Biochem. J. (1969) **114**, 885
Printed in Great Britain

Incorporation of [2-^{14}C,(5*R*)-5-$^{3}H_1$]Mevalonic Acid into Cholesterol by a Rat Liver Homogenate and into β-Sitosterol and 28-Isofucosterol by *Larix decidua* Leaves

By L. J. GOAD, G. F. GIBBONS, LORETTA M. BOLGER, H. H. REES and T. W. GOODWIN
Department of Biochemistry, University of Liverpool, L69 3BX

(*Received 2 June* 1969)

1. Incubation of a rat liver homogenate with 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]mevalonic acid gave cholesterol with $^{3}H/^{14}C$ atomic ratio 6:5. 2. Conversion of the labelled cholesterol into 3β-acetoxy-6-nitrocholest-5-ene or cholest-4-ene-3,6-dione resulted in the loss of one tritium atom from C-6. 3. These results show that during cholesterol biosynthesis the 6α-hydrogen atom of a precursor sterol is eliminated during formation of the C-5–C-6 double bond. 4. Incorporation of 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]mevalonic acid into the sterols of larch (*Larix decidua*) leaves gave labelled cycloartenol and β-sitosterol with $^{3}H/^{14}C$ atomic ratios 6:6 and 6:5 respectively. 5. One tritium atom was lost from C-6 on conversion of the labelled β-sitosterol into either 3β-acetoxy-6-nitrostigmast-5-ene or stigmast-4-ene-3,6-dione, demonstrating that formation of the C-5–C-6 double bond of phytosterols also involves the elimination of the 6α-hydrogen atom of a precursor sterol. 6. The 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]mevalonic acid was also incorporated by larch (*L. decidua*) leaves into a sterol that co-chromatographed with 28-isofucosterol. Confirmation that the radioactivity was associated with 28-isofucosterol was obtained by co-crystallization with carrier 28-isofucosterol and ozonolysis of the acetate to give radioactively labelled 24-oxocholesteryl acetate. 7. The significance of these results to phytosterol biosynthesis is discussed.

The elegant investigations of Cornforth, Popják and their co-workers have strikingly demonstrated the value to biosynthetic studies of stereospecifically labelled MVA* (Popják & Cornforth, 1966, and references cited therein). In our department, 3*R*-[5*R*-5-$^{3}H_1$]MVA has been synthesized and used in studies on carotenoid formation (Williams, Britton, Charlton & Goodwin, 1967). The availability of this substrate has now facilitated our investigations of various problems in animal and plant sterol biosynthesis. Recent reports (Paliokas & Schroepfer, 1968; Akhter & Marsh, 1967; Dewhurst & Akhtar, 1967) have shown that during cholesterol biosynthesis by liver homogenates a *cis*-elimination of the 5α- and 6α-hydrogen atoms occurs during introduction of the C-5–C-6 double bond. We now report the incorporation of 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA (I, Scheme 1) into cholesterol to confirm, by a third independent method, the elimination of the 6α-hydrogen atom from a cholesterol precursor. Also, by using this substrate we show that a similar mechanism is operative in C-5–C-6 double-bond formation in sterols biosynthesized by a higher plant.

* Abbreviation: MVA, mevalonic acid.

EXPERIMENTAL

Nomenclature. Cholesterol is cholest-5-en-3β-ol; cholesteryl acetate, 3β-acetoxy-cholest-5-ene; β-sitosterol, stigmast-5-en-3β-ol; 28-isofucosterol, stigmasta-5,*Z*-24(28)-dien-3β-ol (Frost & Ward, 1968; Blackwood, Gladys, Loening, Petrara & Rush, 1968; Bates, Brewer, Knights & Rowe, 1968); fucosterol, stigmasta-5,*E*-24(28)-dien-3β-ol; 24-oxocholesteryl acetate, 3β-acetoxycholest-5-en-24-one; campesterol, 24*R*-methylcholest-5-en-3β-ol; lanosterol, lanosta-8,24-dien-3β-ol; cycloartenol, 9,19-cyclo-9β-lanost-24-en-3β-ol.

Methods. Methods of saponification, extraction and chromatography were generally as described previously (Goad & Goodwin, 1966, 1967; Williams, Goad & Goodwin, 1967). The i.r. spectra were determined in KBr by using a Perkin–Elmer Infracord spectrophotometer. Melting points were determined on a Kofler block. Radioactivity determinations were performed in a Beckman LS200B liquid-scintillation counter, with the external-standard method to correct for quenching. Counting efficiencies were 58% for ^{14}C and 47% for ^{3}H.

Materials. We thank Dr G. Britton for a generous gift of [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA, synthesized as described by Williams *et al.* 1967), which had specific radioactivity approx. 32 μc of ^{3}H and 3·2 μc of ^{14}C/μmole. Fucosterol was isolated from the marine brown alga *Fucus spiralis*, and 28-isofucosterol was isolated from *Enteromorpha intestinales*

(Gibbons, Goad & Goodwin, 1968). Both compounds were characterised by melting point, and i.r., n.m.r. and mass spectrometry.

Incorporation of 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]*MVA* (*I*, *Scheme* 1) *into cholesterol* (*V*). A rat liver homogenate was prepared by the method of Bucher & McGarrahan (1956) and a portion (8·0ml.) incubated with NADH (16·0mg.), NADPH (4·0mg.), ATP (8·0mg.), GSH (15·0mg.) and 3*R*-[2-^{14}C,-(5*R*)-5-$^{3}H_1$]MVA (I) (2·0μc of ^{14}C) (total volume 10·0ml.) for 3hr. at 37°. The unsaponifiable lipids were then extracted in the usual manner and separated by t.l.c. on silica gel into three fractions containing squalene, lanosterol and cholesterol. The squalene fraction was further purified after addition of carrier squalene (5mg.) by t.l.c. on silica gel developed with hexane. The squalene had $^{3}H/^{14}C$ ratio 9·65:1. The lanosterol fraction after addition of carrier lanosterol (2mg.) was rechromatographed by t.l.c. on silica gel, and contained $9·02\times10^{4}$c.p.m. of ^{14}C, $^{3}H/^{14}C$ ratio 9·25:1. A portion of the fraction containing radioactive cholesterol and companion 4-demethyl sterols was added to 5mg. of carrier cholesterol and the mixture treated with pyridine–acetic anhydride to give the acetates ($9·11\times10^{4}$c.p.m. of ^{14}C). The cholesteryl acetate ($4·77\times10^{4}$c.p.m. of ^{14}C, $^{3}H/^{14}C$ ratio 11·29:1) obtained from the above mixture by t.l.c. on 10%-$AgNO_3$-impregnated silica gel developed with benzene–hexane (2:3, v/v) was added to 200mg. of carrier cholesteryl acetate and crystallized from methanol (m.p. 115°; 185c.p.m. of ^{14}C/mg., $^{3}H/^{14}C$ ratio 11·23:1).

Conversion of [^{3}H,^{14}C]*cholesterol* (*V*, *Scheme* 1) *into* [^{3}H,^{14}C]*cholest-4-ene-3,6-dione* (*VII*). A portion of the [^{3}H, ^{14}C]cholesteryl acetate was saponified with ethanolic 5% KOH to give cholesterol (42mg.), to which was added a further 60mg. of carrier cholesterol. The cholesterol (72c.p.m. of ^{14}C/mg.; $^{3}H/^{14}C$ ratio 11·18:1) was dissolved in 40ml. of diethyl ether, and chromic acid solution (0·1g. of $K_2Cr_2O_7$ in 6·0ml. of water and 1·0ml. of conc. H_2SO_4) was added, with stirring, over a period of 1hr. Stirring was then continued for a further 1hr. at room temperature followed by dilution with water and extraction in the usual manner. Cholest-4-ene-3,6-dione (20mg.) was obtained from the reaction products by t.l.c. on silica gel developed with chloroform followed by two crystallizations from methanol. It had m.p. 121—122° (literature m.p. 125°; Fieser & Fieser, 1959), u.v. $\lambda_{max.}$ 250nm.; i.r. $\nu_{max.}^{KBr}$ 1680, 1600 and 863cm.$^{-1}$, and 54c.p.m. of ^{14}C/mg., $^{3}H/^{14}C$ ratio 9·44:1.

Conversion of [^{3}H,^{14}C]*cholesteryl acetate into* 3*β-acetoxy-6-nitro*[^{3}H,^{14}C]*cholest-5-ene* (*IX*). Labelled cholesteryl acetate (40mg., $^{3}H/^{14}C$ ratio 11·23:1) was mixed with unlabelled cholesteryl acetate (160mg.) and suspended in conc. HNO_3 (6·0ml.) with stirring. $NaNO_2$ (3×50mg.) was then added at 0, 5 and 10min. and the mixture stirred for a further 12min. (Dodson & Riegel, 1948; Akhtar & Marsh, 1967). The resulting 3β-acetoxy-6-nitrocholest-5-ene was isolated in the usual manner and crystallized slowly from methanol at 0° to give white crystals (135mg.), m.p. 102° (lit. m.p. 102°; Mauthner & Suida, 1903), i.r. $\nu_{max.}^{KBr}$ 1750 and 1520cm.$^{-1}$, and 33c.p.m. of ^{14}C/mg., $^{3}H/^{14}C$ ratio 9·34:1.

Incorporation of 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]*MVA into phytosterols*. Twigs of larch (*Larix decidua*) were collected in the spring (1968) as the leaves were starting to appear. The twigs were placed in water and kept for a few days at 20° in the light to promote further leaf development. Fresh leaves (1·0cm. long, 2·7g.) were then chopped into small pieces (1–2mm.), moistened with water (2·0ml.) containing 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA (2·0μc of ^{14}C) and incubated at 18° for 28hr. with illumination. The incubation was terminated by the addition of hot ethanol and the non-saponifiable lipid extracted in the usual manner (45·6mg., $8·9\times10^{5}$c.p.m. of ^{14}C). Preparative t.l.c. on silica gel gave fractions containing squalene ($8·54\times10^{4}$c.p.m. of ^{14}C, $^{3}H/^{14}C$ ratio 9·02:1), 4,4-dimethyl sterols ($3·26\times10^{4}$c.p.m. of ^{14}C), and 4-demethyl sterols ($1·08\times10^{4}$c.p.m. of ^{14}C, $^{3}H/^{14}C$ ratio 10·90:1). Carrier squalene (5mg.) was added to the radioactive squalene fraction and the squalene purified by t.l.c. on silica gel developed with hexane ($6·16\times10^{4}$c.p.m. of ^{14}C, $^{3}H/^{14}C$ ratio 9·14:1). Cycloartenol, 24-methylenecycloartanol, lanosterol and 24,25-dihydrolanosterol were added to the labelled 4,4-dimethyl sterol fraction and a portion of the mixture was subjected to preparative g.l.c. on 3% XE-60. The sterol peaks were trapped as they emerged from the column and assayed for radioactivity (Fig. 1). Most of the radioactivity ($^{3}H/^{14}C$ ratio 9·26:1) was associated with the cycloartenol mass peak. However, significant radioactivity ($^{3}H/^{14}C$ ratio 9·00:1) was also associated with the 24-methylenecycloartanol mass peak. By comparison, only very little radioactivity was trapped in the lanosterol peak. The 4-demethyl sterol fraction (5·8mg.) was analysed by g.l.c. on 1% QF-1 and shown to contain β-sitosterol as the major sterol component (94%) with a small amount of campesterol (2%), as observed in a previous analysis of larch leaf sterols (Goad & Goodwin, 1967). Preparative t.l.c. of this material on 10%-$AgNO_3$-impregnated silica gel developed with chloroform gave material that co-chromatographed with β-sitosterol (3·0mg.; $3·92\times10^{4}$c.p.m. of ^{14}C, $^{3}H/^{14}C$ ratio 10·97:1) and that was identified as β-sitosterol by g.l.c. In addition, the band co-chromatographing with a 28-isofucosterol marker on this system was also eluted and, although negligible in weight was found to be radioactive ($3·18\times10^{4}$c.p.m. of ^{14}C; $^{3}H/^{14}C$ ratio 10·95:1).

Purification of [^{3}H,^{14}C]β-*sitosterol* (*VI*, *Scheme* 1). The radioactive β-sitosterol was added to pure β-sitosterol (268mg.) and crystallized four times from chloroform–methanol (Table 2). Further purification was carried out

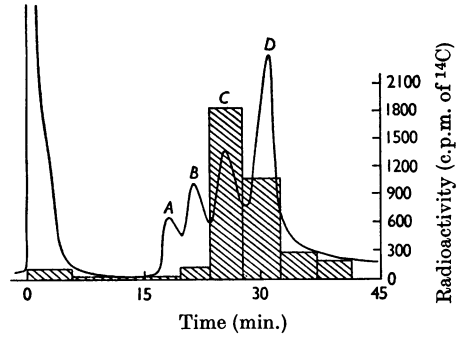


Fig. 1. Preparative g.l.c. of the radioactive 4,4-dimethyl sterol fraction isolated from larch (*L. decidua*) leaves after incubation with 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA. *A*, 24,25-Dihydrolanosterol; *B*, lanosterol; *C*, cycloartenol; *D*, 24-methylenecycloartanol. Hatched areas represent the radioactivity in each fraction.

by the formation of the dibromide (Schwenk & Werthessen, 1952). β-Sitosterol (93·7mg., $^3H/^{14}C$ ratio 10·84:1) was dissolved in diethyl ether (3·0ml.) at 0° and bromine added dropwise until an orange colour persisted; the mixture was then kept for 30min. at 0°. It was then poured into 10% (w/v) $Na_2S_2O_3,5H_2O$ solution and extracted with diethyl ether in the normal manner to give β-sitosteryl dibromide, which was crystallized from methanol (93·1mg., 55c.p.m. of ^{14}C/mg., $^3H/^{14}C$ ratio 10·22:1). The β-sitosteryl dibromide was dissolved in diethyl ether (3·0ml.), and zinc dust (2·0g.) and acetic acid (2·0ml.) were added. After being stirred for 2·5hr. at room temperature the mixture was filtered and the β-sitosterol (75·2mg.) isolated and crystallized from methanol (Table 2). It had m.p. 136–137° and 62c.p.m. of ^{14}C/mg., $^3H/^{14}C$ ratio 10·53:1.

Conversion of [^3H,^{14}C]*β-sitosterol* (*VI, Scheme* 1) *into* [^3H,^{14}C]*stigmast-4-ene-3,6-dione* (*VIII*). The [^3H,^{14}C]-β-sitosterol mother-liquors from the second, third and fourth crystallizations (Table 2) were combined to give β-sitosterol (76·4mg., m.p. 135–137° and 101c.p.m. of ^{14}C/mg., $^3H/^{14}C$ ratio 10·85:1), which was dissolved in diethyl ether (20ml.) and oxidized with chromic acid solution (2·0ml., prepared as described above) by the method outlined above. The reaction products (42·7mg.) were crystallized three times from methanol (Table 2) to give pale-yellow crystals of [^3H,^{14}C]stigmast-4-ene-3,6-dione, m.p. 167–170° (literature m.p. 170–172°; Crabbe, Azpeitia & Djerassi, 1961), u.v. $\lambda_{max.}$ 250nm., i.r. $\nu_{max.}^{KBr}$ 1680 and 1600cm.$^{-1}$, and 57c.p.m. of ^{14}C/mg., $^3H/^{14}C$ ratio 9·17:1. The mother-liquors from the above crystallizations were combined and the [^3H,^{14}C]stigmast-4-ene-3,6-dione purified by t.l.c. on silica gel followed by crystallization from methanol. It had m.p. 168–170° and 67c.p.m. of ^{14}C/mg., $^3H/^{14}C$ ratio 9·41:1.

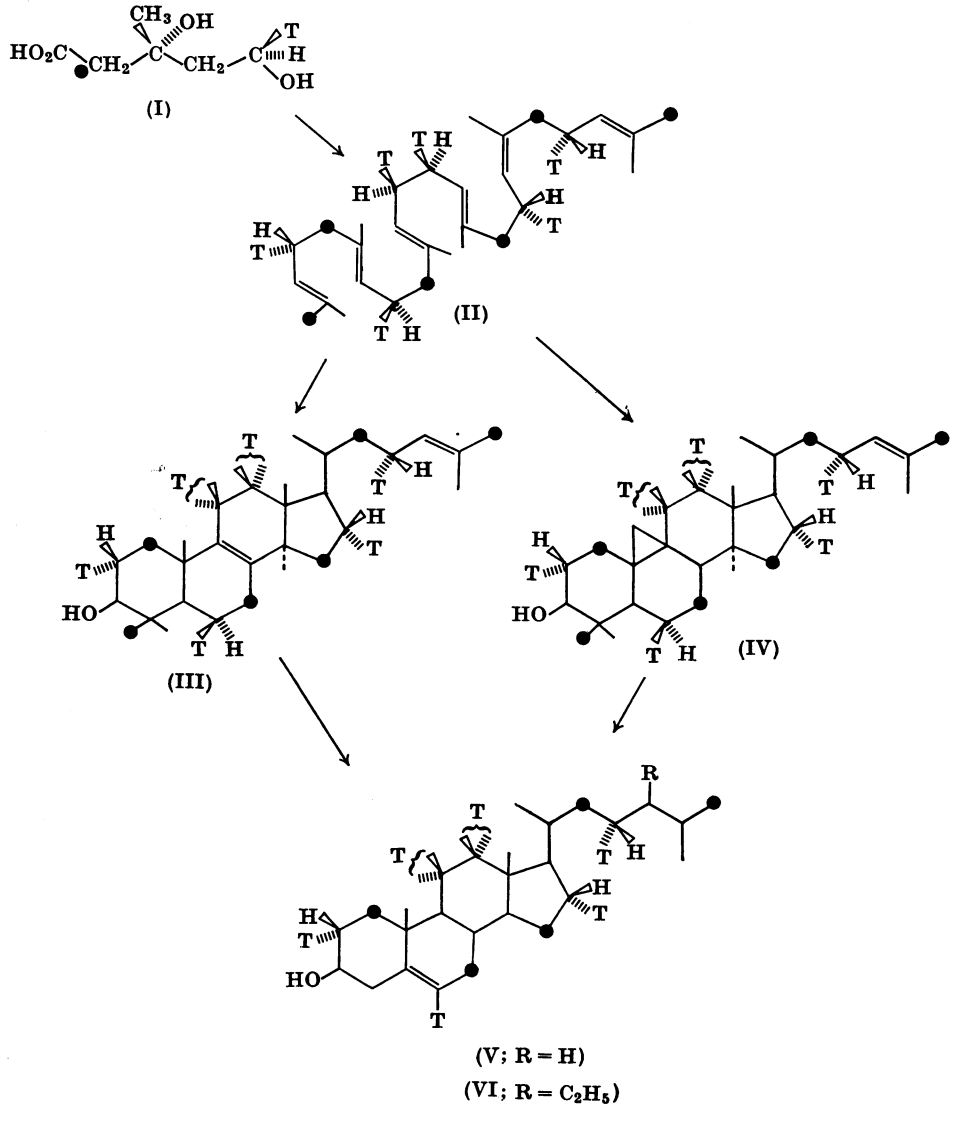
Conversion of [^3H,^{14}C]*β-sitosterol* (*VI, Scheme* 1) *into* 3*β-acetoxy-6-nitro*[^3H,^{14}C]*stigmast-5-ene* (*X*). Labelled β-sitosterol ($^3H/^{14}C$ ratio 10·70:1) was converted into the acetate (77·0mg., $^3H/^{14}C$ ratio 10·96:1), suspended in conc. HNO_3 (2·0ml.) and treated with $NaNO_2$ (3×16mg.) as described above. An oily product (77mg.) was isolated and crystallized twice from methanol to give poorly formed crystals of 3β-acetoxy-6-nitrostigmast-5-ene, which melted over a wide range and had i.r. $\nu_{max.}^{KBr}$ 1745 and 1510cm.$^{-1}$, and 68c.p.m. of ^{14}C/mg., $^3H/^{14}C$ ratio 8·92:1.

Identification of [^3H,^{14}C]28*-isofucosterol* (*XII*). The radioactive material that co-chromatographed with 28-isofucosterol on t.l.c. on 10% $AgNO_3$-impregnated silica gel was divided into two portions. To one part ($2·12\times10^4$c.p.m. of ^{14}C) was added authentic 28-isofucosterol (25·2mg.) and the mixture crystallized from methanol to constant specific radioactivity (Table 3). To the second portion ($1·06\times10^4$c.p.m. of ^{14}C) fucosterol (50mg.) was added, and the mixture crystallized seven times from methanol (Table 3). The mother-liquors from the above fucosterol crystallizations were combined and treated with pyridine–acetic anhydride to give the acetate (42·7mg., $^3H/^{14}C$ ratio 10·80:1). The fucosteryl acetate was dissolved in methylene dichloride (2·0ml.) containing pyridine (0·01ml.), cooled to −30° and treated with ozone. Zinc powder (100mg.) and acetic acid (1·0ml.) were added and the mixture was stirred at room temperature for 2hr. The reaction products (39·4mg.) were extracted with methylene dichloride, and 24-oxocholesteryl acetate (R_F 0·1; 10·5mg.) was separated from unchanged fucosteryl acetate (R_F 0·4; 12·8mg.) by preparative t.l.c. on silica gel developed with benzene–hexane (3:2, v/v). The 24-oxocholesteryl acetate was finally purified by crystallization from methanol (Table 3). It had m.p. 116–117° and 128–128·5° (literature m.p. 115–116° and 126–127°; Bergman & Dusza, 1957), i.r. $\nu_{max.}^{KBr}$ 1725 and 1710cm.$^{-1}$, and 239c.p.m. of ^{14}C/mg. and $^3H/^{14}C$ ratio 10·90:1.

Conversion of [^3H,^{14}C]28*-isofucosterol into* [^3H,^{14}C]-*stigmasta-4,Z-24*(28)*-diene-3,6-dione*. The mother-liquors from the above [^3H,^{14}C]28-isofucosterol crystallizations were combined and the 28-isofucosterol (15·5mg.) was oxidized with chromic acid as described above. The products of the reaction were isolated and subjected to preparative t.l.c. on silica gel developed with chloroform. The material with R_F identical with that of marker cholest-4-ene-3,6-dione was eluted and crystallized from methanol to give pale-yellow crystals of [^3H,^{14}C]stigmast-4,*Z*-24(28)-diene-3,6-dione, m.p. 149–150°, i.r. $\nu_{max.}^{KBr}$ 1695cm.$^{-1}$, and 1015c.p.m. of ^{14}C/mg. (?), $^3H/^{14}C$ ratio 9·20:1.

RESULTS AND DISCUSSION

The enzymic conversion of 3*R*-[2-^{14}C,(5*R*)-5-$^3H_1$]MVA (I, Scheme 1) into squalene (II) involves no loss of tritium (Popják & Cornforth, 1966; Donninger & Popják, 1966; Cornforth, Cornforth, Donninger & Popják, 1966), so the squalene will have the same $^3H/^{14}C$ ratio as the starting MVA. Cyclization (Ruzicka, 1959) of the stereospecifically labelled squalene (II) should produce either lanosterol (III) or cycloartenol (IV), the postulated phytosterol precursor (Benveniste, Hirth & Ourisson, 1966; Goad, 1967), with identical $^3H/^{14}C$ ratios that represent $^3H/^{14}C$ atomic ratio 6:6. This was demonstrated experimentally in the present work for both the lanosterol fraction isolated from the rat liver-homogenate incubation and for cycloartenol from the larch experiment (Tables 1 and 2). The chair–boat–chair–boat folding of squalene that is required for cyclization to produce lanosterol (III) or cycloartenol (IV) (Eschenmoser, Ruzicka, Jeger & Arigoni, 1955; Ruzicka, 1959; Richards & Hendrickson, 1964; Rees, Goad & Goodwin, 1968) will result in tritium occupying the 2α-, 6β-, 16α- and 23-*proR* positions; however, since squalene cyclization can apparently occur from either end of the squalene chain (Samuelsson & Goodman, 1963), the remaining two tritium atoms will be distributed equally between the 11α-, 11β-, 12α- and 12β-positions. The ^{14}C is located as indicated in Scheme 1. The conversion of labelled lanosterol (III) into cholesterol (V) results in the loss of one ^{14}C atom during removal of the C-4-*gem*-dimethyl carbon atoms. In addition, consideration of the now generally accepted biosythetic steps in cholesterol biosynthesis (Clayton, 1965; Frantz & Schroepfer, 1967) reveals that the conversion of the stereospecifically labelled lanosterol (III) into cholesterol (V) apparently involves only one carbon atom with an attached tritium atom. This is at C-6 during



Scheme 1. T, ^3H atom; ● ^{14}C atom.

introduction of the C-5–C-6 double bond (see Scheme 1 for partial pathway) to give the intermediate 5,7-diene, in which the C-7–C-8 double bond is subsequently reduced to give cholesterol (Dempsey, 1965; Wilton, Munday, Skinner & Akhtar, 1968). The cholesterol biosynthesized by the rat liver homogenate from 3*R*-[2-^{14}C,(5*R*)-5-$^3H_1$]MVA had $^3H/^{14}C$ atomic ratio 5·82:5 (Table 1), indicating that the 6β-tritium atom of the cholesterol precursor was retained during introduction of the C-5–C-6 double bond. Conversion of the labelled cholesterol into cholest-4-ene-3,6-dione (VII) and 3β-acetoxy-6-nitrocholest-5-ene (IX) resulted in each case in the loss of one tritium atom (Table 1) from C-6 and therefore confirmed that the 6β-hydrogen (tritium) atom is retained during the enzymic conversion of lanosterol (III) into cholesterol (V). Although the present results provide no evidence on the detailed nature of the mechanism involved in the introduction of the C-5–C-6 double bond of cholesterol, they demonstrate that the overall reaction involves the *cis*-elimination of the

5α- and 6α-hydrogen atoms, in agreement with the previous reports (Paliokas & Schroepfer, 1968; Akhtar & Marsh, 1967; Dewhurst & Akhtar, 1967).

The above result thus proved the feasibility of applying this type of approach to investigate the formation of double bonds in plant sterols. Accordingly leaves of *L. decidua* were incubated with 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA and the labelled phytosterols were isolated. The β-sitosterol fraction, after addition of carrier β-sitosterol, was crystallized four times, followed by purification via the dibromide and a further three crystallizations (Table 2). The β-sitosterol (VI) had $^{3}H/^{14}C$ atomic ratio 5·8:5, which was in agreement with that found for cholesterol (V) (Table 1), showing that the 6β-tritium atom of the β-sitosterol precursor had again been retained on introduction of the C-5–C-6 double bond. This was confirmed by the loss of one tritium atom when the β-sitosterol was converted into either 3β-acetoxy-6-nitrostigmast-5-ene (X) or stigmast-4-ene-3,6-dione (VIII). Similar results have also been obtained for poriferasterol biosynthesis in the phytoflagellate *Ochromonas malhamensis* (Smith, Goad & Goodwin, 1968) and ergosterol biosynthesis in fungi (Akhtar & Parvez, 1968; Bimpson, Goad & Goodwin, 1969). It therefore seems reasonable to assume that in higher plants, algae and fungi a similar enzyme mechanism is operative to that found in animals for the introduction of the sterol C-5–C-6 double bond.

The fall in the specific radioactivity of the β-sitosterol after purification through the dibromide showed the presence of a contaminant sterol of relatively high specific radioactivity that co-crystallized with the β-sitosterol in the four initial crystallizations. The presence of this contaminant sterol was also indicated by the decrease in specific radioactivity of the stigmast-4-ene-3,6-dione during three crystallizations (Table 2). Treatment of the β-sitosterol with either bromine (Copius-Peereboom, 1964) or chromic acid (Fieser & Fieser, 1959; Knights & Laurie, 1967) would remove any labelled Δ^7-sterol present. Such a compound could possibly

Table 1. *Incorporation of* 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]-*MVA into squalene, lanosterol and cholesterol by a rat liver homogenate*

The ratios were normalized by assuming a 6:6 atomic ratio in squalene.

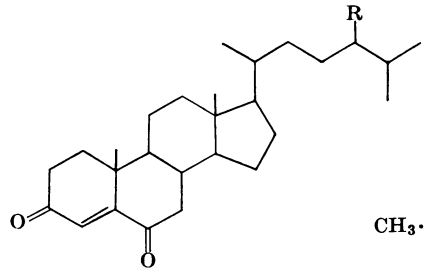
Compound	$^{3}H/^{14}C$ ratio	Normalized $^{3}H/^{14}C$ atomic ratio	Theoretical $^{3}H/^{14}C$ atomic ratio
Squalene	9·65:1	6:6	6:6
Lanosterol*	9·25:1	5·75:6	6:6
Cholesterol	11·18:1	5·80:5	6:5
Cholesteryl acetate	11·23:1	5·82:5	6:5
Cholest-4-ene-3,6-dione	9·44:1	4·89:5	5:5
3β-Acetoxy-6-nitrocholest-5-ene	9·34:1	4·84:5	5:5

* This fraction also contained lanosterol companion sterols having a 4,4-dimethyl group.

Table 2. *Incorporation of* 3*R*-[2-^{14}C,(5*R*)-5-$^{3}H_1$]*MVA into squalene and sterols of larch* (L. decidua) *leaves*

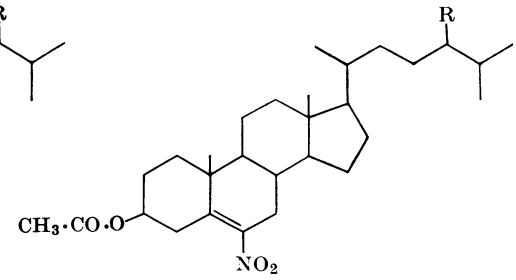
The ratios were normalized by assuming a 6:6 atomic ratio in squalene.

Compound	^{14}C sp. radioactivity (c.p.m. of ^{14}C/mg.)	$^{3}H/^{14}C$ ratio	Normalized $^{3}H/^{14}C$ atomic ratio	Theoretical $^{3}H/^{14}C$ atomic ratio
Squalene	—	9·14:1	6:6	6:6
Cycloartenol	—	9·26:1	6·08:6	6:6
β-Sitosterol				
First crystallization	108	10·93:1	5·98:5	6:5
Second crystallization	102	10·85:1	5·93:5	6:5
Third crystallization	89	10·82:1	5·92:5	6:5
Fourth crystallization	92	10·84:1	5·93:5	6:5
β-Sitosterol after dibromide purification				
Fifth crystallization	57	10·70:1	5·85:5	6:5
Sixth crystallization	61	10·50:1	5·75:5	6:5
Seventh crystallization	62	10·53:1	5·76:5	6:5
Stigmast-4-ene-3,6-dione				
First crystallization	63	9·55:1	5·23:5	5:5
Second crystallization	54	9·50:1	5·19:5	5:5
Third crystallization	57	9·17:1	5·02:5	5:5
3β-Acetoxy-6-nitrostigmast-5-ene	68	8·92:1	4·88:5	5:5



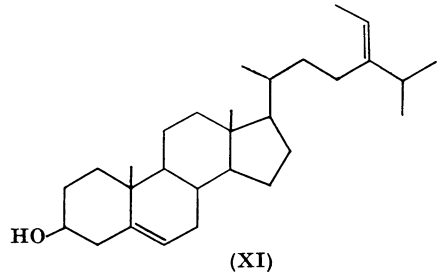
(VII; R = H)

(VIII; R = C_2H_5)

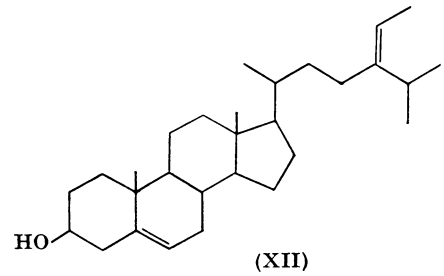


(IX; R = H)

(X; R = C_2H_5)



(XI)



(XII)

be stigmasta-7,Z-24(28)-dien-3β-ol, which has been isolated from higher plants (Knights & Laurie, 1967; Frost & Ward, 1968) and might be expected to occur on biosynthetic grounds in the larch sterol mixture. This compound would be expected to be only slightly more polar than β-sitosterol on t.l.c. on 10%-silver nitrate-impregnated silica gel, and a portion of this material of high specific radioactivity may have been eluted with the β-sitosterol in the present work.

In a previous examination of the sterols of *L. decidua* leaves we observed a small amount of a 4-demethyl sterol, which was tentatively identified on the basis of g.l.c. characteristics as either fucosterol (XI) or 28-isofucosterol (XII) (Goad & Goodwin, 1967). We have been unable to isolate sufficient of this material in pure form to permit a positive identification on the basis of physical properties; however, in the present work we obtained radiochemical evidence that permits the compound to be identified as 28-isofucosterol. Preparative t.l.c. of the labelled 4-demethyl sterols on 10%-silver nitrate-impregnated silica gel gave a highly radioactive fraction with R_F identical with that of 28-isofucosterol but with negligible mass. This was divided into two portions and crystallized several times with, as carrier, either 28-isofucosterol or fucosterol (Table 3). Crystallization with 28-isofucosterol resulted in no loss of specific radioactivity after the first crystallization, whereas with fucosterol the specific radioactivity decreased continually to a very low value. The presence of a C-24–C-28 double bond in the radioactive sterol was proved by acetylation of the combined mother-liquors from the fucosterol crystallizations followed by ozonolysis and crystallization of the resulting 24-oxocholesteryl acetate to constant specific radioactivity (Table 3). The $^3H/^{14}C$ atomic ratio 5·95:5 for the 28-isofucosterol and the fall in the $^3H/^{14}C$ atomic ratio to 5·03:5 on conversion into stigmasta-4,Z-24(28)-diene-3,6-dione was in agreement with the previous results for β-sitosterol and provided additional evidence for the loss of the 6α-hydrogen atom during C-5–C-6 double-bond formation in phytosterols.

A sterol with a 24-ethylidene group produced by a double transmethylation from methionine has been proposed as an intermediate in β-sitosterol biosynthesis in higher plants (Castle, Blondin & Nes, 1963; Bader, Guglielmetti & Arigoni, 1964; Lederer, 1964). The intermediacy of a 24-ethylidene sterol in the formation of poriferasterol by *O. malhamensis* (Smith, Goad, Goodwin & Lederer, 1967) and clionasterol by *Chlorella ellipsoidea* (Patterson & Karlander, 1967) has been demonstrated. Also, our previous work with *L. decidua* (Goad, Hammam, Dennis & Goodwin, 1966; Goad, 1967) provided evidence for the participation of a 24-ethylidene sterol in β-sitosterol production by this plant. The present identification of 28-isofuco-

Table 3. *Crystallization of a radioactive larch* (L. decidua) *sterol with* (*a*) 28-*isofucosterol and* (*b*) *fucosterol and* (c) *the derived* 24-*oxocholesteryl acetate obtained by ozonolysis of the fucosterol mixture* (*b*)

	^{14}C sp. radioactivity (c.p.m. of ^{14}C/mg.)	$^{3}H/^{14}C$ ratio	Normalized $^{3}H/^{14}C$ atomic ratio
(*a*) Crystallization with 28-isofucosterol			
Before crystallization	842	10·95:1	5·99:5
First crystallization	547	10·88:1	5·95:5
Second crystallization	485	10·79:1	5·90:5
Third crystallization	434	10·92:1	5·97:5
Fourth crystallization	480	10·70:1	5·85:5
Fifth crystallization	540	10·79:1	5·91:5
Sixth crystallization	588	10·83:1	5·92:5
(*b*) Crystallization with fucosterol			
Before crystallization	212	—	—
First crystallization	116	—	—
Second crystallization	77	—	—
Third crystallization	77	—	—
Fourth crystallization	35	—	—
Fifth crystallization	30	—	—
Sixth crystallization	16	—	—
Seventh crystallization	20	—	—
		—	—
(*c*) 24-Oxocholesteryl acetate			
First crystallization	180	10·86:1	5·94:5
Second crystallization	225	10·59:1	5·79:5
Third crystallization	217	10·75:1	5·88:5
Fourth crystallization	239	10·90:1	5·96:5

sterol in *L. decidua* leaves and its labelling by MVA under conditions where β-sitosterol also becomes labelled are consistent with this view. 28-Isofucosterol has now been identified in a number of plants (Knights, 1965, and personal communication; van Aller, Chikamatsu, de Souza, John & Nes, 1968; Gibbons *et al.* 1968; J. St Pyrek, personal communication). By contrast, the 24-ethylidene isomer fucosterol, is apparently restricted to the marine brown algae (Carter, Heilbron & Lythgoe, 1939; Ito, Tamura & Matsumoto, 1956; Tsuda, Akagi, Kishida, Hayatsu & Sakai, 1958). There is apparently no evidence for mixtures of 28-isofucosterol and fucosterol occurring in any plant material, indicating a high degree of stereospecificity in the alkylating enzymes of various organisms (Gibbons *et al.* 1968). In conjunction with the brief report that 28-isofucosterol is converted into β-sitosterol in *Pinus pinea* seeds (van Aller *et al.* 1968) the above facts suggest that 28-isofucosterol or possibly other sterols with a C-24–C-28 double bond of the same configuration are normally reduced to give the 24-ethyl sterols typical of most higher plants.

Note added in proof. After completion of this manuscript a similar report on cholesterol appeared (Akhtar, Rahimtula, Watkinson, Wilton & Munday, 1969).

We thank the Science Research Council for financial assistance. G. F. G. was in receipt of a Science Research Council research studentship. We also thank Miss W. Sach for skilled technical help.

REFERENCES

Akhtar, M. & Marsh, S. (1967). *Biochem. J.* **102**, 462.
Akhtar, M. & Parvez, M. A. (1968). *Biochem. J.* **108**, 527.
Akhtar, M., Rahimtula, A. D., Watkinson, I. A., Wilton, D. C. & Munday, K. A. (1969). *Europ. J. Biochem.* **9**, 107.
Bader, A. Gugleilmetti, L. & Arigoni, D. (1964). *Proc. chem. Soc., Lond.*, p. 16.
Bates, R. B., Brewer, A. D., Knights, B. A. & Rowe, J. W. (1968). *Tetrahedron Lett.* no. 59, p. 6163.
Benveniste, P., Hirth, L. & Ourisson, G. (1966). *Phytochemistry*, **5**, 45.
Bergman, W. & Dusza, J. P. (1957). *Liebigs Ann.* **603**, 38.
Bimpson, T., Goad, L. J. & Goodwin, T. W. (1969). *Chem. Commun.* p. 297.
Blackwood, J. E., Gladys, C. L., Loening, K. L., Petrara, A. E. & Rush, J. E. (1968). *J. Amer. chem. Soc.* **90**, 509.
Bucher, N. L. R. & McGarrahan, K. (1956). *J. biol. Chem.* **222**, 1.
Carter, P. W., Heilbron, I. M. & Lythgoe, B. (1939). *Proc. Roy. Soc.* B, **128**, 82.
Castle, M., Blondin, G. & Nes, W. R. (1963). *J. Amer. chem. Soc.* **85**, 3306.
Clayton, R. B. (1965). *Quart. Rev.* **19**, 168.
Copius-Peereboom, J. W. (1964). *Z. anal. Chem.* **205**, 325.
Cornforth, J. W., Cornforth, R. H., Doninger, C. & Popják, G. (1966). *Proc. Roy. Soc.* B, **163**, 492.
Crabbe, P., Azpeitia E. A. & Djerassi, C. (1961). *Bull. Soc. chim. Belg.* **70**, 168.
Dempsey, M. E. (1965). *J. biol. Chem.* **240**, 4176.
Dewhurst, S. M. & Akhtar, M. (1967). *Biochem. J.* **105**, 1187.
Dodson, R. M. & Riegel, B. (1948). *J. org. Chem.* **13**, 424.
Donninger, C. & Popják, G. (1966). *Proc. Roy. Soc.* B, **163**, 465.
Eschenmoser, A., Ruzicka, L., Jeger, O. & Arigoni, D. (1955). *Helv. chim. acta*, **38**, 1890.
Fieser, L. F. & Fieser, M. (1959). *Steroids.* New York: Reinhold Publishing Corp.
Frantz, I. D. & Schroepfer, G. J. (1967). *Annu. Rev. Biochem.* **36**, 691.
Frost, D. J. & Ward, J. P. (1968). *Tetrahedron Lett.* no. 34, p. 3779.
Gibbons, G. F., Goad, L. J. & Goodwin, T. W. (1968). *Phytochemistry*, **7**, 983.
Goad, L. J. (1967). In *Terpenoids in Plants*, p. 159. Ed. by Pridham, J. B. London: Academic Press (Inc.) Ltd.
Goad, L. J. & Goodwin, T. W. (1966). *Biochem. J.* **99**, 735.
Goad, L. J. & Goodwin, T. W. (1967). *Europ. J. Biochem.* **1**, 357.
Goad, L. J., Hammam, A. S. A., Dennis, A. & Goodwin, T. W. (1966). *Nature, Lond.*, **210**, 1322.
Ito, S., Tamura, T. & Matsumoto, T. (1956). *J. Res. Inst. Technol., Nihon Univ.*, no. 13, p. 99.
Knights, B. A. (1965). *Phytochemistry*, **4**, 857.

Knights, B. A. & Laurie, W. (1967). *Phytochemistry*, **6**, 407.
Lederer, E. (1964). *Biochem. J.* **93**, 449.
Mauthner, J. & Suida, W. (1903). *Mh. Chem.* **24**, 648.
Paliokas, A. M. & Schroepfer, G. J. (1968). *J. biol. Chem.* **243**, 453.
Patterson, G. W. & Karlander, E. P. (1967). *Plant Physiol.* **42**, 1651.
Popják, G. & Cornforth, J. W. (1966). *Biochem. J.* **101**, 553.
Rees, H. H., Goad, L. J. & Goodwin, T. W. (1968). *Biochem. J.* **107**, 417.
Richards, J. H. & Hendrickson, J. B. (1964). *The Biosynthesis of Steroids, Terpenes and Acetogenins*, p. 274. New York: W. A. Benjamin Co.
Ruzicka, L. (1959). *Proc. chem. Soc., Lond.*, p. 341.
Samuelsson, B. & Goodman, D. S. (1963). *J. biol. Chem.* **239**, 98.
Schwenk, E. & Werthessen, N. T. (1952). *Arch. Biochem. Biophys.* **40**, 334.
Smith, A. R. H., Goad, L. J. & Goodwin, T. W. (1968). *Chem. Commun.* p. 926.
Smith, A. R. H., Goad, L. J., Goodwin, T. W. & Lederer, E. (1967). *Biochem. J.* **104**, 566.
Tsuda, K., Akagi, S., Kishida, Y., Hayatsu, R. & Sakai, K. (1958). *Chem. Pharm. Bull., Tokyo*, **6**, 724.
van Aller, R. T., Chikamatsu, H., de Souza, N. J., John, J. P. & Nes, W. R. (1968). *Biochem. biophys. Res. Commun.* **31**, 842.
Williams, B. L., Goad, L. J. & Goodwin, T. W. (1967). *Phytochemistry*, **6**, 1137.
Williams, R. J. H., Britton, G., Charlton, J. M. & Goodwin, T. W. (1967). *Biochem. J.* **104**, 767.
Wilton, D. C., Munday, K. A., Skinner, S. J. M. & Akhtar, M. (1968). *Biochem. J.* **106**, 803.

ADDENDUM

Incorporation of [2-^{14}C]Mevalonic Acid into 28-Isofucosterol by Leaves of *Pisum sativum*

By LORETTA M. BOLGER, H. H. REES, L. J. GOAD AND T. W. GOODWIN
Department of Biochemistry, University of Liverpool, L69 3BX

(*Received* 2 *June* 1969)

During a previous examination of the sterols of pea (*Pisum sativum*) leaves we noted a minor component that was tentatively identified as fucosterol on the basis of g.l.c. retention results (Goad & Goodwin, 1966). However, the isomer of fucosterol, 28-isofucosterol, has since been identified as a minor constituent of oat seeds and a few other plants (Knights, 1965; B. H. Knights, personal communication). It therefore became of interest for us to reinvestigate the pea leaf sterol, and the availability of a sample of 28-isofucosterol (Gibbons, Goad & Goodwin, 1968) made this possible. Comparison of the g.l.c. retention times of fucosterol and 28-isofucosterol on the stationary phase cyclohexane dimethanol succinate (CHDMS, also known as Hi-EFF-8B) showed that they can be distinguished when they constitute the major component, as described by Knights (1965). However, when these sterols comprised only a small percentage of the total sterol mixture, as was the case in the pea leaf sterols (Goad & Goodwin, 1966), it was difficult on our column to differentiate between the two components with certainty. We therefore resorted to radiochemical methods to confirm the presence of 28-isofucosterol in pea leaves.

EXPERIMENTAL AND RESULTS

Methods were generally as described previously (Goad & Goodwin, 1966; Williams, Goad & Goodwin, 1967; Goad, Gibbons, Bolger, Rees & Goodwin, 1969). Peas (variety Onward) were germinated for 7 days and 3·0g. of the freshly developed leaves chopped into strips and moistened with 1·0ml. of a solution of [2-^{14}C]mevalonic acid (5·0μc). After 6hr. incubation at 25° with illumination, the non-saponifiable lipids (10·0mg.; $8{\cdot}0 \times 10^5$c.p.m.) were extracted in the usual manner. Preparative t.l.c. on silica gel developed with chloroform gave the 4-demethyl sterol fraction (2·0mg.; $1{\cdot}9 \times 10^5$c.p.m.). To this material 28-isofucosterol (1·0mg.) was added, the mixture was subjected to t.l.c. on 10% silver nitrate-impregnated silica gel developed with chloroform, and the

Table 1. *Crystallization of the labelled 24-ethylidene sterol of pea leaves with 28-isofucosterol or fucosterol*

	Sp. radioactivity (c.p.m./mg.)	
Crystallization no.	28-Isofucosterol	Fucosterol
1	245	256
2	199	188
3	226	137
4	214	90
5	204	90
6	177	77
7	174	67
8	207	28