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STEROLS OF *EUPHORBIA PEPLUS*: THE FATE OF 28-ISOFUCOSTEROL IN PHYTOSTEROL BIOSYNTHESIS

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Abstract—Cholesterol, campesterol, stigmasterol, β-sitosterol, 28-isofucosterol and Δ^7-isofucostenol have been identified by thin-layer and gas chromatography of the sterol fraction of *Euphorbia peplus*. Confirmation of the identity of 28-isofucosterol was obtained from mass spectral data. A time-course study of incorporation of label from mevalonic acid-2-^{14}C into this sterol mixture supports the view that 28-isofucosterol is the precursor of β-sitosterol.

INTRODUCTION

THE INTRODUCTION of the C_2 unit at C-24 of phytosterols is known to occur by two successive transmethylations from methionine.[1-3] Several biosynthetic schemes have been proposed for the formation of 24-ethylsterols (Ic and d, Fig. 1) from a 24-methylenesterol, i.e. a sterol with side-chain (a). The sterol side-chain bearing the carbonium ion (b) can lead to 24-ethylsterols in several ways, some of which are outlined in Fig. 1 (cf. Castle *et al.*,[1] Lederer,[4] Clayton,[5] Goad *et al.*,[6,7] Patterson and Karlander[10]). Goad *et al.*[6,8,9] have shown a 24-ethylidenesterol, i.e. a sterol with side-chain (e) or (f) to be an intermediate in β-sitosterol (Id) synthesis in higher plants and in the phytoflagellate *Ochromonas malhamensis*.[7] However, Lenfant *et al.*[11] found that stigmast-22-en-3-β-ol in the slime mould *Dictyostelium discoideum* contained five deuterium atoms in the 24-ethyl group when the mould was presented with Me-$^2H_3$ methionine. Their data rule against the involvement of a 24-ethylidenesterol intermediate in the formation of this 24-ethylsterol. Patterson and Karlander[10] have shown the 24-ethylidenesterol, fucosterol (Ie) to be converted *in vivo* to clionasterol, 24-β-ethylcholesterol† (Ic) in *Chlorella ellipsoidea*. In the course of our work, van Aller *et al.*[12] reported the *in vivo* conversion of 28-isofucosterol (If) to β-sitosterol (Id) in *Pinus pinea* seedlings. The work described in this paper supports the findings of van Aller *et al.*,[12] i.e. that the

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† The configurations at C-24 of the sterols discussed in this paper are taken from *Chemistry of the Steroids*, by C. W. SHOPPEE, Butterworths, London (1964).

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24-ethylidenesterol, 28-isofucosterol, is metabolized to the 24-α-ethylcholesterol, β-sitosterol.

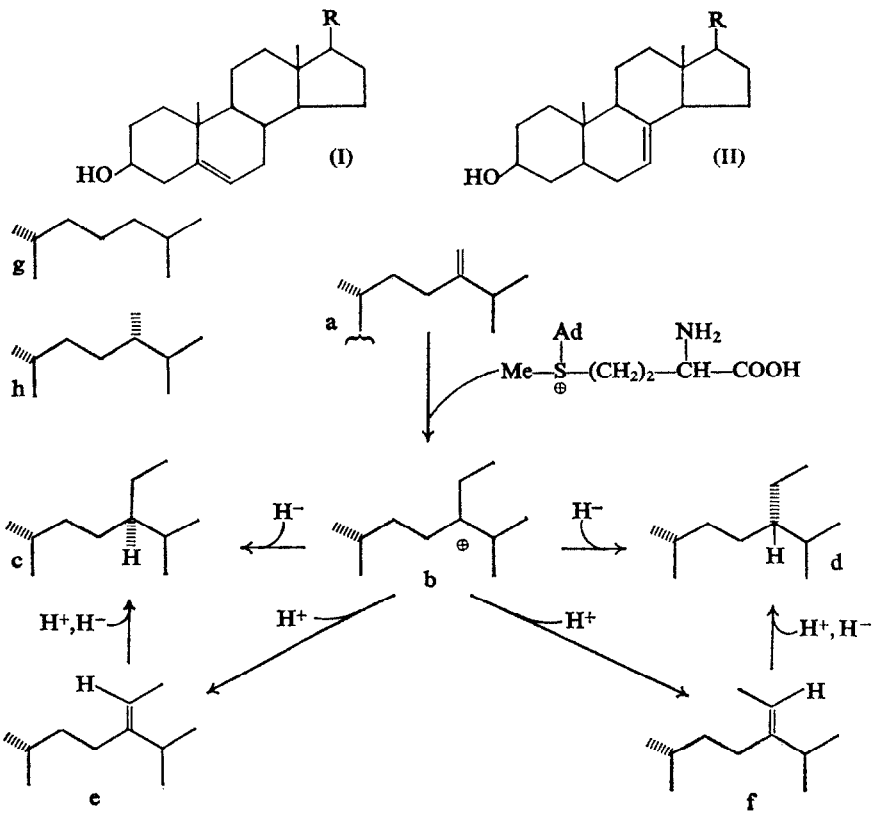

FIG. 1.

RESULTS AND DISCUSSION

The non-saponifiable lipid of *Euphorbia peplus* was isolated from an acetone extract obtained from the aerial parts of freshly cut plants. The sterols were isolated from the non-saponifiable fraction by column chromatography on alumina. Fractions containing sterol, as evidenced by TLC of individual fractions on silica gel G, were combined and acetylated. TLC of the acetates on silica gel plates impregnated with $AgNO_3$ revealed the presence of two spots by spraying with 50% H_2SO_4 and heating at 100° for 5 min. The R_fs coincided with the acetate of β-sitosterol for the faster-moving material and either the acetate of fucosterol or 28-isofucosterol for the other component. Gas–liquid chromatography (GLC) of these acetates on a glass column packed with XE-60 on silanized Anakrom A showed the presence of cholesterol (I; R=g), campesterol (I; R=h), stigmasterol (I; $R=\Delta^{22}$-d), β-sitosterol (I; R=d), fucosterols (I; R=1e and/or 1f) and two slower-moving components. A second glass column packed with HiEFF 8BP on Gas Chrom P pretreated with PVP[13] indicates the acetates to be cholesterol, campesterol, stigmasterol, β-sitosterol, fucosterols

[13] B. A. KNIGHTS, *Phytochem.* **4**, 857 (1965).

and a slower-moving component. We were not able to distinguish which fucosterol was present by the use of these columns.

The mixture of sterol acetates was then subjected to column chromatography on silicic acid treated with $AgNO_3$.[14] Two fractions were clearly separated on this column. TLC on chromagram sheets impregnated with $AgNO_3$ showed the rapidly eluted fraction from the column to correspond to β-sitosterol acetate and the second to 28-isofucosterol acetate (acetate of I; R=f). Chromatography on these sheets cleanly resolved the two fucosterol acetates isomeric at C-28. Confirmation of the presence of 28-isofucosterol acetate was obtained by GLC of this sample on a column of 3% OV-17 on Gas Chrom Q kindly run by Dr. B. A. Knights. In addition to confirming the presence of 28-isofucosterol acetate, a very small amount of a second 24-ethylidenesterol acetate was identified as Δ^7-isofucostenol acetate (acetate of II; R=f). Identification of our sterol with 28-isofucosterol was also obtained by comparison of the mass spectra of authentic fucosterol acetate and our sample. Though the mass spectra of the two acetates were remarkably similar the relative intensities of three peaks, *m/e* 55, 296 and 394 were different for the two compounds.*

The finding of 28-isofucosterol and β-sitosterol in this species suggested the possibility that a precursor–product relationship might exist between the two. A time-course study of the incorporation of ^{14}C from DL-mevalonate-2-^{14}C into the sterol fraction of *E. peplus* cuttings and an analysis of this fraction on chromagram sheets impregnated with $AgNO_3$ was carried out. Each of eight cuttings was placed in a vial, each of which contained DL-mevalonate-2-^{14}C (10^7 dpm) as the sodium salt. The cuttings were exposed to a light intensity of 55,000 lux for 16 hr a day. After 5 hr of light, one pair of cuttings was removed and the non-saponifiable lipid isolated. The remaining six cuttings were transferred to vials containing Hoagland's number 2 nutrient solution.[16] Pairs of cuttings were removed after an additional 24, 48 and 96 hr had elapsed. The non-saponifiable lipid fraction was isolated from each pair in the same way.

TLC of aliquots of non-saponifiable lipids corresponding to each time-interval and containing the same amount of radioactivity were run on an Eastman chromagram sheet. A radioautograph of the developed chromatogram is shown in Fig. 2. The dotted areas show the positions of three standards run on the same sheet: β-sitosterol, β-amyrin and squalene in order of increasing mobility. The radioactive band between the sterol and cyclic triterpene represents the 4-α-methylsterol fraction. It is clear that the hydrocarbon and cyclic triterpene are rapidly synthesized while the sterol fraction does not appear to reach a maximum until after 29 hr have elapsed from the start of the experiment. The incorporation of DL-mevalonate-2-^{14}C into the major fractions of the non-saponifiable lipid of *E. peplus* for these four time-intervals was determined by cutting each freshly run radiochromatogram into lateral strips (0·5 cm wide), from origin to solvent front, and counting them in scintillation vials. The percentage incorporations into the sterols based on the DL-mevalonate-2-^{14}C absorbed by duplicate cuttings were: 0·5, 3·0, 6·6 and 4·7 for the 5, 29, 53 and 101-hr intervals respectively.

Aliquots of the non-saponifiable lipids for each time-interval were submitted to preparative TLC on silica gel G. The bands corresponding in mobility to β-sitosterol were scraped

* We are most grateful to Drs. B. A. Knights and C. J. W. Brooks[15] for informing us of this difference for these two compounds. It is noteworthy that they propose structures If for fucosterol and Ie for 28-isofucosterol.

[14] B. DeVries, *J. Am. Oil Chem.* **40**, 184 (1963).
[15] B. A. Knights and C. J. W. Brooks, *Phytochem.* **8**, 463 (1969).
[16] D. R. Hoagland and D. I. Arnon, *Calif. Univ. Agr. Expt. Sta. Circ.* 347 (1950).

from the plates and the sterols eluted from the adsorbent. The sterols were converted to their acetates and then subjected to TLC on Eastman chromagram sheets impregnated with $AgNO_3$. A quantitative distribution of radioactivity on the sheet for the 5-hr interval is shown in Fig. 3. The standards run on the same sheet clearly show that the bulk of the radioactivity has an R_f coincident with 28-isofucosterol acetate for the 5-hr time-interval. The radiochromatogram also shows that no fucosterol is biosynthesized. This observation is in accord with the absence of this sterol in the earlier TLC and GLC studies. Figure 4 shows the data expressing the radioactivity associated with the zones corresponding to the acetates of 24-methylenecholesterol, (I; $R=a$) 28-isofucosterol and β-sitosterol expressed as a percentage of the total radioactivity on the plate. The difference between 100 per cent and the sum of the radioactivity associated with the three areas shown for each time-interval is accountable in radioactivity remaining at the origin. The Δ^7-isofucostenol identified by GLC would be expected[17] to have an R_f less than that for 28-isofucosterol but its precise

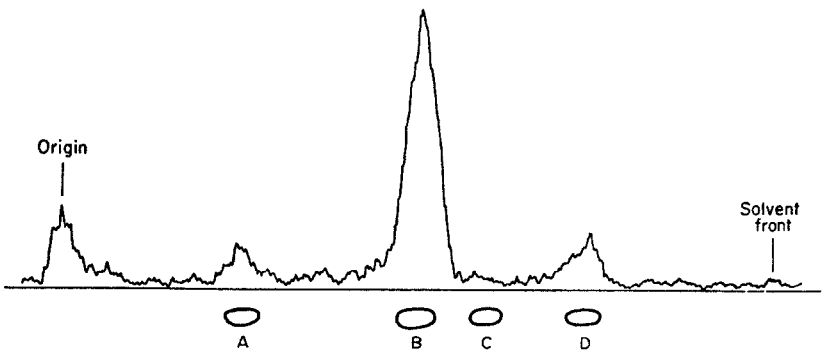

FIG. 3. RADIOCHROMATOGRAPHIC SCAN OF THE STEROL FRACTION (APPROX. 20,000 dpm), CONVERTED TO THE ACETATE, FROM *E. peplus* CUTTINGS ALLOWED TO TAKE UP DL-MEVALONATE-2-^{14}C FOR 5 hr.

The TLC was carried out on an Eastman chromagram sheet impregnated with silver nitrate and run in a benzene–hexane (25:75, v/v) solvent system. The standards: A, 24-methylene cholesterol; B, 28-isofucosterol; C, fucosterol; and D, β-sitosterol; as their acetates were run alongside the radioactive sterol acetate. Visualization of the spots was with rhodamine 6G followed by observation under u.v. light. Scanning was made with a Packard Model 7201 Radiochromatogram Scanner: slit width 2·5 cm, speed 0·2 cm/min.

location in our system is unknown. That it may co-chromatograph with 24-methylenecholesterol is a possibility. Consequently, the radioactivity associated with the 24-methylenecholesterol zone may be due to Δ^7-isofucostenol or 24-methylenecholesterol or both. 24-Methylenecholesterol has been shown to be a precursor of campesterol,[12] a sterol already identified in *E. peplus* while Δ^7-isofucosterol would be expected[8,18] to be a precursor of 28-isofucosterol.

It is evident that during the early stage of sterol synthesis in *E. peplus*, incorporation of ^{14}C into the 24-ethylidenesterol occurs preferentially. The succeeding time-intervals show a considerable turnover of this material and a corresponding increase in the radioactivity content of the 24-ethyl sterol fraction. It should be pointed out that in this TLC system the acetates of β-sitosterol, stigmasterol and campesterol co-chromatograph. Consequently the relationship between 28-isofucosterol and β-sitosterol shown in Fig. 4 probably more

[17] H. E. VROMAN and C. F. COHEN, *J. Lipid Res.* **8**, 150 (1967).
[18] R. B. CLAYTON, *Quart. Rev.* **19**, 201 (1965).

accurately reflects the relationship between the 24-ethylidenesterol and the three sterols mentioned.

In the course of this work, van Aller *et al.*[12] demonstrated the conversion of 28-isofucosterol to β-sitosterol in *Pinus pinea* seedlings. Taking into account the limitations of our own experiments our data agree with those of van Aller, i.e. 28-isofucosterol is metabolized to the 24-ethyl sterol, β-sitosterol.

It is noteworthy that the presence of Δ^7-isofucostenol in this species indicates that the introduction of the C-29 carbon atom has occurred before the Δ^7 double bond has been transformed to the Δ^5 position. van Aller *et al.*[12] have found *in vivo* synthesis of 28-iso-

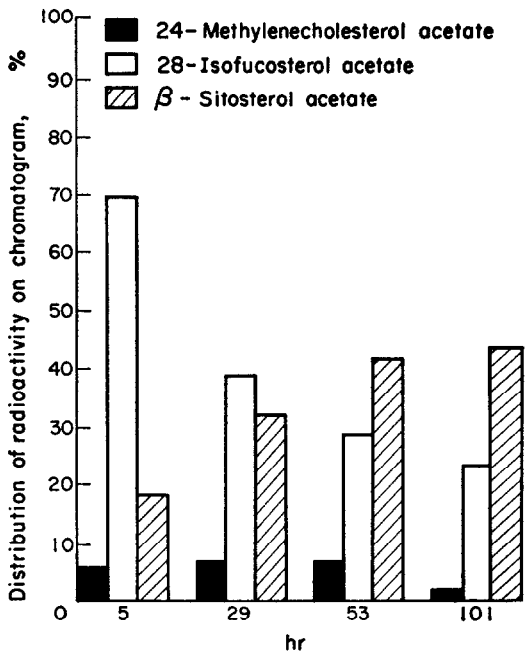

FIG. 4. THIN-LAYER CHROMATOGRAMS OF THE RADIOACTIVE STEROL ACETATE FRACTIONS CORRESPONDING TO EACH TIME INTERVAL RUN ON SILVER NITRATE IMPREGNATED CHROMAGRAM SHEETS.

The chromatoplates were cut into lateral strips, 1 cm wide, and the strips counted in a liquid scintillation spectrophotometer (Packard Tri-Carb, Model 574). Radioactivity associated with the three reference compounds is expressed as a percentage of the radioactivity on the chromatogram.

fucosterol from 24-methylenecholesterol in *P. pinea* seedlings. Clearly, alternate pathways can function for the introduction of the C-29 carbon atom in phytosterol biosynthesis. This lack of specificity in the steroid nucleus for introduction of the alkylidene group and its reduction has been discussed by Goad and Goodwin.[8,9]

The stereochemistry of the reduction at the $\Delta^{24(28)}$ double bond of sterols (e.g. I; R=e and f) is also worthy of mention. Our findings and those of van Aller[12] show 28-isofucosterol to be converted to β-sitosterol (If to Id) in two species of higher plant. Karlander and Patterson[10] have shown the C-28 geometric isomer, fucosterol to undergo reduction to clionasterol (Ie to Ic) in an alga. Thus, the substrates which are geometric isomers about the $\Delta^{24(28)}$ double bond lead to products which are epimerically related at C-24. Gersenghorn *et al.*[23] have speculated that sterols with a 24β-alkyl substituent may be characteristic of

green algae. In contrast, the 24α-sterols are the common sterols of higher plants. Thus a reductase enzyme specific for the step e to c (Fig. 1) may be characteristic of lower plants and another reductase specific for f to d may be characteristic of higher plants. The sterol specificity of the 24-alkylidene reductases in plants is evidently an area offering considerable challenge. Ultimate solutions to the problems must perhaps await the isolation of the reductases themselves.

EXPERIMENTAL

Isolation of Euphorbia peplus *Sterols*

Euphorbia peplus plants were grown from seed* in a greenhouse. Plants (333 g) cut at soil level were macerated in a Waring blendor with acetone and the shredded material extracted with hot acetone in a Soxhlet. The evaporated extract was saponified with 500 ml of 15% (w/v) of KOH in ethanol–water (95–5) for 3 hr. The volume of the mixture was reduced on a rotary evaporator, water added and the non-saponifiable material thoroughly extracted with ether. Evaporation of the dried (anhyd. Na_2SO_4) extract gave a yellow-brown semi-solid (2·4 g) which was poorly soluble in acetone or ether. Large quantities of a waxy solid deposited from a cooled, ether solution of the non-saponifiable lipid. It had m.p. 77–78° and is no doubt ceryl alcohol.[19, 20]

The sterols were isolated by column chromatography of the hexane-soluble material (2·2 g) of the non-saponifiable lipid on alumina, activity II (145 g). Fractions were eluted by step-wise gradient elution using hexane followed by increasing amounts of ether in hexane then ethanol in ether. Contents of the eluted fractions (15 ml) were analysed by TLC on 0·25 mm plates of silica gel G. Three solvent systems: 5% benzene–hexane; benzene, and 5% ethanol in benzene were used to scan the fractions from the least polar to the most polar. In the latter system the sterols had R_f 0·65 and they were located in those fractions eluted from the column with 2–5% ethanol in ether. The spots were visualized with 50% H_2SO_4 followed by heating at 100° for 5 min. The sterol (112 mg) was acetylated with Ac_2O–pyridine.

A small amount of the acetylated sterol was subjected to TLC alongside reference samples of β-sitosterol acetate on silica gel G plates impregnated with $AgNO_3$. Four 20 × 20 cm plates were prepared by spreading 0·25-mm thick layers of adsorbent composed of silica gel G (40 g) slurried with $AgNO_3$ (3·2 g) in water (90 ml). The plates were air-dried in the dark and activated at 100° for 25 min before use. The sterol acetates were chromatographed in benzene–hexane (1 : 1, v/v) and the spots visualized with 50% H_2SO_4 followed by heating at 100° for 5 min. Two spots appeared for the *E. peplus* sample, one with R_f 0·48, corresponding to β-sitosterol acetate and the other with R_f 0·33.

Gas–Liquid Chromatography of the Sterol Acetates

A Beckman GC 4 gas chromatograph fitted with flame ionization detectors was used. Two stationary phases were employed, 1% XE60 on 100–120 mesh silanized Anakrom A packed in glass tubing 2·44 m long and 0·65 cm in dia. The support had been previously water-washed to neutrality and dried before silanizing. The other phase was 1% HiEFF 8BP on 100–120 mesh Gas Chrom P pre-coated with PVP.[13] This support was packed in 2·13 m by 0·65 cm diameter glass tubing. Both packings were prepared by the filtration technique.[21] The XE60 column was run at 230° with a helium flow of 20 ml/min. The *E. peplus* sterol acetate mixture revealed peaks with R_t relative to cholestane of 4·23, 5·57, 6·17, 7·06, 7·60, 9·13 and 10·27 corresponding to the acetates of cholesterol, campesterol, stigmasterol, β-sitosterol, 28-isofucosterol and two unknowns, respectively. Cholestane had R_t of 3·5 min on this column. The peak attributed to 28-isofucosterol acetate appeared as a shoulder on the β-sitosterol acetate peak and a distinction between it and fucosterol acetate could not be made. The HiEFF 8BP column was run at 232° with a helium flow of 20 ml/min. The mixture of sterol acetates had retention times relative to cholestane of 4·43, 5·85, 6·88, 7·54, 8·09 and 9·75 corresponding to the acetates of cholesterol, campesterol, stigmasterol, β-sitosterol, 28-isofucosterol and an unknown, respectively. Cholestane had R_t of 3·3 min on this column. The peak coinciding with 28-isofucosterol acetate again could not be distinguished from fucosterol acetate.

Isolation and Identification of the Unsaturated Sterol

The sterol acetate mixture (105 mg) in hexane was submitted to column chromatography on a mixture of Celite (5 g) and silicic acid (10 g) impregnated with $AgNO_3$. The silicic acid was prepared by treating 20 g of

* Obtained from Harry E. Saier, Diamondale, Michigan, U.S.A.

[19] E. HUPPERT, H. SWIATKOWSKI and J. ZELLNER, *Monatsh.* **48**, 491 (1927)
[20] A. N. STARRAT, *Phytochem.* **5**, 1341 (1965).
[21] E. C. HORNING, W. J. A. VANDEN HEUVEL and B. G. CREECH, *Meth. Biochem. Anal.* **11**, 69 (1963).

silicic acid (Mallinckrodt, 100 mesh) with a solution of water (40 ml) containing $AgNO_3$ (8 g) according to the procedure of DeVries.[14] The acetates were eluted first with increasing amounts of benzene in hexane then ether in hexane. Fractions eluted with 10% ether in hexane gave the unsaturated sterol acetate (7 mg) which, on crystallization from methanol–acetone, gave colorless prisms, m.p. 130·5–131·5° (28-isofucosterol acetate, reported[22] m.p. 130·5–131°).

TLC of the *E. peplus* unsaturated sterol acetate on an Eastman chromagram sheet of silicic acid impregnated with $AgNO_3$ and run in benzene–hexane (1:3, v/v) had R_f 0·59 corresponding to 28-isofucosterol acetate; fucosterol acetate had R_f 0·51. The sheet was impregnated by passing it through a trough containing a solution of $AgNO_3$ (1·58 g) in ethanol–water (1:1, 45 ml). The plates were air dried before use.

A mass spectral analysis* of the sample gave a molecular ion, *m/e* 396, in accord with the loss of acetic acid from a molecule with mol. wt 466. The spectrum was very similar to that of fucosterol acetate but the relative intensities of the peaks at *m/e* 394, 296 and 55 were in accord with the C-28 geometric isomer, 28-isofucosterol acetate.[15]

Incorporation of Radioactivity

Each of eight cuttings of *E. peplus*, approximately 7 cm high, were placed in vials, each containing DL-mevalonate-2-^{14}C (1·64 mc/mmole, 10^7 dpm in 0·1 ml water) as the sodium salt. The cuttings were placed in a growth chamber with a light intensity of 55,000 lux at shelf level and were in the light for 16 hr a day. During the first 5 hr in the light the radioactive solution was taken up and was replaced with water (0·1 ml) several times. One pair of cuttings was removed at this stage and placed in a beaker of acetone. The remaining six were transferred to vials containing Hoagland's[16] number 2 nutrient solution. Measurement of residual radioactivity after the 5-hr uptake revealed $<0{\cdot}1\%$ remained. Pairs of cuttings were removed and placed in acetone 24, 48 and 96 hr after the first pair were taken.

Each duplicate was repeatedly extracted with hot methanol in an homogenizer equipped with a teflon plunger until the plant residue was colorless. The individual evaporated extracts were dissolved in 95% ethanol (5 ml) and saponified by addition of KOH (2·5 g) in water (20 ml) and heated on a steam bath for 2 hr. The volumes were reduced and each mixture thoroughly extracted with ether. The extracts were washed, dried and evaporated to give the non-saponifiable lipids. TLC of the non-saponifiable lipids for radioautography was made on an Eastman chromagram sheet of silica gel 100 μ thick. Aliquots (approx. 32,000 dpm) of each extract and a reference mixture of β-sitosterol, β-amyrin and squalene were applied to the sheet. The chromatography was run in ethyl acetate–heptane (12:88, v/v) and then left exposed to an X-ray film for 1 week before development. The reference compounds were visualized by spraying with rhodamine 6G and observed under u.v. light. They had R_f 0·33, β-sitosterol; 0·55, β-amyrin and 1·00 squalene. Quantitation of the incorporation of mevalonate-2-^{14}C into the sterol fraction was obtained by taking chromatograms run similar to the one used for the radioautography and cutting them into lateral strips, 0·5 cm wide. Each strip was placed in a scintillation vial containing 10 ml of a mixture of 2,5-diphenyloxazole (4 g) and *p*-bis-[2-(5-phenyloxazolyl)]-benzene (30 mg) in toluene (1 l.). Radioactivity was determined in a Packard Tri-Carb, Model 574 liquid scintillation spectrophotometer. Radioactivity associated with the sterol was then expressed as a percentage of the radioactivity measured on the chromatogram. This was taken to represent the percentage of radioactive sterol in the non-saponifiable lipid thereby permitting the incorporation from mevalonate-2-^{14}C to be determined.

The radioactive sterol fractions for each time interval were isolated by preparative TLC of aliquots of the non-saponifiable lipids on silica gel G (0·25 mm thick). The chromatoplates were run in CH_2Cl_2–acetone (99:1, v/v) using β-sitosterol as a reference. The bands associated with the reference compound, visualized with a $KMnO_4$ spray, were scraped from the plates and the sterol eluted with ether–ethanol (5:1). The evaporated extracts were acetylated as before and run on the Eastman chromatogram sheets impregnated with $AgNO_3$ using the acetates of 24-methylene cholesterol, 28-isofucosterol, fucosterol and β-sitosterol as standards. The reference compounds were visualized with rhodamine 6G. Distribution of radioactivity between the sterol acetates was made as for the non-saponifiable lipids but lateral strips, 1·0 cm wide, were counted in this case. The resolution of 28-isofucosterol-^{14}C acetate from the fucosterol acetate standard was made clear by scanning the chromatogram of the 5-hr sample (20,000 dpm) with a Packard Model 7201 Radiochromatogram Scanner using a slit width of 2·5 cm and scanning speed of 0·2 cm/min.

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* Analyses performed by Morgan Schaffer Corporation.

[22] J. P. Dusza, *J. Org. Chem.* **25**, 93 (1960).

[23] M. C. Gersenghorn, A. R. H. Smith, G. Goulston, L. J. Goad, T. W. Goodwin and T. H. Haines, *Biochem.* 7, 1698 (1968).