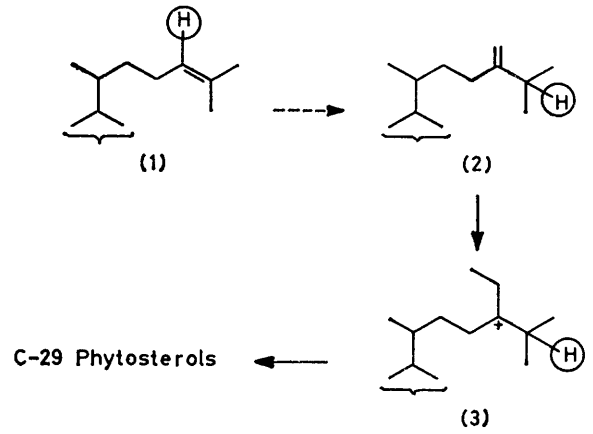
Stereochemical Fate of the Isopropylidene Methyl Groups of Lanosterol during the Biosynthesis of Isofucosterol in *Pinus Pinea*

By **Francesco Nicotra, Fiamma Ronchetti,** and **Giovanni Russo,*** Istituto di Chimica Organica dell'Università di Milano, Centro di Studio per le Sostanze Organiche Naturali del C.N.R., Via Saldini 50, 20133 Milano, Italia
Giuseppe Lugaro and **Marilena Casellato,** Laboratorio di Chimica degli Ormoni del C.N.R., Via M. Bianco 9, Milano, Italia

[26-^{3}H]Lanosterol (16) was administered to *Pinus pinea* and the [26-^{3}H]isofucosteryl acetate (6) obtained was chemically transformed into [26-^{3}H]cholesterol (13). Compound (13) was incubated with rat-liver mitochondria to yield [3-^{3}H] propionic acid. The data obtained are consistent with a biosynthetic pathway in which the *pro*-E isopropylidene methyl group of the Δ^{24}-precursor becomes the *pro*-R isopropyl methyl group in isofucosterol.

PHYTOSTEROLS, the typical plant sterols widely diffused in nature, are characterized by a 24-alkyl substituent (methyl, ethyl, methylene or ethylidene) which arises from *S*-adenosylmethionine by a simple or double trans-methylation reaction on a $\Delta^{24(25)}$ precursor (1).[1,2]

The alkylation mechanism for the formation of C-29 phytosterols involves, as a first stage (Scheme 1), the



SCHEME 1

formation of a 24-methylene sterol intermediate (2) through the loss of one of the C-28 hydrogen atoms, deriving from methionine, and the migration of a hydrogen atom from C-24 to C-25.[3]

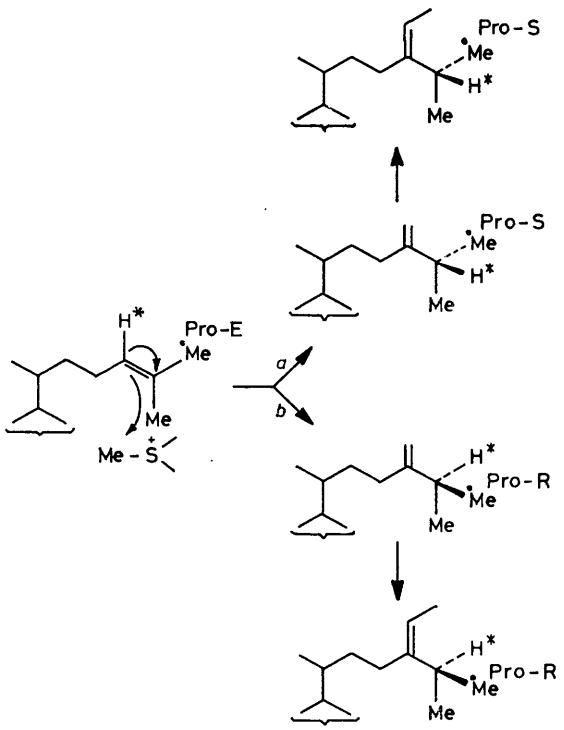
This 24-methylene sterol intermediate (2) undergoes a further alkylation reaction leading to the cation (3), from which C-29 phytosterols with a saturated or unsaturated side-chain originate.

In this paper we report our results about the stereochemistry of one of the most notable features of the alkylation process, the hydrogen transfer from C-24 to C-25. We chose to study this migration in the case of the biosynthesis of isofucosterol,[3] the typical sterol of *Pinus pinea* (the reason why we chose to effect this study on isofucosterol will become evident later).

RESULTS AND DISCUSSION

The migration of the hydrogen atom from C-24 to C-25 can occur in two stereochemically opposite ways (see Scheme 2), the first (path *a*) in which the *pro*-E methyl group of the Δ^{24}-precursor becomes the isopropyl *pro*-S methyl group, the second (path *b*) in which the same methyl group becomes the isopropyl *pro*-R methyl group.

To discover which of the two cases actually occurs it is required: (*i*) to stereospecifically label one of the two isopropylidene methyl groups of a Δ^{24}-precursor; and (*ii*) to analyse the biosynthesized phytosterol through a stereospecific transformation of one of the two methyl

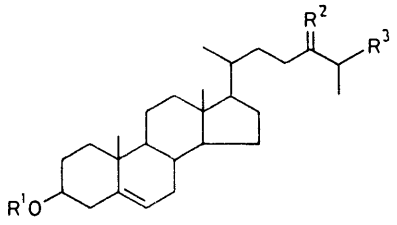


SCHEME 2

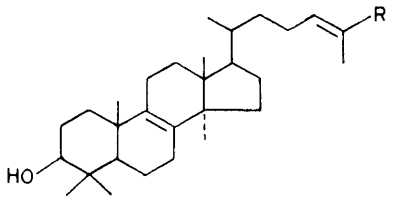
groups of the isopropyl group. As far as (*i*) is concerned, we attempted first to biosynthesize isofucosterol (4) from (3*R*)-[2-^{3}H]mevalonic acid (MVA), from which a Δ^{24}-precursor, like cycloartenol, with tritium in positions 1,7,15,22,26, and 30 should have been obtained; unfor-

tunately, the administration of the labelled MVA to shelled seeds of *Pinus pinea* showed that it was not suitable for our work, owing to poor incorporation, made worse by the fact that only 0.25 of the label is located in the isopropyl group of the phytosterol side-chain.

We then turned to the chemical synthesis of a precursor more advanced than MVA, with all the label in one



(*Z*)-(4) R^1 = H, R^2 = CHMe, R^3 = Me
(*Z*)-(5) R^1 = Ac, R^2 = CHMe, R^3 = Me
(*Z*)-(6) R^1 = Ac, R^2 = CHMe, R^3 = C^3HH_2
(*Z*)-(7) R^1 = H, R^2 = CHMe, R^3 = C^3HH_2
(8) R^1 = Ac, R^2 = O, R^3 = Me
(9) R^1 = Ac, R^2 = H,OH, R^3 = Me
(10) R^1 = H, R^2 = H,H, R^3 = Me
(11) R^1 = H, R^2 = O, R^3 = Me, ^3H at C-23 and C-25
(12) R^1 = H, R^2 = H,OH, R^3 = Me, ^3H at C-23 and C-25
(13) R^1 = H, R^2 = H,H, R^3 = C^3HH_2



(14) R = Me
(15) R = CHO
(16) R = C^3HH_2

position. As lanosterol (14) is known to be incorporated into isofucosterol (4) in *Pinus pinea*,[3] we decided to synthesize this compound tritium-labelled at one of the isopropylidene methyl groups. This synthesis, based on the stereoselective allylic oxidation of the isopropylidene group of lanosterol with selenium dioxide to give the *E*-aldehyde (15), reported elsewhere,[4] yielded a sample of [26-^{3}H]lanosterol (16) with a specific activity of 1.34×10^9 d.p.m. mg^{-1}.

After the problem of the labelled precursor had been solved, we turned to point (*ii*), *i.e.* the stereospecific analysis of the phytosterol isopropyl group. This is not a simple task, since, until now, no processes in which the isopropyl group of the phytosterol side-chain is stereospecifically attacked at one of the two methyl groups were known.

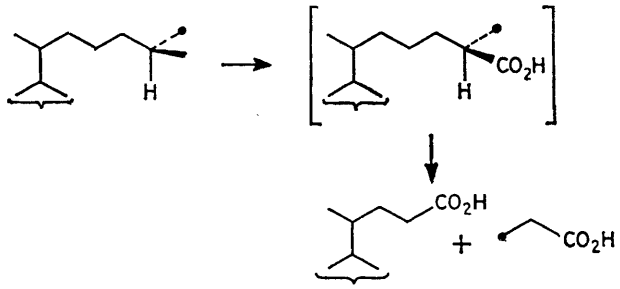
Nevertheless, this task can be achieved, provided that the phytosterol can be transformed into cholesterol; stereospecific reactions on the terminal isopropyl group of this compound are known.[5-7]

On the basis of these considerations, we chose to elucidate the stereochemistry of the hydrogen migration from C-24 to C-25 during the biosynthesis of phytosterols for isofucosterol (4), which, compared with the more common phytosterols, *e.g.* sitosterol, offers the advantage that the 24(28)-double bond allows an easy chemical degradation to cholesterol (10).

We decided also to effect the subsequent analysis of the cholesterol utilizing its conversion to bile acids and propionic acid by rat-liver mitochondria, which occurs through the stereospecific oxidation of the isopropyl *pro*-S methyl group and cleavage between C-24 and C-25 (Scheme 3).[5]

Before starting the labelling experiments we effected the transformation of isofucosterol into cholesterol on an unlabelled sample. As this procedure requires the removal of the 24-ethylidene group by ozonolysis, we attempted first a selective ozonolysis of isofucosteryl acetate (5) with less than the stoicheiometric amount of ozone; however, the yield being unsatisfactory, we decided to effect the ozonolysis after protection of the Δ^5-double bond. To this aim, isofucosterol was transformed into (*Z*)-6β-methoxy-3α,5-cyclostigmast-24(28)-ene (17) by tosylation and treatment of the tosylate with methanol and pyridine. Compound (17) was submitted to ozonolysis to yield the ketone (18), which was reduced with $NaBH_4$ to a diastereoisomeric mixture of the alcohols (19).

To ascertain whether proton exchange had taken place to any extent during the ozonolysis and/or the subsequent reduction, we effected the ozonolysis of a sample



• = C^3HH_2

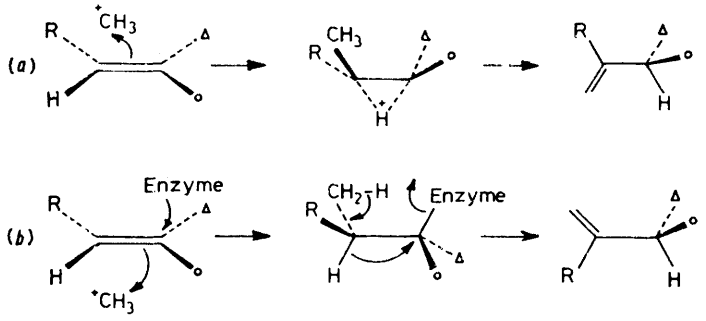
SCHEME 3

of (17) in Pr^iO^3H and the reduction of [23,23,25-$^3H_3$]-3β-hydroxycholest-5-en-24-one (11)[8] with $NaBH_4$. In the first case no appreciable amount of label was introduced, while in the second one no label was lost.

The alcohol (19) was then transformed into 6β-methoxy-3α,5-cyclocholestane (21) by mesylation, reduction of the mesylate (20) with $LiAlH_4$, and purification of the crude product by preparative t.l.c. on Ag-NO_3–silica gel, to remove traces of elimination products (*e.g.* desmosterol) which could interfere during the incubation of cholesterol. Treatment of (21) with acidic

aqueous dioxan yielded the desired cholesterol with a 30% overall yield from isofucosterol.

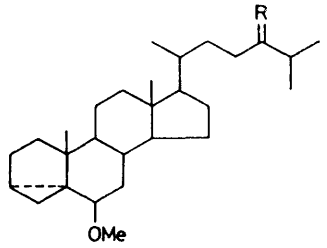
We could then begin the labelling experiments. [26-^{3}H]Lanosterol (16) (1.64 × 10^9 d.p.m.) was administered to 60 shelled seeds of *Pinus pinea*.[3] After the roots had reached a length of 0.5—1 cm (*ca* 10 days), the seeds were macerated in methanol, submitted to alkaline hydrolysis, and the sterol fraction isolated. [26-^{3}H]-Isofucosteryl acetate (6) (2.30 × 10^6 d.p.m.), pure by



SCHEME 4

argentation t.l.c. and g.l.c., was isolated by conventional procedures and transformed into [26-^{3}H]cholesterol (13) by the same sequence of reactions effected on the cold sample.

The tritiated cholesterol was mixed with [26-^{14}C]-cholesterol (8.62 × 10^4 d.p.m. of ^{14}C; ^{3}H : ^{14}C 3.20 : 1) and incubated with rat-liver mitochondria according to Mitropoulos *et al.*[5] After 8 h at room temperature the incubation mixture was quenched with H_2SO_4 and submitted to steam-distillation. The distillate was treated with KOH to neutralize the acidic fraction, which was obtained as the potassium salt by evaporation of the water under reduced pressure. From this crude salt, pure propionic acid was obtained as its *p*-bromophenacyl



(7)-(17) R = CHMe
(18) R = O
(19) R = H,OH
(20) R = H,OSO$_2$Me
(21) R = H,H

derivative, by treatment with *p*-bromophenacyl bromide in the presence of dicyclohexyl-18-crown-6 and purification by chromatography. The *p*-bromophenacylpropionate was repeatedly crystallized and counted (see Table). The data reported in the Table show the retention of 81% of the tritium present in the isopropyl methyl group of cholesterol, and hence of isofucosterol.

These data are consistent with a main biosynthetic pathway leading to isofucosterol in *Pinus pinea* in which the migration of the hydrogen atom from C-24 to C-25 occurs in such a way that the *pro*-E and the *pro*-Z methyl groups of the Δ^{24}-precursor assume, respectively, the *pro*-R and the *pro*-S positions (pathway *b*, Scheme 2).

In principle, the configuration found at C-25 can derive from different mechanisms, according to which *S*-adenosylmethionine enters from opposite sides. Many mechanisms may be envisaged, which can be reduced to the two general cases depicted in Scheme 4. In (*a*) the hydrogen atom migrates from the side opposite to that of the attack of *S*-adenosylmethionine; in (*b*), on the other hand, the attack of *S*-adenosylmethionine and the migration of the hydrogen atom occur from the same side.

Propionic acid (as *p*-bromophenacyl derivative) formed during the incubation of [26-^{3}H,26-^{14}C]cholesterol (8.62 × 10^4 d.p.m. of ^{14}C, ^{3}H : ^{14}C = 3.20 : 1) with rat-liver mitochondria

	^{14}C (d.p.m. mmol^{-1})	^{3}H : ^{14}C Ratio
Third crystallization	7.94 × 10^3	2.57 : 1
Fourth crystallization	8.08 × 10^3	2.62 : 1
Fifth crystallization	7.89 × 10^3	2.60 : 1

Once the stereochemical relation between the methylation by *S*-adenosylmethionine and the hydrogen migration is discovered, our results will allow us to infer the stereochemistry of the transmethylation process.

EXPERIMENTAL

I.r. spectra were recorded on a Perkin-Elmer 157 spectrophotometer as chloroform solutions, unless stated otherwise. N.m.r. spectra were obtained on a Varian NV 14 spectrometer in $CDCl_3$, using $CHCl_3$ as internal reference, unless stated otherwise. Radioactive samples were counted on a Packard Tri-Carb 3 320 liquid scintillator counter. Preparative and analytical t.l.c. was carried out on 0.25-mm Merck HF_{254} silica gel plates; the products were detected by spraying with 50% aqueous sulphuric acid and heating at 110 °C. Column chromatography was effected on silica gel–Celite (1 : 1, w/w). Gas chromatographic (g.l.c.) analyses were carried out with a Carlo-Erba Fractovap 2 400 V in-

strument, with a 2.5% SE 30 column at 235 °C or with a 1% LAC 796 column at 200 °C. Usual work-up refers to dilution with water, extraction with an organic solvent, drying with Na_2SO_4, filtration, and evaporation *in vacuo*. The reactions on the labelled samples were monitored, and the reaction products identified, on analytical t.l.c. by comparison with the cold authentic samples.

Attempted Selective Ozonolysis of Isofucosteryl Acetate (5).—To a stirred and cooled (−78 °C) solution of isofucosteryl acetate (5) (10 mg) in CH_2Cl_2 (5 ml), a half-saturated, cooled (−78 °C) solution of ozone in CH_2Cl_2 (2 ml) was added dropwise [this solution was prepared by passing a stream of ozone through CH_2Cl_2 (50 ml) at −78 °C for 30 min and then diluting the solution obtained with CH_2Cl_2 (50 ml) cooled to −78 °C]. After 5 min at −78 °C, triphenylphosphine (10 mg) was added; the mixture was stirred until room temperature was reached, then the solvent was evaporated *in vacuo* and the crude product was submitted to preparative t.l.c. [benzene–ethyl acetate (9 : 1)] affording 5 mg of isofucosteryl acetate (5) and 4 mg of 3β-acetoxycholest-5-en-24-one (8).[9]

(*Z*)-6β-*Methoxy*-3α,5-*cyclostigmast*-24(28)-ene (17).—From isofucosterol (4) (100 mg) (*Z*)-6β-methoxy-3α,5-cyclostigmast-24(28)-ene (17) (78 mg) was obtained by the method of Hey *et al.*[10] as an oil: $\nu_{max.}$ (liquid film) 2 900, 1 475, 1 385, and 1 105 cm^{-1}; δ 5.10 (q, *J* 6 Hz, 28-H), 3.28 (s, OMe), 2.78 (sept, *J* 6 Hz, 25-H), 2.72 (m, 6-H), 1.53 (d, *J* 6 Hz, 29-Me), 0.95 (s, 19-Me), 0.92 (d, *J* 6 Hz, 21-, 26-, and 27-Me), 0.65 (s, 18-Me), and 0.7—0.3 (cyclopropyl).

6β-*Methoxy*-3α,5-*cyclocholestan*-24-*one* (18).—To a stirred and cooled (−78 °C) solution of (17) (50 mg) in CH_2Cl_2 (100 ml), a half-saturated cooled (−78 °C) solution of ozone in CH_2Cl_2 (20 ml) was added. After 10 min at −78 °C, triphenylphosphine (50 mg) was added and the stirred solution was maintained for 10 min at −78 °C. The solvent was then removed *in vacuo*; usual work-up afforded 98 mg of crude reaction product, from which 42 mg of pure (18) were obtained after chromatography (benzene). 6β-Methoxy-3α,-5-cyclocholestan-24-one (18) was crystallized from methanol, m.p. 83—84 °C; $\nu_{max.}$ 2 900, 1 720, 1 475, 1 385, and 1 105 cm^{-1}; δ 3.30 (s, OMe), 2.71 (m, 6-H), 1.04 (d, *J* 6 Hz, 21-, 26-, and 27-Me), 0.98 (s, 19-Me), 0.68 (s, 18-Me), and 0.7—0.3 (cyclopropyl).

6β-*Methoxy*-3α,5-*cyclocholestan*-24-*ol* (19).—Compound (18) (40 mg) in 95% ethanol (50 ml) was reduced with $NaBH_4$ (50 mg) to yield, after purification by chromatography [benzene–ethyl acetate (9 : 1)], 6β-methoxy-3α,5-cyclocholestan-24-ol (19) (32 mg) as an oil; $\nu_{max.}$ 3 300, 2 900, 1 475, 1 385, and 1 105 cm^{-1}; δ 3.3 (m, 24-H), 3.28 (s, OMe), 2.74 (m, 6-H), 1.01 (s, 19-Me), 0.90 (d, *J* 6 Hz, 21-, 26-, and 27-Me), 0.69 (s, 18-Me), and 0.7—0.3 (cyclopropyl).

Ozonolysis of (*Z*)-6β-*Methoxy*-3α,5-*cyclostigmast*-24(28)-*ene* (17) *in the Presence of Tritiated Propan*-2-*ol*.—Compound (17) (5.9 mg) was dissolved in CH_2Cl_2 (10 ml) and iso-$C_3H_7O^3H$ (120 μl) (3.3 mCi $mmol^{-1}$) was added. The ozonolysis was effected as described before and the recovered product, purified by preparative t.l.c. [benzene–ethyl acetate (9 : 1)] afforded 3.5 mg of 6β-methoxy-3α,5-cyclocholestan-24-one (18) (0.1 μ Ci $mmol^{-1}$).

Reduction of [23,23,25-^{3}H]-3β-*Hydroxycholest*-5-*en*-24-*one* (11) *with* $NaBH_4$.—[23,23,25-^{3}H]-3β-Hydryoxycholest-5-en-24-one (11) (40 mg, 1.32×10^5 d.p.m. $mmol^{-1}$), obtained according to the method of Nicotra *et al.*,[8] dissolved in 95% ethanol (40ml), was reduced with $NaBH_4$ (40 mg) yielding 36 mg of [23,23,25-$^3H_3$]cholest-5-ene-3β,24-diol (12) which was crystallized to constant specific activity (1.33×10^5 d.p.m. $mmol^{-1}$).

6β-*Methoxy*-3α,5-*cyclocholestane* (21).—Freshly distilled methanesulphonyl chloride (0.5 ml) was added to a cooled (0 °C) solution of (19) (32 mg) in dry pyridine (2 ml). After 16 h at 0 °C, usual work-up afforded the crude mesylation product (20) which was dissolved in dry ethyl ether (5 ml) and stirred overnight at room temperature with $LiAlH_4$ (30 mg). The excess of $LiAlH_4$ was then destroyed with water and the precipitate filtered off. The filtrate was evaporated *in vacuo* and the product obtained was purified by chromatography (benzene) affording 28 mg of 6β-methoxy-3α,5-cyclocholestane (21), m.p. 79—80 °C (methanol) (lit.,[11] 79 °C); $\nu_{max.}$ 2 900, 1 475, 1 385, and 1 105 cm^{-1}; δ 3.27 (s, OMe), 2.71 (m, 6-H), 1.00 (s, 19-Me), 0.90 (d, *J* 6 Hz, 21-Me), 0.87 (d, *J* 6 Hz, 26- and 27-Me), 0.70 (s, 18-Me), 0.7—0.3 (cyclopropyl).

Cholest-5-*en*-3β-*ol* (10).—Compound (21) (25 mg) was dissolved in dioxan (7.5 ml) and stirred for 6 h at 75—80 °C with water (5 ml) and toluene-*p*-sulphonic acid (10 mg). The solvent was then removed *in vacuo*; usual work-up afforded 27 mg of crude product which was purified by preparative t.l.c. [benzene–ethyl acetate (8 : 2)] yielding 20 mg of cholest-5-en-3β-ol (10), the identity and purity of which were confirmed by t.l.c., g.l.c., i.r., and n.m.r. analysis, and by comparison with an authentic sample.

Administration of [26-^{3}H]*Lanosterol* (16) *to Shelled Seeds of* Pinus pinea *and Isolation of* [26-^{3}H]*Isofucosterol* (7).—[26-^{3}H]Lanosterol (16) (1.64×10^9 d.p.m.) was dissolved in acetone (0.5 ml); a few drops of Tween-80 were added and acetone was evaporated under a stream of nitrogen. Water (1 ml) was then added and the solution was deposited dropwise onto 60 shelled seeds of *Pinus pinea* disposed into boxes filled with paper. After one day a drop of water was deposited on each seed; the paper was maintained moist during all the germination, which was allowed to proceed until the roots were 0.5—1 cm long. The germinated seeds were macerated in methanol and the sterol fraction (564 mg), isolated as described by Raab *et al.*,[3] was chromatographed [benzene–hexane (1 : 1)] to yield a mixture of tritiated isofucosterol and sitosterol (140 mg), which was acetylated. From the crude aceylation product [26-^{3}H]isofucosteryl acetate (6) (7 mg, 2.30×10^6 d.p.m. of ^{3}H) pure by g.l.c. (LAC 796 1%) was recovered after chromatography on 20% $AgNO_3$–silica gel [benzene–hexane (1 : 1)]. This compound was treated overnight at room temperature with 5% methanolic KOH (4 ml); the solvent was removed *in vacuo*, and the usual work-up afforded [26-^{3}H]isofucosterol (7) (6 mg, 2.22×10^6 d.p.m.).

[26-^{3}H]*Cholest*-5-*en*-3β-*ol* (13).—[26-^{3}H]Isofucosterol (7) (6 mg, 2.22×10^6 d.p.m.) was submitted to the degradation procedure described for the cold sample to yield [26-^{3}H]-cholest-5-en-3β-ol (13) (2.76×10^5 d.p.m.) pure by g.l.c. (SE 30, 2.5%).

Administration of [26-^{3}H,26-^{14}C]*Cholest*-5-*en*-3β-*ol to Rat-liver Mitochondria and Isolation of Propionic Acid*.—[26-^{3}H]Cholest-5-en-3β-ol (13) (2.76×10^5 d.p.m.) was mixed with [26-^{14}C]cholest-5-en-3β-ol (8.62×10^4 d.p.m.; ^{3}H : ^{14}C = 3.20 : 1). Potassium propionate was added (12 mg), and the mixture was incubated with 6 g of rat-liver mitochondria as described by Mitropoulos *et al.*[5] After 8 h the incubation mixture was acidified with H_2SO_4 and submitted to steam-distillation. To the distillate (1.5 l) 0.1N KOH was added to pH 8.9. The water was evaporated *in vacuo* and the residue (175 mg), diluted with cold potassium

propionate (1 mg), was dissolved in acetonitrile (15 ml) and refluxed for 1.5 h with *p*-bromophenacyl bromide (423 mg) and dicyclohexyl-18-crown-6 (9.3 mg). The solvent was removed *in vacuo* and the residue (606 mg) was chromatographed (benzene) yielding 48 mg of crude *p*-bromophenacyl propionate. This was purified by preparative t.l.c. [benzene–ethyl acetate (97 : 3)] and the recovered *p*-bromophenacyl propionate (41 mg) was crystallized from hexane and counted (see Table).

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