mmol) of the mixture of **51** and **52** in 5 ml of absolute ethanol was heated in a sealed ampoule at 108° for 24 hr and then at 125° for 16 hr. Tlc (6% ethanol–benzene) showed the presence of only two products, the starting material and a product with larger R_f. The solvent was removed and the brown oily residue subjected to preparative tlc (developed three times with 5% ethanol–benzene), to yield 26 mg (65%) of "starting material" and 14 mg (35%) of isomerized product: tlc (6% ethanol–benzene) only one spot, R_f 0.50; ir ($CHCl_3$) 3400 (sharp, NH), 2980, 2940, 2860, 1730 (ester), 1640 (amide), 1600, 1525, 1480, 1460, 1395, and 1370 cm^{-1}; nmr δ 8.15 (mult, 1 H, C_7–H), 7.20 (mult, 3 H, C_{4-6}–H), 5.50 (broad singlet, 1 H, C_2'–H), 4.17 (q, 2 H, CH_2CH_3), 3.23 and 3.13 (2 singlets, separated 5 Hz, 3 H, N–CH_3), 2.0 (broad S, 1 H, NH), 1.47 and 1.29 (2 singlets, 6 H, CH_3CCH_3), 1.29 (t, 3 H, CH_2CH_3); mass spectrum (160°), *m/e* 320 (M^+), identical with that for **51** and **52**, except for a stronger signal at *m/e* 304 ($M^+ - CH_4$) and a weaker one at *m/e* 247 ($M^+ - CO_2C_2H_5$) and 245.

The nmr spectrum of the isolated "starting material" showed a change in that the ratio of the two signals from the C_9-proton was reversed (now δ 5.65/5.29 = 1:2), indicating that only the cis enantiomers **51** have been isomerized.

Acknowledgment. We are indebted to Professor R. J. F. Nivard for reading the manuscript.

Reduction of Δ^{24} of Lanosterol in the Biosynthesis of Cholesterol by Rat Liver Enzymes. II. Stereochemistry of Addition of the C-25 Proton

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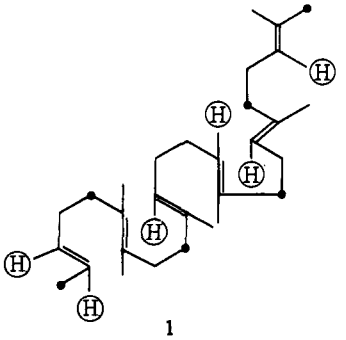
Contribution from the Worcester Foundation for Experimental Biology, Inc., Shrewsbury, Massachusetts 01545. *Received September 13, 1972*

Abstract: *Mycobacterium smegmatis* converts cholesterol to (25*S*)-26-hydroxycholest-4-en-3-one. It was shown, with the use of [$^{14}C_5$]cholesterol biosynthesized from [2-^{14}C]mevalonic acid in the S-10 fraction of rat livers, that the 26-^{14}C atom bore the oxygen atom. Evidence is provided that the 26-hydroxylation by *M. smegmatis* proceeds without loss of the C-25 hydrogen. Barring rearrangement and migration of this hydrogen, it may be inferred that the C-26-hydroxylation proceeds without epimerization at C-25. Previously, we demonstrated that in the reduction of Δ^{24} of lanosterol a 24-*pro-S* hydrogen is added. It follows that the reduction of Δ^{24} of lanosterol in the biosynthesis of cholesterol in the S-10 fraction of rat liver entails the overall cis addition of two hydrogen atoms.

It is now well established that the biosynthesis of squalene (**1**) proceeds with the incorporation of six

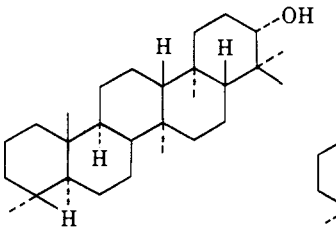


1

•, derived from C-2 of MVA
Ⓗ, derived from 4-*pro-R* hydrogen of MVA

C-2 carbon atoms of mevalonic acid[2] (MVA) and six 4-*pro-R* hydrogen atoms of MVA. The location of the C-2 and 4-*pro* H atoms of MVA in squalene is as shown in **1**. The terminal methyls of squalene are derived from C-2 and C-3′ carbons of MVA. The cis geometry of the methyl derived from C-2 of MVA and of the hydrogen on *each* of the terminal double bonds of **1** has been proven.[3]

The operation of both oxidative and proton-initiated mechanisms of enzymatic cyclization of squalene are now firmly established. The 3-deoxytriterpenes are thought to be formed *via* a nonoxidative proton initiated attack on a terminal double bond of squalene.[4,5] This mechanism was shown to operate in the biosynthesis of the pentacyclic triterpene tetrahymanol (**2**) in the proto-



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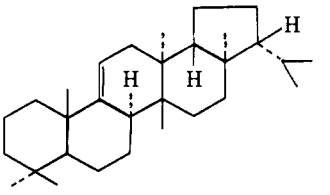

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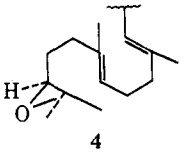

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zoan *T. pyriformis*. The overall process is equivalent to the acquisition by squalene of the elements of water (*loc. cit.*). It seems likely that in the fern *Oleoandra*

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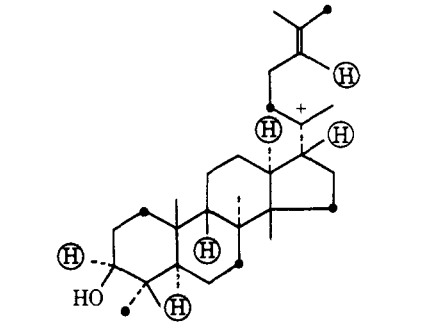
wallichii, tetrahymanol (**2**) is formed in as imilar manner.[6] Evidence consistent with the operation of a nonoxidative mechanism in the biosynthesis of fern-9-ene (**3**) has also been presented.[7]

The oxidative cyclization of squalene involves the initial formation of (3*S*)-2,3-oxidosqualene[8,9] (**4**). Enzymatic opening of the oxide is assumed to generate an electron deficiency at C-2, and there ensues cyclization.[10,11] In the rat liver enzyme system, the cyclization is thought to produce the C-20 cation **5** in a free[10] or transiently stabilized form,[11] which after rearrangement and loss of the C-9 proton yields lanosterol[12-17] (**6**). It is accepted that the geometry at C-24 of lanosterol is the same as that of the terminal double bonds of squalene (**1**).



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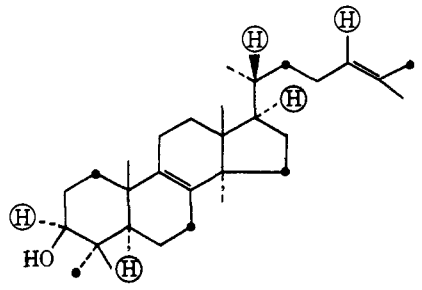

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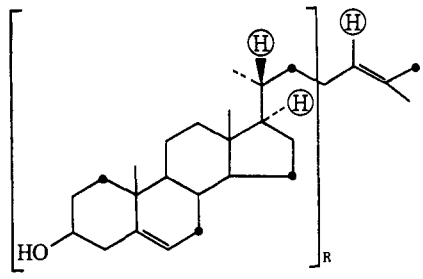

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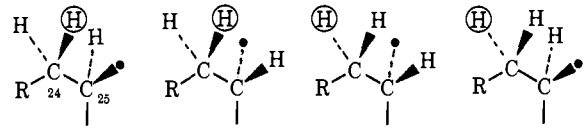
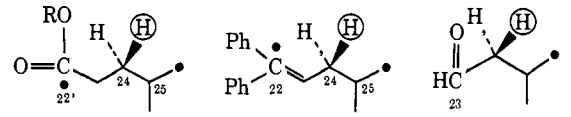
The biosynthetic conversion of lanosterol (**6**) to cholesterol requires the removal of three methyl groups, transposition of the 8(9) double bond to C-5, and reduction of the C-24 double bond. The question of the overall mechanism of reduction of the Δ^{24} by enzymes of the S-10 fraction of rat livers is the subject of the present paper.

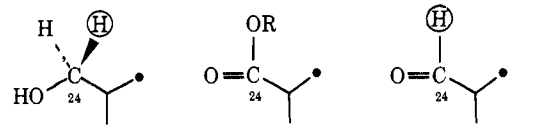
By definition, an ionic reduction of a double bond requires the acquisition by the olefinic carbons of a hydride ion and of a proton. The process may involve a cis or a trans addition of the two ionic species of hydrogen. Without implying a biosynthetic sequence, and solely for the sake of simplification of the current discussion, we will assume that desmosterol (**7**) is the immediate precursor of cholesterol. It follows that acquisition by desmosterol (**7**) of two hydrogen atoms at C-24 and C-25 will result in cholesterol. Should the addition of a hydrogen at C-24 occur from the backside, a cis reduction will result in cholesterol **8**, while trans



8 **9** **10** **11**



12a, R = H
b, R = CH_3 **13** **14**



15 **16a**, R = H
b, R = CH_3 **17**

reduction will yield cholesterol **9**. On the other hand, a front-side attack at C-24 will give, *via* a cis reduction **10**, and, *via* a trans reduction, **11**. Thus, for the definition of the mode of reduction of Δ^{24}, it is necessary to determine the prochirality at C-24 and C-25 in the resulting cholesterol.

Previously, we have established the chirality at C-24 of [$^3H_3$,$^{14}C_5$]cholesterol, biosynthesized from (3*RS*,4*R*)-[2-^{14}C,4-^{3}H]MVA by rat liver enzymes.[16,18] The side chain of the [$^3H_3$,$^{14}C_5$]cholesterol was cleaved by incubation with a bovine adrenal preparation, and the [$^3H_1$,-$^{14}C_2$]-4-methylpentanoic acid (**12a**) was isolated. The methyl ester **12b** was converted to the diphenyl olefin **13**, and this in turn was cleaved to the aldehyde **14**. Baeyer–Villiger oxidation of **14** and treatment of the product with $LiAlH_4$ resulted in [1-^{3}H,3-^{14}C]-2-methylpropan-1-ol (**15**). Oxidation of **15** with RuO_4 gave [3-^{14}C]-2-methylpropionic acid (**16**) devoid of tritium. On the other hand, oxidation of **15** with NAD^+ and yeast alcohol dehydrogenase (YADH) to aldehyde **17** proceeded without loss of tritium. The NAD^+–YADH oxidation of 2-methylpropan-1-ol proceeds with the abstraction of the 1-*pro*-*R* hydrogen.[19] Hence, it can

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be concluded that the [1-^{3}H,3-^{14}C]-2-methylpropan-1-ol has the 1-*S* configuration. Since C-1 of **15** corresponds to C-24 of cholesterol, it follows that the 24-*pro-R* hydrogen (tritium atom) of cholesterol originates from the 4-*pro-R* hydrogen (tritium atom) of MVA, while the newly introduced 24-*pro-S* hydrogen is derived from the water in the medium.[20] In view of this result, the possibility of a front-side addition of a hydrogen at C-24 as shown in **10** and **11** need not be considered.

With the completion of the work on C-24, we turned to the question of the prochirality at C-25 of cholesterol. As mentioned earlier, the two chemically equivalent terminal methyls differ in that one originates from C-2 and the other from C-3′ of MVA. That the addition of the hydrogen at C-25 is stereospecific could be deduced from experiments involving incubation of $^{14}C_5$-rat liver cholesterol with liver enzymes[21,22] and of [2-^{14}C]MVA with *Digitalis lanata*.[23] Thus, incubation of [$^{14}C_5$]cholesterol, biosynthesized from [2-^{14}C]MVA in rat livers, with a mouse liver enzyme system resulted in 26-hydroxycholesterol in which the methyl derived from C-3′ of MVA bore the oxygen atom.[21] Similarly, in tigogenin biosynthesized from [2-^{14}C]MVA in *Digitalis lanata*, the oxygen function resided on the terminal carbon atom derived from C-3′ of MVA.[23] It should be noted that the 25-*R* configuration of tigogenin has been proven.[24]

The definition of the prochirality at C-25 of the rat liver cholesterol and evidence for the overall cis reduction of the 24(25) double bond of lanosterol are presented. This work has been subject to preliminary communications.[25,26]

Experimental Section

Physical Measurements. Melting points were taken on a hot-stage apparatus and are corrected. Infrared (ir) spectra were recorded on a Perkin-Elmer 237 spectrophotometer as KBr wafers.[27] Ultraviolet spectra were measured on a Perkin-Elmer 202 spectrophotometer. Nuclear magnetic resonance (nmr) spectra were recorded on a Varian DA60 spectrometer at 60 MHz. Peaks are quoted in hertz (cycles per second) downfield from the tetramethylsilane internal standard. Mass spectra were measured on Varian Associates M-66 or Du Pont 21-491 instruments. A Hilger MK-III manual and O. C. Rudolph and Sons photoelectric polarimeter were used for the measurements of optical rotation.

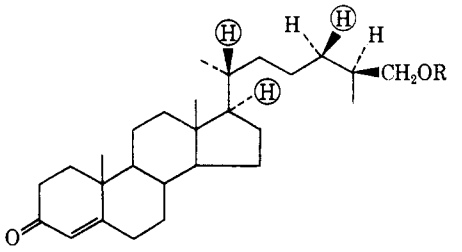
Chromatography. Gas chromatographic (glc) analyses were performed on a Perkin-Elmer Model 811 instrument equipped with a flame ionization detector. A 3-ft silanized glass column of 3% XE-60 on Chromosorb at 240–250° was used for all analyses. Silica gel (Merck $HF_{254+366}$) was used for preparative and analytical tlc in the indicated solvent systems. The products were detected under ultraviolet light and by color reactions with phosphomolybdic acid. Radiochromatograms were scanned on a Vanguard automatic chromatogram scanner (Model 880) with a tlc attachment.

Radioactivity Counting. Samples were counted on a Nuclear-Chicago Mark I automatic liquid scintillation counter. The samples were dissolved in 15 ml of a scintillation solution of toluene containing 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per 1000 ml.

Incubation of cholesterol with *Mycobacterium smegmatis* SG 346.[28] *Mycobacterium smegmatis* was grown in 3-l. Fernbach flasks containing 1 l. of nutrient medium: glucose 1%, proteose peptone 1%, casamino acids 1%, yeast extract 0.2%, and NaCl 0.6%. The pH of the medium was adjusted to 7.0 with 2 *N* NaOH, and the cultures were incubated at 37° with vigorous rotary shaking. After 84 hr the cells were harvested by centrifugation, each flask yielding 10–15 g of wet cells. This cake was immediately resuspended in a second medium containing NH_4NO_3 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, glycerin 2%, and a micronutrient solution[29] (1 ml/1000 ml of medium) at 7.0. Fresh cells (7.5 g) were suspended in the above medium (160 ml) in 1-l. flasks, and cholesterol (200 mg) in acetone (8 ml) was added to the broth. The flasks were shaken vigorously at 37° for 5–7 days. The transformation of cholesterol to 26-hydroxycholestenone was monitored on aliquots (10 ml) removed from *one* flask at intervals. The aliquot was worked up by refluxing with 20% KOH in methanol (10 ml) under nitrogen for 3 hr. The mixture was diluted with water and extracted with ether–ethyl acetate (1:1). The organic phase was washed with NaCl and water. After drying (Na_2SO_4) and evaporation of the solvent, the residue was analyzed by tlc (ethyl acetate–benzene (1:3)). Scanning of the cultures showed that cholest-4-en-3-one was produced for 3–4 days. The cholestenone was gradually metabolized and three more polar products appeared, one of which had the same R_f as 26-hydroxycholestenone. When most of the cholestenone had disappeared, the cells were harvested and refluxed with 10% KOH in 50% aqueous methanol for 4 hr under nitrogen. The saponification mixture was worked up as described above. The total yield of conjugated ketones (estimated by uv spectroscopy) varied from 4 to 15% in a series of incubations.

The nonsaponifiable material from several incubations was combined (2.25 g) and chromatographed on a silica column (200 g). Fractions eluted with benzene–ether (4:1) were combined (286 mg) and purified by preparative tlc (benzene–ethyl acetate (4:1)). Three major bands, detected by uv absorption, were eluted with ethyl acetate: band 1, 26-hydroxycholest-4-en-3-one (110 mg); band 2, 88 mg; band 3, 3.6 mg.

The product from band 1 was dissolved in ethyl acetate and decolorized with animal charcoal. Two crystallizations from ethyl acetate–hexane gave 26-hydroxycholest-4-en-3-one (**18a**) having



18a, R = H
b, R = OC—C_6H_4—*p*-Br

mp of 129–131°; $[\alpha]^{23}D$ +87.1 ± 1.2° (*c* 2.50, $CHCl_3$) and +86.1° (*c* 1.9, $CHCl_3$) (measured on a Hilger MK-III polarimeter). The product was homogeneous when tested by tlc (ethyl acetate–benzene (1:4)) and glc (3-ft XE-60 (3%) glass column at 260°; R_t 14 min).

The homogeneity of this product was further evaluated by preparative tlc (ethyl acetate–hexane (3:2), developed eight times). The recovered **18a** (26 mg) was crystallized twice (ethyl acetate–hexane): mp 146–147° (lit. mp 138–139.5;[30] 162–163.5;[28] in general, considerable variations of melting point were noted[28]); ir ν_{max} (KBr) 3380, 1660, and 1615 cm^{-1}; $[\alpha]^{25}D$ +89.4° (*c* 0.64, $CHCl_3$) (measured on a Rudolph & Sons photoelectric polarimeter);

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(28) K. Schubert, G. Kaufmann, and C. Hörhold, *Biochim. Biophys. Acta*, 176, 163, 170 (1969).
(29) Micronutrient solution: $FeSO_4 \cdot 7H_2O$, 0.5 g/l.; $ZnCl_2$, 0.2 g/l; $MnSO_4 \cdot H_2O$, 0.15 g/l.; $CaCl_2 \cdot 2H_2O$, 18.5 g/l.
(30) I. I. Zaretskaya, L. M. Kogan, O. B. Tikhomirova, J. D. Sis, N. S. Wulfson, V. I. Zaretskii, V. G. Zaikin, G. R. Skryabin, and I. V. Torgov, *Tetrahedron*, 24, 1595 (1968).

m/e 400 (M^+), 382 (M − 18), 367 (382 − 15), 297 (M − 103), 269 (M − 131), 124. An $[\alpha]D$ +95° ($CHCl_3$) was reported for a sample obtained by incubation of cholesterol with the organism *Mycobacterium*, sp 2104.[30]

The product was homogeneous when tested by tlc (ethyl acetate–benzene (1:4)) and glc (3-ft XE-60 (3%) glass column at 240°).

Incubation of [25-^{3}H,$^{14}C_5$]Cholesterol with *Mycobacterium smegmatis* 346.[28] Incubation of [2-^{14}C]mevalonic acid dibenzylethylenediamine salt (22 μCi) with a rat liver homogenate and the isolation and purification of the [$^{14}C_5$]cholesterol (5 μCi) were performed as described previously.[18] The synthesis of [25-^{3}H]cholesterol has also been reported.[31] The two radioactive samples were mixed and diluted with nonradioactive cholesterol (1.5 g) giving a sample containing 1.1×10^7 dpm of ^{14}C (^{3}H:^{14}C ratio 10.8).

M. smegmatis was grown on the nutrient medium described above yielding a total of 115 g of wet cells. Two portions of cells (50 g) were added to two 3-l. Fernbach flasks containing 1 l. of the second medium, and the remainder was divided into two 1-l. flasks each containing 100 ml of the second medium. The sample of [25-^{3}H,-$^{14}C_5$]cholesterol was dissolved in acetone (60 ml) and distributed into the four flasks (24 ml per 3-l. flask and 6 ml per 1-l. flask). The cultures were shaken at 37° for 84 hr, after which the cells were harvested by centrifugation and saponified with 10% KOH in aqueous methanol (1:1, 500 ml) under reflux for 3 hr (N_2 atmosphere). After evaporation of methanol, the alkaline solution was extracted with ether (three times) and with chloroform (once). To the broth recovered from the centrifugation, solid KOH was added (10% w/v), and the mixture was refluxed for 3 hr under nitrogen. The alkaline solution was continously extracted with ether; the ether was washed, dried, and concentrated to a residue. The residues from all the extracts were combined and fractionated by preparative tlc (ethyl acetate–benzene (1:3)). The chromatograms showed three major bands. Bands A and B contained cholest-4-en-3-one and cholesterol, respectively, while band C contained 26-hydroxycholest-4-en-3-one. The extracts of bands A and B were combined (200 mg, 7.7×10^6 dpm of ^{14}C) and dissolved in acetone (48 ml). The solution was distributed equally into six 1-l. flasks each containing the second medium (150 ml) and fresh cells of *M. smegmatis* (7.5 g). After shaking for 7 days at 37°, the cells were harvested and processed as described above, resulting in a recovery of 3.1×10^6 dpm of ^{14}C. The mixture was resolved by preparative tlc into bands A, B, C. The product of band C from both experiments was purified by chromatography on a column of silica (15 g). Elution with benzene–ether (4:1, 1600 ml) gave 26-hydroxycholest-4-en-3-one which was finally purified by preparative tlc (ethyl acetate–benzene (1:4)). The product was diluted with nonradioactive 26-hydroxycholest-4-en-3-one (total 80 mg) and crystallized once from ethyl acetate to give 40 mg of **18a**, mp 144–146° (sp act 5.24×10^5 dpm of ^{14}C per mmol; ^{3}H:^{14}C ratio 10.0).

Oxidation of [25-^{3}H,$^{14}C_5$]-26-Hydroxycholest-4-en-3-one to the Aldehyde[32] **19a.** [25-^{3}H,$^{14}C_5$]-26-Hydroxycholest-4-en-3-one (48 mg) was treated with Ag_2CO_3–Celite (700 mg, 10 equiv[32]) in toluene (20 ml) under reflux for 18 hr. The mixture was filtered and the residue washed with benzene. The filtrate and washings were combined and the solvent was removed *in vacuo*. A portion of the product was purified by preparative tlc (ethyl acetate–benzene (1:4)) to give [25-^{3}H,$^{14}C_5$]cholest-4-en-3-on-26-al (**19a**): mp 153–154°; ir ν_{max}^{KBr} 3020, 2690, 1720, 1675, 1620, 1265, 1235, 1225, 1180, 1025, 950, 925, 850, 750 cm^{-1}; mass spectrum *m/e* 398 (M^+), 370 (M − 28), 368 (M − 30), 356, 353, 328, 269, 229 (base peak).

[25-^{3}H,$^{14}C_4$]-26-Norcholest-4-en-3-one (19b). A solution of [25-^{3}H,$^{14}C_5$]cholest-4-en-3-on-26-al (**19a**) (30 mg) in toluene (10 ml) was refluxed with tris(triphenylphosphine)rhodium chloride (100 mg) under nitrogen for 6 hr.[33] The solvent was removed *in vacuo* and the dark red residue was dissolved in ether. The solution was passed through a column of grade 1 neutral alumina (10 g) and the column washed with several portions of ether to ensure complete removal of the steroids. The product obtained was purified by preparative tlc (ethyl acetate–hexane (1:3)) to give [25-^{3}H,$^{14}C_4$]-26-norcholest-4-en-3-one (**19b**) (18 mg) which was crystallized from methanol: mp 95.5–96° (sp act. 4.37×10^5 dpm of ^{14}C per mmol; ^{3}H:^{14}C ratio 11.87); ir ν_{max}^{KBr} 3020, 1675, 1615, 1265, 1225, 1180, 1100, 950, 925, 855 cm^{-1}; nmr spectrum 42.5 (singlet, 3 H; 18-CH_3), 53.0, 57.0 (doublet, 21-CH_3), 71.0 (singlet, 3 H, 19-CH_3), 345 (multiplet, 1 H, C-4 H); mass spectrum, *m/e* 370 (M^+) (base peak), 355 (M − 15), 328 (M − 42), 313, 247, 229.

Cholest-4-en-3-on-26-ol *p*-Bromobenzoate. A mixture of the microbially prepared 26-hydroxycholestenone (10 mg), pyridine (3 drops), and *p*-bromobenzoyl chloride (94 mg) in dry CH_2Cl_2 (1 ml) (freshly distilled from P_2O_5) was stored for 16 hr at ambient temperature. Water and ethyl acetate were added, the phases were separated, and the aqueous residue was extracted with ethyl acetate. The combined ethyl acetate solution was washed with ice-cold dilute HCl and a saline solution and then magnetically stirred with a saturated solution of sodium carbonate. The ethyl acetate solution was washed with a little saline, dried ($MgSO_4$), and concentrated to a residue. The residue was leeched with CCl_4 (4 × 1 ml). The CCl_4 solution was concentrated and the residue chromatogaphed on a silica gel column (4 g) prepared with hexane, and 20-ml fractions were collected. The column was first percolated with hexane and then with increasing amounts of benzene in hexane (30 fractions were collected). The product was eluted with a mixture of benzene–ethyl acetate (199:1) in fractions (31–64). The combined residue (17 mg) was dissolved in CH_2Cl_2 (2 ml) and stirred with a saturated solution of sodium carbonate for 1 hr. Ether was added and the ether solution was washed with a little saline, dried, and concentrated. The residue was homogeneous when tested by tlc (silica gel, benzene–ethyl acetate (17:3)). The solid was slowly crystallized several times from ethyl acetate–methanol: mp 107–109°; nmr 42 (3 H, 18-CH_3) 52, 57, 76 (19-, 21-, and 27-CH_3), 250 (d, CH_2OH), 344 (1 H, C-4 H), 456, 474 (d, aromaticprotons); *m/e* 582 and 584 (M^+).

Results and Discussions

The definition of the overall mechanism of the *in vitro* enzymatic reduction of Δ^{24} of lanosterol by rat liver enzymes requires the determination of the prochirality at C-24 and C-25 of the resulting cholesterol. The prochirality at C-24 as determined on [$^3H_3$,$^{14}C_5$]-cholesterol biosynthesized from (3*R*,4*R*)-[2-^{14}C,4-^{3}H]-mevalonic acid in the S-10 fraction of rat livers has been described in detail. It was shown that a 24-*pro-S* hydrogen is added resulting in (17α,20*R*,24*R*)-[$^3H_3$,-$^{14}C_5$]cholesterol.[16,18]

The definition of the situation at C-25 was more complex and posed considerable technical difficulties. In essence, there are two separate problems that required solution. First, it was necessary to differentiate the terminal methyls, one of which originates from C-2 and the other from C-3′ of mevalonic acid.[2,3] Secondly, the configuration at C-25 had to be established.

From the outset we realized that differentiation of the 26 and 27 carbons could best be accomplished enzymatically, *e.g.*, *via* stereospecific hydroxylation of *one* of the terminal methyls of [$^{14}C_5$]cholesterol biosynthesized from [2-^{14}C]mevalonic acid in the S-10 fraction. Removal of the oxygenated carbon and determination of its ^{14}C content would provide the required information on its origin with respect to mevalonic acid. We also envisaged the use of the 26-hydroxylated analog for the determination of the C-25 configuration.

We considered two enzymatic systems capable of hydroxylating cholesterol specifically at the terminal methyl derived from C-3′ of mevalonic acid. The hydroxylation by mouse or rat liver enzymes could be employed for the determination of the origin of the oxygen bearing carbon.[21,22] Unfortunately these enzymatic systems yield at best minute amounts of 26-oxygenated material totally insufficient for the determination of C-25 configuration by the available conventional means. An alternative route involved the administration of [$^{14}C_5$]cholesterol biosynthesized from [2-^{14}C]mevalonic acid in the S-10 fraction of rat liver

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(32) M. Fetizon and M. Golfier, *C. R. Acad. Sci., Ser. C*, **276**, 900 (1968).
(33) Y. Shimizu, H. Mitsuhashi, and E. Caspi, *Tetrahedron Lett.*, 4113 (1966).

to a suitable plant, *e.g.*, *Digitalis*, and isolation of tigogenin and diosgenin which are known to have the 25*R* configurations.[23,24] Unfortunately, even though this route seemed more promising, it had a number of pitfalls. Firstly, the conversion of cholesterol to sapogenins in *Digitalis* plants is a capricious process depending on many unknown variables and proceeds at best in rather poor yield.[31] Secondly, the feeding of relatively large amounts of biosynthetic cholesterol of comparatively low specific activity to such plants caused additional concern.

Fortuitously, a much more direct approach became available when Schubert, *et al.*,[28] and Torgov, *et al.*,[30] independently reported on the conversion of cholesterol to 26-hydroxycholestenone by species of *Mycobacterium*. They have incubated up to 10 g of cholesterol and reported the isolation of about 100 mg of the 26-hydroxycholestenone.[30] Clearly this approach, if successful, provided a very distinct advantage because it allowed for the preparation of sufficient amounts of material for our chemical manipulations. We therefore opted for this route. It should be kept in mind, however, that at this stage it was not known whether the hydroxylation by the *Mycobacterium* is stereospecific, although the fact that other enzymatic systems hydroxylated the terminal carbons of cholesterol stereospecifically gave us some confidence that the same might occur in the present case.

Through the kind cooperation of Professor Kurt Schubert of the Zentral Institut für Mikrobiologie und Experimentelle Therapie in Jena (DDR), we obtained a specimen of *M. smegmatis*. In our hands, the conversion of cholesterol to 26-hydroxycholestenone proceeded in only about 0.1–0.2% yield. However, after considerable efforts, we accumulated about 110 mg of material which was extensively purified and crystallized (mp 129–131°). The rotation of the product $[\alpha]^{23}D$ 87.1 ± 1.2° (in chloroform) was determined on a Hillger MK-III manual polarimeter. Subsequently, the purity of this material was further tested by chromatography and crystallization (mp 146–147°). The rotation of the reprocessed sample, $[\alpha]^{25}D$ 89.4° (in chloroform), was determined on a Rudolph and Sons photoelectric polarimeter. The specimen from the first and second purification was homogeneous when analyzed on thin layer and gas–liquid chromatography and its mass spectrum showed a peak *m/e* 400 (M^+). Considerable variations in the melting point of the product have been noted previously.[28]

The twice purified material was converted to cholest-4-en-3-on-26-ol 26-*p*-bromobenzoate (mp 107–109°). The mass spectrum of the *p*-bromobenzoate showed peaks at *m/e* 582 and 584 for (M^+). It was proven by X-ray crystallography that the cholest-4-en-3-on-26-ol 26-*p*-bromobenzoate has the 25*S* configuration.[25] Details of the X-ray studies will be published elsewhere.

Initially, attempts were made to define the C-25 configuration on the basis of apparent variations of specific $[\alpha]D$ rotations of several specimens of 26-hydroxycholestenone prepared by different routes. A specimen derived from kryptogenin, which is known to have the 25*R* configuration, was used as a reference. Unfortunately, the rotational differences lead to an erroneous conclusion,[26] and the problem is currently under study.

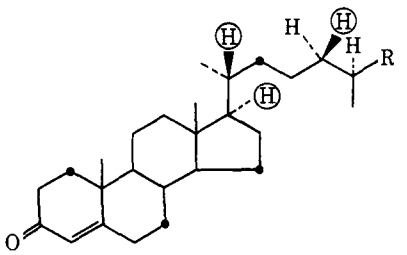
Having established the 25*S* configuration of the microbially prepared 26-hydroxycholestenone, we addressed ourselves to the question of the origin of the carbon bearing the oxygen function. For this purpose [$^{14}C_5$]cholesterol biosynthesized from [2-^{14}C]mevalonic acid in the S-10 fraction of rat livers was used. We have previously proven that oxygenation of a terminal carbon of cholesterol in the biosynthesis of tigogenin in *D. lanata* proceeds without the loss of the C-25 hydrogen.[31] However, since the mechanism of hydroxylation of primary methyls is not yet fully understood, it is conceivable that a different mechanism (*e.g.*, *via* Δ^{25}, etc.) might operate in *M. smegmatis*. For this reason, we considered it advisable to add to the biosynthetic [$^{14}C_5$]cholesterol, [25-^{3}H]cholesterol,[31] and use the double-labeled specimen (^{3}H:^{14}C ratio 10.8) for the incubation with *M. smegmatis*. Indeed, incubation of this sample gave 26-hydroxycholestenone which showed a ^{3}H:^{14}C ratio of 10.0. Unchanged cholesterol (^{3}H:^{14}C ratio 10.3) was also recovered (see Table I).

Table I. Specific Activity and ^{3}H:^{14}C Ratios of Products Derived from the Incubation of [25-^{3}H,$^{14}C_5$]Cholesterol with *M. Smegmatis*

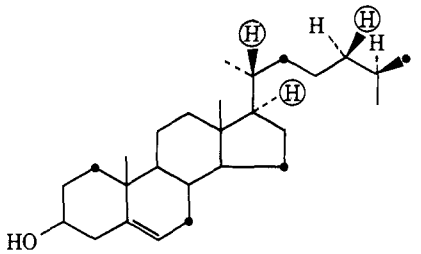
	Specific activity of ^{14}C × 10^5 dpm/mmol	—^{3}H–^{14}C ratio— Isotopic	Atomic
[25-^{3}H,$^{14}C_5$]Cholesterol (incubated)		10.8	
[25-^{3}H,$^{14}C_5$]Cholesterol (recovered)		10.3	
[25-^{3}H,$^{14}C_5$]-26-Hydroxycholestenone	5.24	10.0	1:5
[25-^{3}H,$^{14}C_4$]-26-Norcholestenone	4.37	11.9	1:4.17

It is clear that nearly all the tritium was retained in the [25^{3}H,$^{14}C_5$]-26-hydroxycholestenone. *This proves that the C-25 proton is not lost in the process of C-26 hydroxylation of cholesterol by M. smegmatis.* Barring a rearrangement and migration of this hydrogen, it seems likely that the hydroxylation proceeds without *epimerization at C-25*. The [25^{3}H,$^{14}C_5$]-26-hydroxycholestenone was diluted with nonradioactive material to a specific activity of 5.24 × 10^5 dpm/mmol of ^{14}C and oxidized with silver carbonate on Celite to the 26-aldehyde.[32] The aldehyde was decarbonylated by refluxing with tris(triphenylphosphine)rhodium chloride[33] to yield [25^{3}H,$^{14}C_4$]-26-norcholestenone (specific activity 4.37 × 10^5 dpm/mmol of ^{14}C; ^{3}H:^{14}C ratio 11.9; atomic ratio 1:4.17) (see Table I).

It is apparent that removal of the terminal carbon bearing the oxygen function of **19a** involved a 16.6% drop in ^{14}C specific activity in **19b**. This corresponds to the loss of 0.83 atom of ^{14}C. The drop in the ^{14}C specific activity was accompanied by a parallel increase of 16% in the ^{3}H:^{14}C ratio (11.9) of the 26-norcholestenone (**19b**) as expected. The reason for the loss of only 0.83 atom of ^{14}C instead of one atom is not clear and multiple factors of chemical or enzymatic nature may be responsible for it. It is worthy of note that ^{3}H specific activities of the 26-hydroxycholestenone (5.24 × 6 10^6 dpm/mmol) and 26-norcholestenone (5.2 × 10^6 dpm/mmol) are the same, indicating that the decarbonylation of the 26-aldehyde proceeded without loss of tritium.[25,26] The results subsequently reported by



19a, R = CHO
b, R = H



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Walborsky and Allen[34] on the mechanism of decarbonylation of aldehydes with tris(triphenylphosphine)-rhodium chloride confirm our observations.

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In any event, it is abundantly clear that the terminal carbon of cholesterol, derived from C-2 of mevalonic acid, bears the oxygen function. Since the 26-hydroxycholestenone has the 25*S* configuration as in **18a**, it follows that cholesterol must have configuration **20**. This taken together with the proven addition of a 24-*pro-S* hydrogen indicates that reduction of the Δ^{24} of lanosterol by the enzymes of the S-10 fraction of rat livers can be formally considered as a cis addition of two hydrogens. In contrast the reduction of Δ^{24} of the sterol precursor of tigogenin in *Digitalis lanata* was recently shown to be equivalent to a trans reduction involving the addition of a 24-*pro-R*-hydrogen.[35]

Acknowledgment. This work was supported by grants from the National Institutes of Health, AM12156, HE10566, CA-K3-16614, and the National Science Foundation, GB8277. We are greatly indebted to Professor Kurt Schubert of the Zentral Instut für Mikrobiologie und Experimentelle Therapie of the Deutsche Akademie der Wissenschaften zu Berlin in Jena (DDR) for the specimen of *M. smegmatis*. Thanks are due to Professor Ch. Tamm of the University of Basel and Professor D. Arigoni of the E. T. H. in Zurich for letting us know their results prior to their publication.

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Crystalline Chain Conformation of Mycodextran[1a]

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Abstract: Mycodextran, also known as nigeran, is a linear polysaccharide consisting of α-D-glucose units joined by alternating α-1→4′ and α-1→3′ linkages. X-Ray analysis of polymer single crystals of mycodextran with the folded-chain structure led to an orthorhombic unit cell of dimensions a = 17.6 Å, b = 6.85 Å, and c (fiber axis) = 13.4 Å, with a twofold screw axis along the chain, for the crystalline polymer. A conformational analysis method based on minimum energy considerations and allowing four rotatable bonds shows that a hydrogen bond is possible between the O-2 and O-3′ hydroxyls of contiguous residues in the α-1→4′ linkage and another between the O-2 O-4′ hydroxyls of the residues in the α-1→3′ linkage. The results obtained by means of two different approaches, namely the one which analyzes conformations in terms of the angles ϕ and ψ and the other which considers the rotation of the residues around a virtual bond, are compared. The crystalline conformation of mycodextran is shown to be a "corrugated ribbon." An energetically favored scheme of chain folding in mycodextran is proposed.

Mycodextran, also known as nigeran, is a naturally occurring fungal polysaccharide. It was first isolated by Dox and Niedig[2] from the species *Penicillium expansum* and *Aspergillus niger*. More recently, Reese and Mandels[3] found that mycodextran constitutes up to 40% of the dry weight of the mycelium of some species of *Aspergillus* and *Penicillium* genera. Tung and Nordin[4] have shown that mycodextran is a major constituent of the mycelium cell wall of *Aspergillus niger*. Reese and Mandels[3] also isolated an α-D-glucanase having high specificity for mycodextran.

Chemical investigations[5-7] have shown that mycodextran is an exclusively α-D-glucan, composed of units linked together alternately through 1→3′ and 1→4′ linkages, as shown in Figure 1. The polymer may be considered either as a polymaltose with α-1→3′ linkages or as a polynigerose with α-1→4′ linkages. The repeating unit of this polysaccharide is therefore

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