Biosynthesis of Cholesterol by Seedlings of *Digitalis purpurea*

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Abstract: Three- and four-week old seedlings of *Digitalis purpurea* were incubated with mevalonic acid-2-^{14}C and found to incorporate 4.8 and 3.6% of the label into a mixture of sterols. Of the radioactivity of the sterol fraction 24–31% was found associated with cholesterol. Cholesterol comprises about 3% of the weight of all sterols. The high incorporation of radioactivity into cholesterol and its small weight compared to other sterols support the hypothesis that cholesterol is an intermediate in plant sterol biosynthesis. The sterols were found as water-soluble conjugates.

For many years cholesterol was thought to be a sterol peculiar to animals. This view could no longer be maintained when Tsuda, *et al.*,[1] found cholesterol in red algae; Johnson, *et al.*,[2] found the sterol in *Solanum tuberosum* and *Dioscorea spiculiflora;* Devys and Barbier[3] found it in the pollen of *Hypochoris radicata;* and Linde, *et al.*,[4] found it as constituent of "γ"-sitosterol from *Digitalis canariensis.* Recently, Caspi, *et al.*,[5] have shown that cholesterol can be degraded to pregnenolone by whole *D. purpurea* plants, and Tschesche and Lilienweiss[6] have demonstrated that the steroid in pregnenolone-β-D-glucopyranoside can be converted to digitoxigenin by excised leaves of *D. lanata.* These reports have led to speculation that cholesterol represents an intermediate in the biosynthesis of plant steroids. In the present paper we describe the synthesis of radiocholesterol from mevalonic acid-2-^{14}C by seedlings of *D. purpurea.*

Experimental Section

Materials. Seeds of *D. purpurea* were acquired from Burnett-Seedsmen, New York, N. Y. *dl*-Mevalonic acid-2-^{14}C, as the dibenzylethylenediamine salt, was purchased from New England Nuclear Corp. All chemicals were reagent grade. Methylene chloride, alcohols, benzene, and water were distilled.

Growth and Incubation of Seedlings. Two experiments with separate batches of seeds were performed. Experiment 1 was run 8 months prior to the other and will be described in detail. Experiment 2 differed from the first in age and amount of seedlings and quantity of administered mevalonic acid-2-^{14}C, as indicated below.

Seeds of *D. purpurea* were grown on nutrient agar plates for 21 days at room temperature and in the dark. Seeds were briefly soaked in 95% ethanol, transferred to a 1% sodium hypochlorite solution, washed with water, and blotted on several layers of filter paper under aseptic techniques. Approximately 2400 seeds were spread over the surface of an agar plate in a glass dish, 10 cm in diameter and 8 cm high. The agar was prepared by adding 1% of Difco "Noble" agar to White's standard medium,[7] without the color indicator. Of this medium, 150 ml was placed into each glass dish which was then autoclaved for 30 min. The dishes, after setting of the gel and seeding, were covered and placed in a dark cabinet without further attendance for 21 days at room temperature. Of each batch of 12 dishes, one or two were usually found contaminated with microorganisms. These were discarded as soon as noticed. If no contamination occurred during the first week after seeding, none would occur at a later time.

The seedlings were pulled from the agar after softening the latter by heating to 35°. To each of three 250-ml erlenmeyer flasks provided with silicone rubber stoppers were added 150 ml of inorganic salt solution prepared according to White,[7] 13.8 g of seedlings, and 1 ml of water containing 0.196 mg of mevalonic acid-2-^{14}C as the dibenzethylenediamine salt (specific activity 8.5 μcuries/mg). The flasks were shaken slowly for 5 days and were opened daily for 1 hr. At the end of the time period the salt solution was decanted and the seedlings were washed three times with 100 ml of water each time and blotted on filter paper.

Isolation of Cholesterol. All seedlings were combined, 150 ml of water was added, and the mixture was homogenized first in a Waring blender at high speed for 30 sec and then in a Potter-Elvehjem tissue grinder.

To remove fatty substances, the product was shaken thoroughly for 1 hr with 200 ml of petroleum ether (bp 60–90°) and separated. This extraction was repeated three more times. The aqueous phase was acidified to pH 4 with hydrochloric acid, and 40 g of sodium chloride was added and dissolved with stirring. The mixture was extracted four times with an equal volume of 1-butanol each time. The pooled alcoholic extracts were taken to dryness by water-pump suction at 60–70°. The dry residue was refluxed for 2 hr with 30 ml of 4 *N* hydrochloric acid made up by diluting one volume of concentrated hydrochloric acid with one volume of water and one volume of methanol.[8] After cooling to room temperature, the hydrolysate was extracted three times with 30 ml of benzene each time. The pooled benzene extracts were washed once with 10 ml of 1 *N* sodium hydroxide and five times with 10 ml of water and evaporated to dryness under vacuum. The residue was chromatographed on four thin layer plates of 0.50-mm-thick silica gel in the system 5% petroleum ether in methylene chloride so that the solvent front ran up a distance of 15 cm from the origin. Material corresponding to sterols by comparison with standards moved with an R_f value between 0.13 and 0.23. Unidentified radioactivity remained at the origin and, except for the sterol spot, no significant counts were present elsewhere on the plate. The silica gel in the region between 2 and 3.5 cm was scraped off, and substances were eluted exhaustively with methanol and ethyl ether and dried.

The residue contains sterols and unidentified substances. It was subjected to a second thin layer separation on three similar plates in the system 8.5% ethanol in methylene chloride. All of the radioactivity was localized in an area between R_f values of 0.60 and 0.73, corresponding to authentic sterols. The rest of the plate contained only background counts. The silica with the radioactive spot was scraped off and exhaustively extracted, as before. A product weighing 3.5 mg and having a total radioactivity of 23,350 cpm was obtained. One-thousandth of this material was subjected to gas chromatography.

Gas chromatography was performed on a Barber-Colman Model 10 instrument, equipped with a 150-cm U-shaped column 4 mm in diameter. The supporting medium consisted of Gas Chrom Q, 100–120 mesh, coated with 0.8% of SE-52. The column was bled at 255° for 114 hr and used for analysis at 219°. The sample injection port temperature was 270°, and the detector temperature

(1) K. Tsuda, S. Akagi, and Y. Kishida, *Chem. Pharm. Bull.* (Tokyo), 6, 101 (1958).
(2) D. F. Johnson, R. D. Bennett, and E. Heftmann, *Science*, **140**, 198 (1963).
(3) M. Devys and M. Barbier, *Compt. Rend.*, **261**, 4901 (1965).
(4) H. Linde, N. Ergenc, and K. Meyer, *Helv. Chim. Acta*, **49**, 1246 (1966).
(5) E. Caspi, D. O. Lewis, D. M. Piatak, K. V. Thimann, and A. Winter, *Experientia*, **22**, 506 (1966).
(6) R. Tschesche and G. Lilienweiss, *Z. Naturforsch.*, **19**, 265 (1964).
(7) P. R. White, "The Cultivation of Animal and Plant Cells," The Ronald Press, New York, N. Y., 1963, p 60.
(8) This method was found optimal for the hydrolysis of saponins by E. S. Rothman, M. E. Wall, and H. A. Walens, *J. Am. Chem. Soc.*, **74**, 5791 (1952).

Table I. Specific Activities[a] of Crystallized Sterols in Experiment 1

No. of crystallizations	Stigmasterol		Cholesterol acetate		Cholesterol		Cholesterol benzoate	
	Crystals	Supernatant	Crystals	Supernatant	Crystals	Supernatant	Crystals	Supernatant
1	6912	6120	359	1000	462	637	179	
2	4860	7550	588	559	323	477	190	82
3	4220	6200	738	569	311	498	225	128
4	3450	6160	727	705	337	467	266	278
5	2775	6400	734	Iq[b]	318	402	295	315
6	2178	6750			302	350	303	328
7	1710	3000			297	Iq[b]	305	301
8							302	Iq[b]

[a] Counts per minute per milligram. [b] Iq = insufficient quantity.

was 267°. A β-ionization chamber with ^{90}Sr was the detecting device. A flow rate of 52–60 cc/min of argon was maintained. The retention times were 15, 18.5, 20.5, and 24 min, respectively, for cholesterol, campesterol, stigmasterol, and β-sitosterol. The respective acetates showed peaks at 20.5, 27.5, 30, and 35 min.

Two milligrams of authentic stigmasterol was added to the sterol residue obtained from the final thin layer chromatography plates. The stigmasterol was recrystallized seven times from methanol–ether. The mother liquor of each stigmasterol crystallizate, except the last, showed a small cholesterol peak on the gas chromatogram. With the residue of the seventh supernatant the cholesterol peak had disappeared and the specific activity was reduced to less than one-half. The specific activity of crystals decreased with each succeeding crop, but the rate of decrease slowed down. It is likely that counts were associated with stigmasterol. All supernatants were pooled, taken to dryness, and acetylated. One-fiftieth was subjected to thin layer chromatography according to the procedure of Waters and Johnson,[9] and it was found that the major amount of radioactivity was associated with the cholesterol acetate area. Gas chromatography of the acetates indicated peaks which coincided with peaks for standard cholesterol, campesterol, stigmasterol, and β-sitosterol acetates. Thereupon 10 mg of cholesterol acetate was added to the combined residue, which weighed 1.3 mg, and one-quarter of the material was recrystallized five times.

The rest of the cholesterol acetate mixture was divided into two portions. Portion 1 was hydrolyzed with 6% potassium hydroxide in ethanol for 0.5 hr in a boiling water bath. Water was added and the hydrolysate was extracted with ether. After evaporation of ether a quantity of standard cholesterol approximately equal to the weight of the residue was added and recrystallized seven times. Portion 2, remaining from the above, was also hydrolyzed and then benzoylated in the usual manner. Standard cholesterol benzoate was added and recrystallized eight times.

Seedlings used in expt 2 were 29 days old and weighed 66.3 g. They were incubated with a total of 0.30 mg of mevalonic acid-2-^{14}C, as the dibenzylethylenediamine salt (specific activity 7.0 μcuries/mg). The seedlings were homogenized, as described, sonicated for 1 min, homogenized again, and resonicated. This procedure was found necessary in order to break up all of the cells, as determined by low-power microscopy. To the residue, after thin layer chromatography in both systems described, was added 10 mg of cholesterol. The latter was crystallized five times.

Scintillation counting was done on a Nuclear Chicago Series 720 spectrometer at room temperature with enough radioactive material for a net count at least double the background rate and for most samples appreciably more. The scintillation fluid consisted of 10 ml of a solution of toluene, containing 5 g of 2,5-diphenyloxazole and 300 mg of 1,4-bis(2-(5-phenyloxazolyl))benzene per liter. The counting time varied between 20 and 100 min. The efficiency was 78%. The mevalonic acid-2-^{14}C was dissolved in 0.5 ml of methanol before the scintillation fluid was added.

Results

Table I shows the specific activity of the sterol crystals and the mother liquors from each crystallization step of expt 1, obtained after incubation with mevalonic acid-2-^{14}C. In three instances residues from the final supernatants were too small to be transferred to a microbalance. The radioactivity of stigmasterol decreased

(9) J. A. Waters and D. F. Johnson, *Arch. Biochem. Biophys.*, **112**, 387 (1965).

Table II. Specific Activities[a] of Cholesterol in Experiment 2

No. of crystallizations	Crystals	Supernatant
1	1701	4173
2	1283	1475
3	1297	1325
4	1295	1307
5	1271	1315

[a] Counts per minute per milligram.

throughout the crystallization steps, but the rate of decrease appears to approach asymptotically a level above 1200 cpm/mg. The pooled petroleum ether extracts of the defatting stage contained a total of 38,500 cpm. Analysis by gas chromatography showed a number of peaks appearing during the first 17 min. No peaks corresponding to the four sterols assayed here could be detected in this fraction. The aqueous phase contained 453,000 cpm. Gas chromatography of the sterol mixture in the aqueous phase after hydrolysis and separation in both thin layer systems indicated the presence of 0.075 mg of cholesterol, 0.43 mg of campesterol, 1.44 mg of stigmasterol, and 1.64 mg of β-sitosterol, by comparison with standards.

The petroleum ether extracts from expt 2 contained 1360 cpm and the aqueous phase 1,460,000 cpm. Quantitation of sterols by planimetry of gas chromatograph peaks gave the following values: 0.175 mg of cholesterol, 0.80 mg of campesterol, 1.69 mg of stigmasterol, and 2.00 mg of β-sitosterol. The specific activities of crystallized cholesterol and the residues from the mother liquors are indicated in Table II.

Discussion

Incubation with mevalonic acid-2-^{14}C permits the isolation of cholesterol acetate, cholesterol, and cholesterol benzoate of constant specific activities after the second, first, and fourth crystallizations, respectively, as indicated in Tables I and II. It took somewhat longer for the benzoate to reach constant specific activity, presumably because of the presence of benzoic acid in the extract. The specific activities of the residues from the terminal supernatants coincided with that of the crystals. The substrate was added as the dibenzylethylenediamine salt. Rilling and Bloch[10] found no difference in results in squalene formation in yeast from mevalonate as the potassium salt or as the dibenzylethylenediamine salt.

(10) H. C. Rilling and K. Bloch, *J. Biol. Chem.*, **234**, 1424 (1957).

The thin layer chromatographic systems for free sterols do not separate campesterol, stigmasterol, β-sitosterol, and cholesterol. The system for the acetates[9] separates these sterols, although β-sitosterol and campesterol acetates run together. Chromatography of the acetates presented the first evidence that cholesterol was labeled. The gas chromatographic method separates all four sterols, but the cholesterol peak of the plant extract was so small that at first no significance was attached to it.

From the specific activity of the cholesterol acetate of expt 1 it can be calculated that 7300 cpm, or 31% of the total activity of the sterol mixture, was due to cholesterol. No more than 0.075 mg of cholesterol by weight was present in this mixture, and thus the specific activity of cholesterol was at least 97,000 cpm/mg in the native extract. The specific activity of stigmasterol can be roughly estimated at 5000 cpm/mg and, since 1.44 mg of this sterol was present, 7200 cpm or one-third of the total sterol activity can be accounted for by this sterol. Similarly, from expt 2, it can be calculated from the specific activity of cholesterol that 24% of the radioactive counts of the mixture was due to cholesterol. Because of the large amount of endogenous substance, radioactive stigmasterol produced during the course of the experiment has been extensively diluted.

Cholesterol was found in the aqueous phase after defatting with petroleum ether. It became soluble in a nonpolar solvent only after hydrolytic treatment with mineral acid. It is supposed that cholesterol can be conjugated at C-3 and that it may occur as glycoside. It is also possible that cholesterol can be conjugated with sulfuric acid. This speculation is reinforced by the recent findings of cholesterol sulfate in the human organism.[11,12] No other water-soluble conjugate of cholesterol has been discovered in nature.

With the finding of cholesterol in plants,[1-4] increased attention has been drawn to the intermediate role of this substance in plant sterol biosynthesis. Cholesterol comprises but 3% by weight of the total sterol mixture, yet its rate of formation is rapid. One-quarter to one-third of the radioactivity found in all sterols became associated with cholesterol during the incubation period. The high rate of incorporation of label into cholesterol is consonant with the expectation that cholesterol fulfills an intermediary role in formation of other sterols. Data indicate that the turnover rate of cholesterol must be high.

Animals seem to be restricted in their ability to degrade cholesterol, and on a weight basis this compound is the most important of all steroid-nucleus-containing substances. Plants appear to convert cholesterol rapidly to a number of compounds which occur side by side. Bergmann[13] has pointed out that cholesterol has achieved a position of dominance at higher levels of evolution until it has become the principal sterol of vertebrate animals. It remains to be seen whether higher animals have achieved an advantage by limiting the means of biochemical modification of the steroid side chain. Lipid-soluble cholesterol and its esters in the circulatory system of animals have never been shown to be of advantage to the organism and have in fact been blamed for certain types of heart disease when present in excessive amounts. Unsaturation at C-22, a reaction not possible in animals, may render the side chain vulnerable to oxidative attack with subsequent loss of a number of carbon atoms. This and other transformations may increase water solubility. It was pointed out that we found cholesterol in the water-soluble portion of the plant homogenate. Conjugation at C-3 and modification of the side chain may act together to increase polarity. Perhaps a deficiency in the ability to transform cholesterol to water-soluble compounds is peculiar to higher animals and is responsible for organic malfunctions due to high circulating levels of the sterol.

Reports in the literature suggest that sterols in higher plants arise by a pathway similar to the one known for animals and yeast. Carbons from mevalonic acid can be incorporated into squalene in soybeans,[14] peas,[15,16] *Ocimum basilicum*,[17] and carrots and tomatoes;[18] into stigmasterol in peas,[9] *Dioscorea spiculiflora*,[19] and tomatoes;[20] into β-sitosterol in peas,[9,21] *Rauwolfia serpentina*,[22] *Dioscorea spiculiflora*,[19] and *Salvia officinalis* and *S. sclarea*;[23] into α- and β-amyrin in peas;[16] and into digitoxigenin in *Digitalis purpurea* and *D. lanata*.[24-26] In several instances the mode of incorporation of carbons or hydrogens from mevalonic acid into steroids has been examined and found to coincide with the expected location on the carbon skeleton or with the expected $^{14}C/^{3}H$ ratio.[16,22,25] When administered as free compound or as glycoside to excised leaves of *Digitalis lanata*, cholesterol is converted to tigogenin, gitogenin, and diosgenin.[27] Free cholesterol applied to the surface of intact leaves of *Dioscorea spiculiflora* appears as diosgenin.[28] The present report describes an extensive incorporation of mevalonic acid-2-^{14}C into cholesterol when compared with other sterols. The small concentration of cholesterol in a mixture of all plant sterols is indicative of the rapid conversion of cholesterol to other compounds. Cholesterol appears to fulfill the same role in plants and animals: that of key intermediate in sterol biosynthesis.

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