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IN VIVO CONVERSION OF SQUALENE TO β-SITOSTEROL

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Abstract—Squalene-^{14}C was converted to β-sitosterol by *Pharbitis nil* seedlings. The β-sitosterol was isolated by chromatography and its radiochemical purity established by dilution with carrier material and crystallization to constant specific activity.

INTRODUCTION

WORK in recent years has indicated that steroids are formed in plants by the same biosynthetic pathway as in animals. The role of acetate and mevalonate as precursors of plant steroids is now well established,[1] and degradation studies on β-sitosterol synthesized from sodium acetate-1-^{14}C and mevalonolactone-2-^{14}C have shown that the pattern of labelling is consistent with that observed in the biosynthesis of cholesterol from these same compounds.[2] However, no direct evidence exists for the steps beyond mevalonic acid. The incorporation of mevalonic acid into squalene, the immediate precursor of the steroid ring system in animals, has been demonstrated in two different plants,[3,4] and a time study showed that the radioactivity of β-sitosterol increased as that of squalene decreased, but efforts to prove this conversion directly were unsuccessful.[3] We have now been able, by use of an efficient technique for incorporating non-polar lipids into plants, to show conclusively that squalene is a precursor of β-sitosterol.

RESULTS

Squalene-^{14}C, prepared biosynthetically from mevalonate-2-^{14}C, was applied to the cotyledons of *Pharbitis nil* Chois seedlings (Japanese morning glory). The cotyledons were then sprayed with a petroleum ether solution of silicone oil, which was absorbed within a few hours and carried most of the squalene along with it. After 2½ days the cotyledons were removed and the lipids were extracted. Figure 1 shows a scan of the radioactivity of a thin-layer chromatogram of an aliquot of this extract. The major peaks correspond to unchanged squalene, lanosterol, β-sitosterol, and unknown polar material which remained at the origin. The β-sitosterol was separated from other radioactive material by preparative thin-layer chromatography (TLC) in two different solvent systems and then acetylated. β-Sitosterol acetate was purified by preparative TLC. The methods used up to this point do not separate sterols differing only in number of carbon atoms or degree of unsaturation in the side chain.

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[1] E. HEFTMANN, *Ann. Rev. Plant Physiol.* **14**, 225 (1963).
[2] A. R. BATTERSBY and G. V. PARRY, *Tetrahedron Letters*, 787 (1964).
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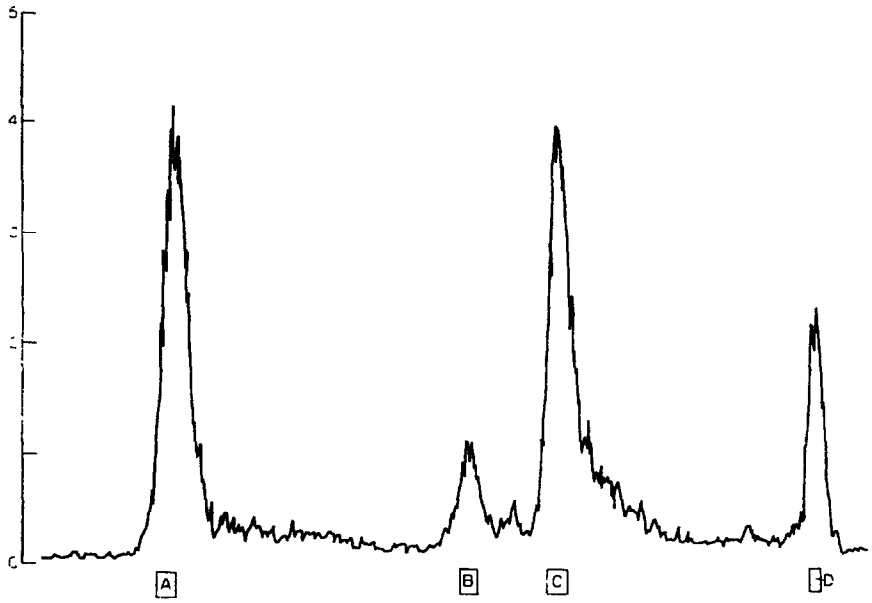

FIG. 1. RADIOCHROMATOGRAM OF LIPID EXTRACT.

Letters indicate positions of zones corresponding to standards: A, squalene; B, lanosterol; C, β-sitosterol; D, origin, A Silica Gel G plate was developed with dichloromethane:acetone (97·3) and scanned at 1·5 in./hr, using a time constant of 30 sec and a slit width of 2 mm.

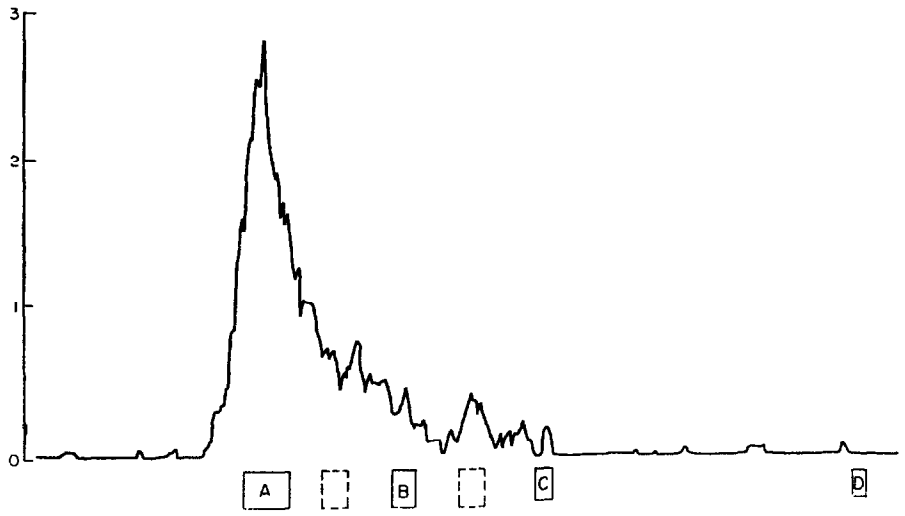

FIG. 2. RADIOCHROMATOGRAM OF STEROL ACETATES.

Letters indicate positions of zones corresponding to standards: A, β-sitosterol acetate; B, cholesterol acetate; C, stigmasterol acetate; D, origin. An Anasil B plate was developed continuously with hexane: ether (99:1) for 5 hr and scanned at 1·5 in./hr, using a time constant of 100 sec and a slit width of 3 mm.

To resolve these sterols continuous development is necessary, as shown in Fig. 2. This scan of a thin-layer chromatogram of an aliquot of the sterol acetates shows the presence of several components, of which β-sitosterol acetate is by far the major one, both in amount and radioactivity. Some material was also concentrated in the zones corresponding to reference samples of cholesterol acetate and stigmasterol acetate, which were co-chromatographed, but contained little radioactivity. β-Sitosterol acetate was finally isolated in chromatographically pure form by preparative TLC under the same conditions. An aliquot of this material was diluted with pure β-sitosterol acetate and recrystallized to constant specific activity. It is evident from Table 1 that the β-sitosterol isolated by chromatography was almost radiochemically pure.

TABLE 1. PURIFICATION OF β-SITOSTEROL ACETATE TO CONSTANT SPECIFIC ACTIVITY*

Compound	Solvent used for crystallization	Cpm/μM
β-Sitosterol acetate		173 ± 10
	Methanol	166 ± 9
	Ethanol	156 ± 8
	Acetone–methanol	160 ± 8
β-Sitosterol†	Methanol	164 ± 8
	Ethanol	160 ± 8

* 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 counter with micromil window mounted in a Model C110B sample changer, to the 0·9 level of confidence.

† By treatment of β-sitosterol acetate with lithium aluminum hydride.[8]

DISCUSSION

It is now established that the biosynthesis of β-sitosterol proceeds from mevalonic acid via squalene. Furthermore, the radioactive peak corresponding to lanosterol (Fig. 1) suggests that this trimethylsterol may be a precursor of β-sitosterol, in accord with its relationship to cholesterol in animals. The main point still to be clarified in the biosynthesis of C_{29} sterols is the stage at which carbon atoms 28 and 29, now known to originate from methionine,[5,6] are introduced.

EXPERIMENTAL

Methods

The thin-layer plates were 50 × 200 mm, with adsorbent layers 0·3 mm thick. Samples were applied as bands, 20 mm long, 15 mm from the bottom of the plate. The bands were centered and reference compounds were co-chromatographed as spots 10 mm from each side of the plate. All solvent systems used on Silica Gel G plates were saturated with water to prevent the formation of undesirable activity gradients in the absorbent layer[7] and were allowed to ascend

[5] M. CASTLE, G. BLONDIN and W. R. NES, *J. Amer. Chem. Soc.* **85**, 3306 (1963).
[6] S. BADER, L. GUGLIELMETTI and D. ARIGONI, *Proc. Chem. Soc.* 16 (1964).
[7] G. HESSE and G. ROSCHER, *Z. Anal. Chem.* **200** 3 (1964).
[8] R. D. BENNETT, E. HEFTMANN, W. H. PRESTON and J. R. HAUN, *Arch. Biochem. Biophys.* **103**, 74 (1963).

the layer to a line drawn 15 mm from the top. Chromatograms were scanned for radioactivity, using a Strip Scanner I apparatus with TLC holder.* For preparative thin-layer chromatograms 200 × 200 mm plates were used with layers 1 mm thick. Rhodamine 6G was used to locate the zones, which were scraped off the plate and eluted in a chromatographic column with acetone.

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

Materials

Squalene-^{14}C was prepared from mevalonic acid-2-^{14}C by the biosynthetic method of Capstack *et al.*[4] It was purified by chromatography on a silica gel column and then by preparative TLC on Silica Gel G, first with hexane: benzene (9:1) and subsequently by continuous development[9] with hexane for 2½ hr. The squalene thus obtained had a specific activity of $1{\cdot}1 \times 10^7$ cpm/mg and showed only a single radioactive peak, corresponding to an authentic sample of squalene, by TLC in the systems used above.

Procedure

Seedlings of *Pharbitis nil* Chois, strain Violet, were germinated as described by Zeevaart.[10] On the fifth day after germination 10 μl(180,000 cpm) of squalene-^{14}C in acetone solution was applied to one cotyledon of each of four seedlings. The cotyledons were then sprayed with a petroleum ether solution of Silicone DC-200 (Chromatospray†). The spray treatment had little or no injurious effect upon subsequent growth of the plants, provided the process was slow enough to allow rapid evaporation of the petroleum ether and only a thin film of silicone was deposited. The seedlings were kept in continuous artificial light for 64 hr. The cotyledons to which squalene had been applied were removed and the radioactivity remaining on their surfaces (35,000 cpm) washed off with dichloromethane. The cotyledons were then homogenized with 5 ml of 75% methanol in a tissue grinder with Teflon pestle.§ The homogenate was refluxed for 4 hr and the solution was separated by centrifuging. The residue was washed with three 3-ml portions of hot 75% methanol, which were combined with the solution above, and then it was extracted with one 5-ml and two 2-ml portions of boiling benzene. The methanolic extract was concentrated to 3 ml and this aqueous solution extracted with four 3-ml portions of hexane, which were combined and washed with 0·5 ml of water. The hexane extract was combined with the benzene extracts from above (lipid fraction)—total radioactivity, 489,000 cpm. The aqueous layer contained 128,000 cpm. Thus 617,000 cpm (86 per cent) of the radioactivity originally administered was found in the cotyledons.

TLC of an aliquot of the lipid fraction showed zones corresponding, in mobility and fluorescence on spraying with 50% sulfuric acid, to co-chromatographed standards of squalene, lanosterol, and β-sitosterol, respectively. Each of these zones was radioactive (Fig. 1). The whole fraction was then subjected to preparative TLC on Silica Gel G with dichloromethane: acetone (97:3). Elution of the zone corresponding to β-sitosterol gave 167,000 cpm. The β-sitosterol was freed of a small amount of slightly more polar radioactive material by preparative TLC on Silica Gel G with hexane: ether (3:7). It was then acetylated, using pyridine:

* Atomic Accessories, Inc., Valley Stream, N.Y.
† Research Specialties Co., Richmond, Calif.
§ Arthur H. Thomas Co., Philadelphia, Pa.

[9] R. D. BENNETT and E. HEFTMANN, *J. Chromatog.* **12**, 245 (1963).
[10] J. A. D. ZEEVAART, *Plant Physiol.* 37, 296 (1962).

acetic anhydride (1:1) for 16 hr at 25°, and an aliquot of the acetate chromatographed with hexane:dichloromethane (2:3) on Silica Gel G. This showed the presence of a small amount of radioactivity (about 5 per cent that of the β-sitosterol acetate) with a mobility similar to β-sitosterol, which was removed by preparative TLC in the same system. The β-sitosterol acetate (62,000 cpm) was freed of other sterol acetates (see Fig. 2) by preparative TLC on Anasil B* through continuous development with hexane:ether (99:1) for 5 hr. The chromatographically pure β-sitosterol acetate thus obtained (estimated at 60 μg by comparison with known amounts of standard by TLC) had a radioactivity of 38,000 cpm. An aliquot representing 10,000 cpm was diluted with 20·9 mg of pure β-sitosterol acetate and recrystallized to constant specific activity as shown in Table 1.

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* Analabs, Inc., Hamden, Conn.