§ to promote rapid absorption of cholesterol.[9]

Without silicone treatment absorption of cholesterol was very slow, and after 3 weeks about 50% of the radioactivity remained on the leaf surface. When an ethanol rinsing of the leaf surface after 4 days was subjected to TLC with System B, only a trace of the radioactivity was found to be associated with cholesterol; the major peak was due to more polar material, which remained at the origin. Apparently, when cholesterol is spread on the leaf, the surface area exposed is so large that it is very susceptible to air oxidation. Although destruction of the cholesterol could be partially prevented by covering the surface of the leaf with a thin film of lanolin, absorption into the leaf was still very slow. When mineral oil was used in place of silicone, the uptake of cholesterol was only about half as efficient.

Biosynthesis of Diosgenin

Cholesterol-4-^{14}C ($3{\cdot}6 \times 10^5$ cpm per plant) was applied to the leaf of each of two *Dioscorea* seedlings, A and B, growing in soil, about 2 weeks after germination. The leaves were then sprayed with silicone. The plants were treated separately as follows. After 4 days the leaves were rinsed free of radioactivity with 80% ethanol, and aliquots of the rinsings were counted: Plant A, $3{\cdot}1 \times 10^5$ cpm; Plant B, $3{\cdot}0 \times 10^5$ cpm. Thus, the radioactivity absorbed by the leaves was 5×10^4 cpm for Plant A and 6×10^4 cpm for Plant B.

After 19 days (Plant A) and 36 days (Plant B) each seedling was homogenized with 1 ml of water in a tissue grinder. After 20 min, 3 ml of methanol was added and the mixture refluxed for 4 hr. The solution was centrifuged, the residue re-extracted with two 1-ml

* Analabs, Inc., Hamden, Conn.

† Brinkmann Instruments, Inc., Westbury, N.Y.

‡ Kindly supplied by Dr Joseph R. Haun, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

§ Research Specialties Co., Richmond, Calif.

[9] R. D. BENNETT and E. HEFTMANN, *Phytochemistry* 4, 475 (1965).

portions of 75% methanol, and the methanol extracts combined. The methanol was removed by azeotropic distillation with excess benzene, the benzene layer separated, the aqueous layer extracted with two 1-ml portions of benzene, and the benzene solutions combined (lipid fraction): Plant A, 3000 cpm; Plant B, 4000 cpm. TLC showed that this fraction contained cholesterol and cholesterol esters but no sapogenins (Fig. 1).

The aqueous layer, containing steroid glycosides, was diluted to 3·5 ml with water; 0·7 ml of concentrated hydrochloric acid was added, and the solution overlaid with 3 ml of benzene and refluxed for 2 hr. The benzene layer was separated, the aqueous layer extracted with two 1-ml portions of benzene, and the benzene solutions combined: Plant A, $3{\cdot}3 \times 10^4$ cpm; Plant B, $1{\cdot}5 \times 10^4$ cpm. This fraction was evaporated and subjected to TLC with System D. In this system the three sterols and the sapogenins diosgenin and yamogenin ran as a single zone which was well separated from the more polar sapogenins (Fig. 5). The sterol-sapogenin zone was removed and eluted: Plant A, 4100 cpm; Plant B, 4000 cpm.

This material was acetylated, using pyridine:acetic anhydride (1:1) for 16 hr at 25°, and rechromatographed, developing with System G. In this system the three sterol acetates run as a single zone near the solvent front, while diosgenin acetate is completely separated from the isomeric yamogenin acetate in the middle of the plate (Fig. 5). The diosgenin acetate zone was removed and eluted: Plant A, 600 cpm; Plant B, 900 cpm. The remainder of the plate was scanned for radioactivity. In neither case were peaks corresponding to yamogenin acetate observed, although at the level of sensitivity used as little as 100 cpm could have been detected.

The two diosgenin acetate fractions were combined (< 0·5 mg) and diluted with 10·8 mg of pure diosgenin acetate. Purification to constant specific activity was carried out as shown in Table 1.

Biosynthesis of Kryptogenin

Four seedlings were treated with two $1{\cdot}86 \times 10^5$-cpm doses of cholesterol per plant as above. The first dose was given 4 weeks and the second one 6 weeks after germination. A total of $3{\cdot}04 \times 10^5$ cpm was taken up by the four plants. Two weeks after the second treatment, the plants were homogenized with 1 ml of water, and 3 ml of methanol was added after 20 min. The mixture was refluxed for 4 hr, centrifuged, the residue extracted with four 3-ml portions of methanol, and the methanolic solutions combined and evaporated to dryness under nitrogen. The residue was taken up in 1 ml of water and extracted with five 2-ml portions of hexane. The hexane solutions were combined, concentrated to 5 ml, and extracted with 0·5 ml of water which was combined with the aqueous layer above. The hexane solution (lipid fraction) contained $4{\cdot}6 \times 10^4$ cpm.

The aqueous layer was evaporated to dryness under nitrogen and the residue extracted with three 2-ml portions of boiling methanol. The combined methanol solutions (glycoside fraction) contained $1{\cdot}62 \times 10^5$ cpm. An aliquot representing 4×10^4 cpm was evaporated to dryness and the residue refluxed with 2 ml of 3N hydrochloric acid in 30% ethanol overlaid with 2 ml of benzene (equilibrated with 30% ethanol) for 3 hr. The benzene layer was separated and the aqueous layer extracted with three 2-ml portions of benzene. The benzene solutions were combined, washed with 0·5 ml of water, and evaporated, leaving a residue that gave $3{\cdot}4 \times 10^4$ cpm. Figure 4 shows a radiochromatogram of an aliquot of this material.

The whole extract was subjected to TLC in the same system and the zone corresponding to kryptogenin removed and eluted, giving 6200 cpm. This was rechromatographed with System D and the kryptogenin zone was again isolated (900 cpm). After addition of 100 μg

of pure carrier kryptogenin,* acetylation, and TLC with System F, kryptogenin acetate having radioactivity of 900 cpm was isolated. This was diluted with 9·9 mg of authentic kryptogenin acetate and purified to constant specific activity as shown in Table 2.

The kryptogenin obtained by the final recrystallization (3·2 mg) was dissolved in 1·5 ml of dry tetrahydrofuran and a solution of 2·0 mg of sodium borohydride in 0·5 ml of isopropyl alcohol was added. After 48 hr at 25°, 5 drops of concentrated hydrochloric acid were added cautiously, followed by 0·5 ml of water. The solution was placed in a water bath at 50° for 2 min and was then extracted with three 3-ml portions of benzene. The benzene extracts were combined, washed with 1 ml of 2% sodium bicarbonate solution and 1 ml of water, and evaporated. The residue was chromatographed on a 200 × 200 mm Silica Gel G plate, 1 mm thick, developed with System *E*. In this system kryptogenin remains near the origin, while diosgenin moves more than halfway up the plate (Fig. 5). The zone corresponding to diosgenin was removed and eluted. This material was recrystallized from methanol, giving 0·9 mg, m.p. 205–208°; mixed melting point with authentic diosgenin, 206–208°. The specific activity was 6·72 ± 0·74 cpm/μmole. This figure is of the same order of magnitude as, but not directly comparable with those in Table 2 because a different weight of sample was counted and therefore the self absorption was different.

Radioactivity of Sterols

A lipid fraction was obtained by the methods described in the diosgenin experiment. Figure 1 shows the radiochromatogram of an aliquot of this material. The remainder of the fraction (2×10^4 cpm) was subjected to TLC in the same system and the zone corresponding to cholesterol palmitate removed and eluted, giving 2200 cpm. These sterol esters were converted to the free sterols by treatment with lithium aluminium hydride[1] and then acetylated. Figure 3 shows a radiochromatogram of the sterol acetates.

* Gift of Dr. A. Zaffaroni, Syntex Laboratories, Inc., Palo Alto, Calif.