Reduction of the 24,25-Double Bond of Lanosterol *in vivo* in the Rat. Stereochemistry of the Addition of the C-25 Proton in the Biosynthesis of Cholesterol

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The stereochemistry of addition of the 25-proton in the Δ^{24} reduction of lanosterol to cholesterol *in vivo* in the rat is shown to be the same as that in the S-10 fraction of rat liver homogenates.

It is also shown that oxidation of [1,2-$^3H_2$]octan-1-ol by the Pfitzner–Moffatt method proceeds with complete removal of tritium from C-2 of octanol. A similar oxidation of [25-^{3}H]-5α-cholestane-3β,26-diol to 3-oxo[25-^{3}H]-5α-cholestan-26-al resulted in the loss of 23% of tritium. In contrast, Collins oxidation of [1,2-$^3H_2$]octan-1-ol to octanal did not involve any loss of tritium from C-2.

ONE of the steps in the biosynthetic transformation of lanosterol (1) to cholesterol (2) is the reduction of the 24,25-double bond of lanosterol. We have previously shown [1,2] with the use of (24*R*)-[$^{14}C_5$,17α,20,24-$^3H_3$]-cholesterol (2), biosynthesized from (3*RS*,4*R*)-[2-^{14}C,4-^{3}H]mevalonic acid (MVA) in the S-10 fraction of a rat liver preparation,[3] that a 24-*pro*-*S*-proton is added.[1,2]

The addition of the proton at C-25 was shown [4,5] to occur from the *si*-face, resulting in cholesterol having the stereochemistry shown in (2). For this determination [$^{14}C_5$]cholesterol biosynthesized from [2-^{14}C]MVA in the rat liver enzyme preparation was mixed with [25-^{3}H]-cholesterol [6] and incubated with *M. smegmatis* [1,2,7] to yield (25*S*)-26-hydroxy[$^{14}C_5$,25-^{3}H]cholest-4-en-3-one,[4,5] and not the (25*R*)-stereoisomer as initially thought.[8] The microbial hydroxylation proceeded without loss (or epimerization) of the C-25 hydrogen, and it was

[1] J. B. Greig, K. R. Varma, and E. Caspi, *J. Amer. Chem. Soc.*, 1971, **93**, 760.

[2] E. Caspi, K. R. Varma, and J. B. Greig, *Chem. Comm.*, 1969, 45.

[3] J. W. Cornforth, R. H. Cornforth, C. Donninger. G. Popjak, Y. Shimizu, S. Ichii, E. Forchielli, and E. Caspi, *J. Amer. Chem. Soc.*, 1965, **87**, 3224.

[4] D. J. Duchamp, C. G. Chidester, J. A. F. Wickramasinghe, E. Caspi, and B. Yagen, *J. Amer. Chem. Soc.*, 1971, **93**, 6283.

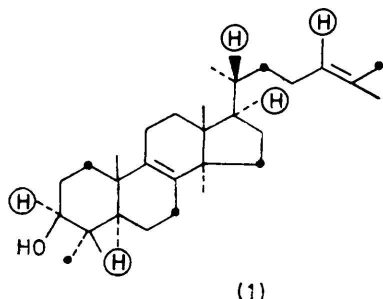
[5] M. G. Kienle. R. K. Varma, L. J. Mulheirn, B. Yagen, and E. Caspi, *J. Amer. Chem. Soc.*, 1973, **95**, 1996.

[6] K. R. Varma, J. A. F. Wickramasinghe, and E. Caspi, *J. Biol. Chem.*, 1969. **244**, 3951.

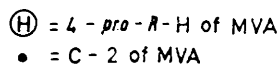
[7] K. Schubert, G. Kaufman, and C. Hörhold, *Biochim. Biophys. Acta*, 1969, **176**, 163, 170.

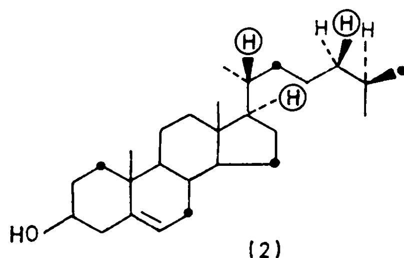
[8] E. Caspi, M. G. Kienle, K. R. Varma, and L. J. Mulheirn, *J. Amer. Chem. Soc.*, 1970, **92**, 2161.

shown that the C-26 methyl group of cholesterol derived from C-2 of MVA bore the oxygen function.[4,5,8] Consequently the reduction of the 24,25-double bond in the



(1)





(2)

S-10 fraction of rat livers proceeded by the *cis* acquisition of two hydrogens.

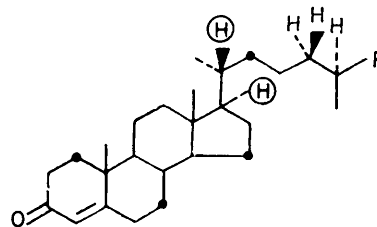
It has been demonstrated [9] that tigogenin has the (25*R*)-configuration and that the methyl derived from C-3′ of MVA bears the oxygen function.[10] Thus it may be inferred that the addition of the C-25 hydrogen in the formation of this sapogenin in *D. lanata* occurred from the same side of the double bond of its Δ^{24}-precursor as in the case of reduction of the 24,25-double bond in rat liver homogenates. However, Canonica *et al.*[11] have reported that in contrast to rat liver, the addition of a C-24 *pro-R*-hydrogen takes place in the biosynthesis of tigogenin in *D. lanata*. Hence in the biosynthesis of tigogenin a *trans*-addition of two hydrogens occurs in the reduction of the 24,25-double bond of the plant precursor, and the overall mechanism differs from that in the S-10 fraction of rat livers.

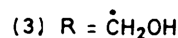
The question then arose whether the differences in Δ^{24} reduction are species dependent and/or system dependent (*in vivo vs. in vitro*). Such variations were previously noted for catabolic and anabolic transformations of other steroids.[12] For example, investigations concerning the metabolism of steroid hormones having a 3-oxo-4-ene system revealed the presence of 5α- and 5β-reductases.[13] Occasionally, *e.g.* in rat liver incubations [13,14] and perfusions [15,16] of the 4,5-saturated metabolites produced, only those having the 5α-configuration were obtained. It was demonstrated that the stereochemistry of the 3-oxo-4-ene reduction was species,[14] sex,[13,14] and system (*in vivo* and *in vitro*) dependent.[15,17] In some instances the 5α- and 5β-reductases have been separated.[18,19]

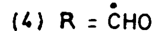
In view of these and other precedents [20-23] we considered the possibility of the operation of alternative courses of Δ^{24} reduction *in vitro* and *in vivo*.

RESULTS AND DISCUSSION

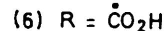
Biosynthetic [^{14}C]cholesterol (2) obtained from *in vivo* administration of [2-^{14}C]MVA to rats was mixed with a synthetic sample of [25-^{3}H]cholesterol (2) [6] (^{14}C specific activity $9{\cdot}0 \times 10^{6}$ disint. min^{-1} mmol^{-1}; ^{3}H : ^{14}C, 7·1) and the doubly labelled specimen was incubated with *M. smegmatis*. The recovered sterols were fractioned by t.l.c., and the zone corresponding to 26-hydroxycholestenone (3) was saved, and those corresponding to cholestenone and unchanged cholesterol were combined and reincubated twice as above. From the three incubations a total of $4{\cdot}9 \times 10^{4}$ disint. min^{-1} of ^{14}C of a crude 26-hydroxycholestenone (3) was obtained which corresponds to 0·54% conversion of the administered [$^{14}C_5$]-cholesterol. The product was purified by t.l.c. and




(3) R = $\dot{C}H_2OH$


(4) R = $\dot{C}HO$

(5) R = H


(6) R = $\dot{C}O_2H$

diluted with non-radioactive (25*R*)-26-hydroxycholestenone (3), obtained from kryptogenin.[8] The alcohol (3)

[9] L. Fieser and M. Fieser, ' Steroids,' Reinhold, New York, 1959, p. 837.
[10] R. Joly and Ch. Tamm, *Tetrahedron Letters*, 1967, 3535.
[11] L. Canonica, F. Ronchetti, and G. Russo, *J.C.S. Chem. Comm.*, 1972, 1309.
[12] R. I. Dorfman and F. Ungar, in ' Metabolism of Steroid Hormones,' Academic Press, New York, 1965, pp. 289, 405.
[13] E. Forchielli, K. Brown-Grant, and R. I. Dorfman, *Proc. Soc. Exptl. Biol. Med.*, 1958, **99**, 594.
[14] F. E. Yates, A. L. Herbst, and J. Urquhart, *Endocrin.*, 1958, **63**, 887.
[15] E. Caspi, H. Levy, and O. M. Hechter, *Arch. Biochem. Biophys.*, 1953, **45**, 169.
[16] E. Caspi and O. Hechter, *Arch. Biochem. Biophys.*, 1956, **61**, 299.
[17] G. M. Tomkins, *J. Biol. Chem.*, 1957, **225**, 13.
[18] E. Forchielli and R. I. Dorfman, *J. Biol. Chem.*, 1956, **223**, 443.
[19] J. S. McGuire, jun., and G. M. Tomkins, *J. Biol. Chem.*, 1960, **235**, 1634.
[20] D. C. Wilton, K. A. Munday, S. J. M. Skinner, and M. Akhtar, *Biochem. J.*, 1968, **106**, 803.
[21] E. Caspi, J. B. Greig, P. J. Ramm, and K. R. Varma, *Tetrahedron Letters*, 1968, 3829.
[22] J. M. Zander and E. Caspi, *J. Biol. Chem.*, 1970, **245**, 1682.
[23] L. J. Mulheirn and P. Ramm, *Chem. Soc. Rev.*, 1972, **1**, 259.

showed a constant ^{14}C specific activity of $1{\cdot}01 \times 10^5$ disint. min^{-1} $mmol^{-1}$, and a $^3H : ^{14}C$ ratio of 6·72. Two specimens of the 26-*p*-bromobenzoate were prepared which showed $^3H : ^{14}C$ ratios of 6·8 and 6·65 (see Table 1).

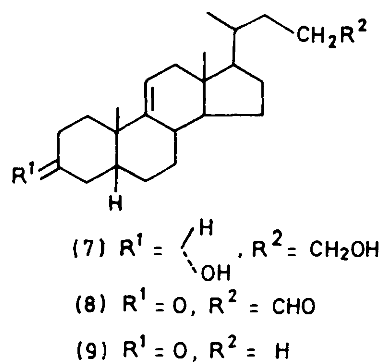
TABLE 1

Specific activities and $^3H : ^{14}C$ ratio of products derived from [$^{14}C_5$,25-^3H]cholesterol ($^3H : ^{14}C$, 7·1)

Compound and crystallization	^{14}C Specific activity $\times 10^5$ (disint. min^{-1} $mmol^{-1}$)	$^3H : ^{14}C$ Ratio	
		Isotopic	Atomic
26-Hydroxy[$^{14}C_5$,25-^3H]cholest-4-en-3-one *p*-bromobenzoate			
1	0·9	6·7	
2	1·0	6·9	
Average	0·95	6·8	
26-*p*-Bromobenzoate (after further dilution of alcohol)			
1	0·15	6·35	
2	0·15	6·65	
3	0·15	7·00	
Average	0·15	6·67	
26-Hydroxy[$^{14}C_5$,25-^3H]cholest-4-en-3-one (3)			
1	1·05		
2			
3	0·96	6·55	
4		6·9	
Average	1·01	6·72	1·00 : 5
[$^{14}C_4$,25-^3H]-26-Norcholest-4-en-3-one (5)			
1	0·84	7·20	
2	0·84	7·35	
3	0·82	7·60	
Average	0·83	7·38	0·88 : 4

Previously we employed [4,5,8] the Fetizon and Golfier [24] procedure for the conversion of 26-hydroxycholestenone (3) into the aldehyde (4). However, in our hands the method proved capricious. In a search for a more reliable oxidation procedure we explored the method of Pfitzner and Moffatt.[25] The efficacy of the oxidation was first tested on 5β-chol-9(11)-ene-3α,24-diol (7) which was converted into the 3-oxo-24-al (8) in acceptable and reproducible yield. The aldehyde (8) was then decarbonylated with tris(triphenylphosphine)rhodium chloride [5,26] to give the 24-nor-3-ketone (9).

In view of these encouraging results we used the Pfitzner–Moffatt method for the oxidation of the microbially prepared 26-hydroxycholestenone (3): this gave the aldehyde (4) which was extensively purified by t.l.c. Attempts at crystallization of an aliquot portion (10%) of the recovered aldehydic fraction failed.*



Decarbonylation of the chromatographic fraction of aldehyde (4) with tris(triphenylphosphine)rhodium chloride [26] gave 26-norcholestenone (5) which showed a ^{14}C specific activity of $0{\cdot}83 \times 10^5$ disint. min^{-1} $mmol^{-1}$ and a $^3H : ^{14}C$ ratio of 7·38 (see Table 1). The results indicate that the decarbonylation proceeded with a loss of nearly one fifth of the ^{14}C radioactivity initially present in the 26-hydroxy[$^{14}C_5$]cholestenone. However, it is also apparent that the *ca.* 20% drop in ^{14}C specific activity *was not accompanied by a parallel rise in the* $^3H : ^{14}C$ *ratio*. The calculated $^3H : ^{14}C$ ratio for (5), corresponding to 1 atom of ^3H and 4 atoms of ^{14}C, was 8·4 We have previously established that the described decarbonylation of the aldehyde (4) proceeds without loss of ^3H from C-25.[4,5,8] In view of these observations we considered the possibility that some ^3H was lost during the Pfitzner–Moffatt oxidation, *e.g.* by a keto–enol equilibration of the 26-aldehyde (4). We decided to evaluate this hypothesis and in the preliminary tests the readily accessible [1,2-$^3H_2$]octan-1-ol was used.

A modified Cornforth [27] procedure was used for the reduction of octanal to [1,2-$^3H_2$]octanol. For the determination of the distribution of the isotopic hydrogen,† the [1,2-$^3H_2$]octanol (11) was oxidized with RuO_4 [28] to [2-^3H]octanoic acid (19) which retained 9% of the ^3H originally present (see Table 2). We have also demonstrated that the remaining tritium is located at C-2 of the octanol (see below).

With the information on the distribution of the isotopic hydrogens in the alcohol at hand, two oxidation experiments were carried out as outlined in the Scheme. Oxidation of [1,2-^3H]octanol (11) with the modified Collins reagent [29] gave [1,2-$^3H_2$]octanal (12). The aldehyde was then treated with trifluoroperacetic acid and the resulting heptyl formate (13) reduced with

* It was noticed, however, that during the attempted crystallization a considerable amount of the aldehyde was oxidized, apparently to the 26-acid (6). In view of this, the homogeneity of the remaining 90% of the aldehydic fraction which had been stored at −15° under N_2 was re-examined by t.l.c. Unfortunately, in spite of these precautions, a considerable amount of 26-acid (6) was formed. Consequently, the product was rechromatographed and the recovered noncrystalline aldehyde (4) was immediately subjected to decarbonylation.

† Specific activities of the radioactive alcohols, aldehydes, and acids were determined for their phenylurethane, semicarbazone, and amide derivatives respectively. The derivatives were identical (m.p. and R_F) with authentic derivatives.

24 M. Fetizon and M. Golfier, *Compt. rend.*, 1968, **276**, 900.
25 K. E. Pfitzner and J. G. Moffatt, *J. Amer. Chem. Soc.*, 1965, **87**, 5670.
26 Y. Shimizu, H. Mitsuhasi, and E. Caspi, *Tetrahedron Letters*, 1966, 4113.
27 R. H. Cornforth, *Tetrahedron*, 1970, **26**, 4635.
28 D. M. Piatak, H. B. Bhat, and E. Caspi, *J. Org. Chem.*, 1969, **34**, 112; D. M. Piatak, G. Herbst, J. Wicha, and E. Caspi, *ibid.*, p. 116.
29 R. Ratcliffe and R. Rodehorst, *J. Org. Chem.*, 1970, **35**, 4000.

TABLE 2

Specific activities of transformation products of $[1,2\text{-}^3H_2]$octanol (11)

Compound and recrystallization	Derived from	^3H Specific activity (disint. min^{-1} $mmol^{-1}$)
n-Octyl phenylurethane	(11)	
1st		1.72×10^7
2nd		1.76×10^7
3rd		1.76×10^7
n-Octanal semicarbazone	(16)	
1st		1.29×10^7
2nd		1.07×10^7
3rd		1.04×10^7
4th		1.04×10^7
n-Octanal semicarbazone	(12)	
1st		1.46×10^7
2nd		1.38×10^7
3rd		1.34×10^7
n-Heptyl phenylurethane	(18)	
1st		3.3×10^4
2nd		3.1×10^4
3rd		3.8×10^4
n-Heptyl phenylurethane	(14)	
1st		1.69×10^6
2nd		1.52×10^6
3rd		1.65×10^6
n-Heptyl phenylurethane after dilution with heptanol	(14)	
1st		8.45×10^4
2nd		8.67×10^4
3rd		8.45×10^4
n-Heptanamide	(15)	
1st		No significant counts
2nd		No significant counts
3rd		No significant counts
n-Hexanamide	(19)	
1st		1.50×10^6
2nd		1.60×10^6
3rd		1.56×10^6

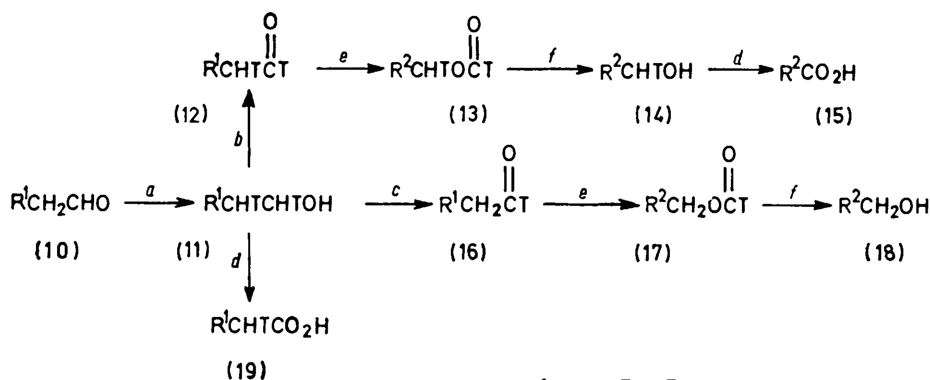
$LiAlH_4$ [1] to yield $[1\text{-}^3H]$heptan-1-ol (14). The results are summarized in Table 2. The $[1\text{-}^3H]$heptanol (14) retained 9% of the ^3H originally present in the $[^3H_2]$-octanol. Oxidation of the $[1\text{-}^3H]$heptanol with RuO_4 [28] to heptanoic acid (15) proceeded with the loss of all the tritium present in the $[1\text{-}^3H]$heptanol (Table 2).

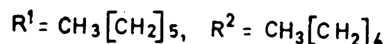
Two conclusions can be drawn from these experiments. Clearly 9% of the ^3H initially present in the $[1,2\text{-}^3H_2]$octanol (11) is located at C-2 of the tritiated octanol. It is also evident that none of the ^3H located at C-2 of $[1,2\text{-}^3H_2]$octanol was lost during the Collins oxidation.

We then oxidized the $[1,2\text{-}^3H_2]$octanol (11) employing the Pfitzner–Moffatt procedure.[25] The resulting $[1\text{-}^3H]$-octanal (16) was subjected to Baeyer–Villiger oxidation with trifluoroperacetic acid as described above. The obtained heptyl formate (17) after reduction with $LiAlH_4$ gave heptanol (18) essentially devoid of ^3H. The results are summarized in Table 2. It has been shown that the Baeyer–Villiger oxidation proceeds without loss of tritium from the α-carbon [1] and with retention of asymmetry.[30] This therefore confirmed our hypothesis that ^3H can be lost from C-2 of the aldehyde during the course of the Pfitzner–Moffatt oxidation.[25]

It is reasonable to assume that the degree of exchange of the hydrogen from the α-carbon will depend on the rate of enolization of an aldehyde under a particular set of reaction conditions. Consequently we undertook to determine the percentage loss of tritium from a steroidal $[25\text{-}^3H]$-26-alcohol during the Pfitzner–Moffatt oxidation to the 26-aldehyde. As a model we chose $[25\text{-}^3H]$-5α-cholestane-3β,26-diol [6] (20) which was oxidized as described for (3) to the aldehyde (21). The aldehyde was fractionated by preparative t.l.c. and an aliquot portion of the crude product was immediately decarbonylated to 26-nor-5α-cholestan-3-one (22). The formation of the 26-norcholestanone (22) *via* the 26-aldehyde (21) involved the loss of 23% of the ^3H (see Table 3).

We conclude therefore that the lack of increase in the ^3H : ^{14}C ratio of the $[^{14}C_4,25\text{-}^3H]$-26-norcholestenone (5) derived from the $[^{14}C_5]$cholesterol biosynthesis *in vivo* in rats can be attributed to a partial loss of ^3H from C-25 in the course of the Pfitzner–Moffatt oxidation of the 26-ol to the 26-al. As mentioned earlier the decarbonylation was accompanied by an 18% decrease in ^{14}C specific activity which is equivalent to a loss of *ca.* one ^{14}C atom from 26-hydroxy$[^{14}C_5]$cholestenone (3). Hence the microbial hydroxylation of the $[^{14}C_5]$cholesterol biosynthesized *in vivo* in the rat from $[2\text{-}^{14}C]$MVA again

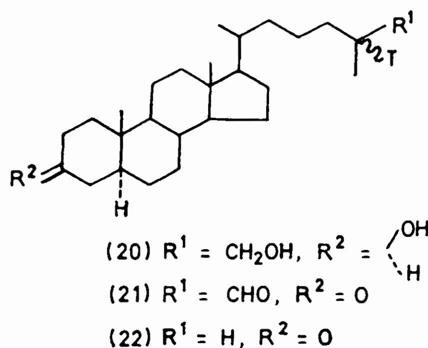




SCHEME Reagents: *a*, $LiBH_4$–T_2O; *b*, CrO_3–pyridine; *c*, DCC–DMSO; *d*, RuO_4; *e*, Baeyer–Villiger; *f*, $LiAlH_4$

[30] K. Mislow and J. Brenner, *J. Amer. Chem. Soc.*, 1953, **75**, 2318.

occurred on the carbon derived from C-2 of MVA. Since the stereospecificity of the 26-hydroxylase of *M. smegmatis* most certainly remained unchanged, it may be



inferred that the product (3) has the (25*S*)-configuration. These results lead to the conclusion that the addition of the hydrogen at C-25 in the Δ^{24} reduction of lanosterol

TABLE 3

Loss of ^{3}H during Moffatt oxidation [25] of [25-^{3}H]-5α-cholestane-3β,26-diol

Compound and recrystallization	^{3}H Specific activity $\times 10^5$ (disint. min^{-1} mmol^{-1})
26-Hydroxy[25-^{3}H]cholesterol	
2nd	2·08
3rd	2·12
4th	2·15
[25-^{3}H]-5α-Cholestane-3β,26-diol (20)	
1st	2·12
2nd	2·12
3rd	2·13
[25-^{3}H]-26-Nor-5α-cholestane-3-one (22)	
1st	1·61
2nd	1·64
3rd	1·62

(1) *in vivo* in the rat occurred from the same side as in the *in vitro* biosynthesis in the S-10 fraction of rat liver.

EXPERIMENTAL

For general procedures see ref. 5. Gas chromatographic (g.l.c.) analyses were performed either on an F & M model 720 instrument using a 6 ft × 2 mm glass column of 1% OV101 on Chromosorb Q, or a Hewlett–Packard 7620A instrument using a 6 ft × 2 mm glass column of 5% SE-30 on Chromosorb. Compounds were identified by the peak enhancement technique with authentic samples.

Dry column chromatography was carried out using Silica gel 60 (Merck, 70—230 mesh) which had been pre-equilibrated sequentially with 3% by weight of H_2O and 10% by weight of the appropriate solvent system in nylon casings of various diameters. The distribution of the products on the columns was evaluated by the puncture technique [31] and products were eluted with ethyl acetate.

Materials.—Octan-1-ol, octanal, and octanoic acid were purchased from Aldrich Chemical Co. Tritiated water, 90 mCi mmol^{-1} (5 Ci ml^{-1}), and 1 Ci ml^{-1} was purchased from New Enfland Nuclear Corp., Boston ,Mass.

Incubation [5,7] *of* [$^{14}C_5$,25-^{3}H]*Cholesterol* (2) *with* Mycobacterium Smegmatis SG 346.—[$^{14}C_5$]Cholesterol (2) biosynthesized *in vivo* in rats from [2-^{14}C]mevalonic acid was mixed with a sample of [25-^{3}H]cholesterol [6] (2), yielding a sample containing 9·0 × 10^6 disint. min^{-1} of ^{14}C (^{3}H : ^{14}C ratio 7·1). The sample (0·3 g) of [$^{14}C_5$,25-^{3}H]cholesterol (2) was dissolved in acetone (12 ml) and distributed equally into three flasks of fermenting *Mycobacterium smegmatis* (see ref. 7). The flasks were shaken vigorously at 37° for 3 days. The course of the reaction was followed on aliquot portions taken at intervals from an analogous incubation experiment with non-radioactive cholesterol.[8] At the termination of the experiment the cells were harvested by centrifugation and treated with 20% KOH in 40% aqueous methanol and refluxed for 3 h under nitrogen. The methanol was evaporated and the mixture diluted with water and extracted with ether–ethyl acetate (1 : 1). The organic phase was washed with saturated NaCl, dried ($MgSO_4$), and evaporated, and the residue was fractionated by preparative t.l.c. (benzene–ethyl acetate, 4 : 1). The zone with mobility analogous to 26-hydroxycholest-4-en-3-one (3) was eluted with ethyl acetate and purified twice on t.l.c. plates in the system described above. The product was homogeneous by g.l.c. analysis (HP-7620A, trimethylsilyl ether of 26-hydroxycholest-4-en-3-one, column temperature 250°; R_t 12 min); *m/e* 400 (M^+). The sample was identical by chromatographic and mass spectral analysis with the 26-hydroxycholest-4-en-3-one (3) synthesized from kryptogenin.[8]

The bands containing cholest-4-en-3-one and cholesterol were recovered and reincubated twice. The total yield of 26-hydroxycholest-4-en-3-one (3) (estimated by ^{14}C-radioactivity) was 0·54%. Part of the product was diluted with non-radioactive (25*R*)-26-hydroxycholest-4-en-3-one (3) (total 0·100 g) and crystallized from ethyl acetate–hexane (1 : 4) to give (3) (0·080 g), m.p. 130—131° (^{14}C specific activity 1·01 × 10^5 disint. min^{-1} mmol^{-1}, ^{3}H : ^{14}C ratio 6·72); *p*-bromobenzoate, *m/e* 582/584 (M^+) (^{14}C specific activity 0·95 × 10^5 disint. min^{-1} mmol^{-1}, ^{3}H : ^{14}C ratio 6·8). A portion of the alcohol was diluted further and converted into its 26-*p*-bromobenzoate (^{14}C specific activity 0·15 × 10^5 disint. min^{-1} mmol^{-1}, ^{3}H : ^{14}C ratio 6·65).

3-Oxo-5β-chol-9(11)-en-24-al (8).—To 5β-chol-9(11)-en-3α,24-diol [32] (7) (0·140 g, 0·39 mmol) in dimethyl sulphoxide (DMSO) (0·92 ml) and benzene (2·0 ml) was added pyridine (0·045 ml), trifluoroacetic acid (TFA) (0·024 ml), and dicyclohexylcarbodi-imide (DCC) (0·42 g, 2·0 mmol).[25] After stirring for 18 h at 23°, the solid was removed by filtration and washed with ethyl acetate. The ethyl acetate portion was washed with H_2O (2 × 25 ml), dried (Na_2SO_4), and evaporated to a syrup (0·340 g). The residue was placed on a dry column (1 × 8 in) of silica and the column was developed with ethyl acetate–hexane (1 : 3). The fraction from R_F 0·19 to 0·52 yielded 3-oxo-5β-chol-9(11))en-24-al (8) (0·076 g, 55%), $\nu_{max.}$ (KBr) 2680 (aldehyde C–H- and 1717 (C=O) cm^{-1}, δ ($CDCl_3$) 9·72 (1H, t, *J* 2 Hz, 24-H) and 5·50br (1H, 11-H), *m/e* 356 (M^+).

24-Nor-5β-chol-9(11)-en-3-one (9).—To 3-oxo-5β-chol-9(11)-en-24-al (8) (0·076 g, 0·211 mmol) in toluene (60 ml) was added tris(triphenylphosphine)rhodium chloride (0·250 g, 0·270 mmol) and the mixture was refluxed under N_2 for 6 h. The solvent was evaporated and the orange residue dissolved in hot ethanol (100 ml), filtered, and the filtrate evaporated to dryness (procedure repeated thrice). The product

[31] H. E. Hadd and E. Caspi, *J. Chromatog.*, 1972, **71**, 353.
[32] T. Komeno, *Chem. and Pharm. Bull. (Japan)*, 1960, **8**, 668.

(0·160 g) was placed on a dry column (1 × 13 in) of silica gel which was then developed with ethyl acetate–hexane (1 : 3). The fraction from R_F 0·46 to 0·63 yielded 24-nor-5β-chol-9(11)-en-3-one (9) (0·037 g, 52%), m.p. 90—92°, $\nu_{max.}$ (CCl_4) 1720 (C=O), 1458, and 1375 cm^{-1}, *m/e* 328 (M^+), 313 (*M* − 15), 295, and 271.

3-*Oxo*[$^{14}C_5$,25-^{3}H]*Cholest*-4-*en*-26-*al* (4).—26-Hydroxy-[$^{14}C_5$,25-^{3}H]cholest-4-en-3-one (3) (0·080 g, 0·2 mmol) (^{14}C specific activity 1·01 × 10^5 disint. min^{-1} $mmol^{-1}$) was added to a stirred solution of DMSO (0·5 g, 6·4 mmol) and benzene (1·0 ml) containing DCC (0·21 g, 1 mmol), pyridine (0·024 ml, 0·3 mmol), and TFA acid (0·012 ml, 0·1 mmol) and kept at room temperature overnight. Ethyl acetate (50 ml) was added, and the solution was extracted with H_2O (3 × 5 ml), dried ($MgSO_4$), and the solvents evaporated to yield a semicrystalline residue that was extracted with ethyl acetate (5 × 10 ml). The solvent was evaporated and the residue purified by preparative t.l.c. (ethyl acetate–hexane 1 : 4) to yield 3-oxo[$^{14}C_5$,25-^{3}H]cholest-4-en-26-al (4), which had chromatographic properties identical with (4) described previously.[4,5,8] Attempts to crystallize an aliquot portion (10%) of the aldehyde failed. During the unsuccessful crystallization attempts, most of the aldehyde was oxidized to the 26-acid (6) (indicated by t.l.c. in ethyl acetate–hexane, 1 : 4).

The remainder (90%) of the 26-aldehyde (4) had been stored under nitrogen at −15°. However, even under these conditions after *ca.* 10 days the previously homogeneous compound also showed the presence of the 26-acid (6). Consequently the material was resolved by t.l.c. (ethyl acetate–hexane 1 : 3) to give 3-oxo[$^{14}C_5$,25-^{3}H]cholest-4-en-26-al (4) (0·020 g), which was used for decarbonylation.

[$^{14}C_4$,25-^{3}H]-26-*Norcholest*-4-*en*-3-*one* (5).—To a solution of the [$^{14}C_5$,25-^{3}H]aldehyde (4) (0·020 g, 0·05 mmol) in toluene (10 ml) was added tris(triphenylphosphine)rhodium chloride (0·115 g), and the mixture refluxed under nitrogen for 2 h. More reagent was added (0·080 g) and the refluxing continued for 8 h. The solvent was evaporated under reduced pressure and the dark red residue extracted with ether. The product was purified by preparative t.l.c. (ethyl acetate–hexane 1 : 3) to give [$^{14}C_4$,25-^{3}H]-26-norcholest-4-en-3-one (5) (0·007 g) which was crystallized from ethyl acetate–methanol, m.p. 96—97°, and counted (Table 1). The compound was identical in spectral and chromatographic properties with (5) described previously.[4,5,8]

[1,2-$^3H_2$]*Octanol* (11).—To a stirred suspension of $LiBH_4$ (1·4 g, 64·5 mmol) (more than the quantity used by Cornforth [27]) in tetrahydrofuran (THF) (10 ml) (freshly distilled from $LiAlH_4$), tritiated water (5 mmol, 90 μl; specific activity 90 mCi $mmol^{-1}$) was added and the mixture was refluxed for 2 h under nitrogen.[27] After cooling to room temperature, octanan (10) (1·35 g, 10·5 mmol), was added and the mixture was refluxed for 2 h. THF was evaporated, water added, the solution acidified with dilute H_2SO_4 and warmed briefly on a steam-bath. The solution was then saturated with NaCl and extracted with hexane. The hexane extract was washed (aqueous NaCl), dried ($MgSO_4$), and the solvent evaporated. The residue (1·24 g) (92% yield, and 7·2% incorporation of radioactivity from the tritiated water) was distilled at 60° *in vacuo*. To the residue, cold octanol (0·420 g) was added and the mixture distilled in the same system. After repeating this procedure once more, the combined distillates yielded a total of 1·110 g of octanol (11) containing 3·5% of the tritium of the water used.

An aliquot portion was treated with phenyl isocyanate and the octanol phenylurethane [33] isolated and crystallized to constant specific activity, m.p. 74—75°.

[1,2-$^3H_2$]*Octanal* (12).—Chromium trioxide (12·0 g, 120 mmol) was added to a mechanically stirred solution of pyridine (18·9 g, 240 mmol) in CH_2Cl_2 (300 ml, redistilled from P_2O_5).[29] To this reagent was added dropwise a solution of [1,2-$^3H_2$]octanol (11) (1·839 g, 13·9 mmol) in CH_2Cl_2 (20 ml). After 1 h at 23° the solution was decanted and the black residue washed with ether (3 × 100 ml). The combined CH_2Cl_2 and Et_2O extract was washed with aqueous NaOH (5%; 3 × 100 ml), aqueous HCl (5%; 100 ml), aqueous $NaHCO_3$ (5%, 100 ml), and saturated NaCl, dried (Na_2SO_4), and evaporated to a syrup (3·27 g). The product was purified by dry column chromatography (silica gel, 2 × 16 in, pre-equilibrated with 10% v/w of ether–hexane, 1 : 2). The column was developed with ether–hexane (1 : 2). The fraction (R_F 0·13—0·88) yielded [1,2-$^3H_2$]octanal (12) (1·31 g, 72%). G.l.c. showed a single peak identical with octanal, $\nu_{max.}$ (neat) 1710 cm^{-1}; semicarbazone,[34] m.p. 100—101° (from ether–hexane).

[1-^{3}H]*Octanal* (16).—To a stirred solution of [1,2-$^3H_2$]-octanol (11) (3·274 g, 25 mmol) in DMSO (6·0 ml) and benzene (12 ml) was added sequentially, pyridine (0·28 ml), TFA (0·144 ml), and DCC (2·52 g).[25] After 24 h at 23°, the solid formed was removed by filtration and the filtrate washed with aqueous HCl (10%), water, dried (Na_2SO_4), and evaporated to a syrup. The product (2·2 g) was purified by dry column chromatography (silica gel, 2 × 16 in, pre-equilibrated with 10% by weight of ether–hexane 1 : 2). The column was developed with ether–hexane (1 : 2). The fraction (R_F 0·13—0·37) yielded unchanged octanol (0·32 g) (by g.l.c.), the fraction (R_F 0·37—0·62) yielded a 3 : 1 mixture (by g.l.c.) of octanol and octanal (1·22 g), and the fraction (R_F 0·62—1·0) yielded [1-^{3}H]octanal (16) (homogeneous by g.l.c.) (0·560 g) (42% yield, based on recovered starting material); semicarbazone,[34] m.p. 99—100°.

[1-^{3}H]*Heptan*-1-*ol* (14).—To a stirred mixture of [1,2-$^3H_2$]octanal (12) (0·450 g, 3·5 mmol) in CH_2Cl_2 (5 ml) at 0° and disodium hydrogen phosphate (2·5 g; dried in a crucible with a Bunsen burner for 30 min) a solution of trifluoroperacetic acid [prepared by mixing at 0° $(CF_3CO_2)O$ (1·33 g, 6·45 mmol, 0·90 ml), CH_2Cl_2 (5 ml; distilled from P_2O_5), and 90% H_2O_2 (0·27 g, 7·15 mmol, 0·2 ml)] was added dropwise.[1] An aliquot portion showed no octanal (by g.l.c.) after 3 h. A saturated solution of Na_2CO_3 (5 ml) was added and then after 1 h, Na_2SO_4 (40 g) was added. The mixture was filtered through Celite and evaporated to a syrup (0·398 g). The resulting heptyl formate (13) was dissolved in ether (5 ml) and added to $LiAlH_4$ (0·200 g) in ether (5 ml) at 0°. After 1 h at 0° a solution of saturated Na_2SO_4 was added, the solid removed by filtration through Celite, and the filtrate evaporated. The resulting syrup (0·352 g) was fractionated by preparative g.l.c. (1% OV 101, 6 ft) to yield [1-^{3}H]heptan-1-ol (14) (0·133 g); phenylurethane,[33] m.p. 62—63° (from ether–hexane).

Heptan-1-*ol* (18).—Baeyer-Villiger oxidation of [1-^{3}H]-octanal (16) (0·460 g) from the Pfitzner–Moffatt oxidation was carried out as for (14) to yield heptan-1-ol (18) (67 mg) after preparative g.l.c.; phenylurethane,[33] m.p. 62—63° (from ether–hexane).

[33] R. L. Shriner, R. C. Fuson, and D. Y. Curtin, in 'The Systematic Identification of Organic Compounds,' 5th edn., J. Wiley, New York, 1964, p. 317.
[34] Ref. 33, p. 320.

[2-^{3}H]*Octanoic Acid* (19).—To a stirred suspension of ruthenium dioxide (1·09 g) in acetone (125 ml) was added $NaIO_4$ (7·5 g) in water (35 ml) and [1,2-$^3H_2$]octanol (11) (1·00 g, 7·73 mmol) in acetone (150 ml).[28] A solution of $NaIO_4$ (11·0 g) in water–acetone (1 : 1; 100 ml) was employed to regenerate RuO_4 and was added dropwise as needed. After 30 min at 23° isopropyl alcohol was added to the suspension, which was then filtered through Celite, and the filtrate evaporated to give a syrup (1·22 g). A portion of the acid (10 mg) was treated with diazomethane, and g.l.c. analysis of the product showed one peak corresponding to methyl octanoate.

To the remaining [2-^{3}H]octanoic acid (19) (1·21 g) in benzene (95 ml) was added $SOCl_2$ (2·5 g) and the mixture refluxed for 0·5 h. The solvent was evaporated, NH_4OH (30 ml) was added to the acid chloride at 0°, and the amide was removed by filtration. Recrystallization from acetone–hexane gave octanamide,[35] m.p. 100—101°.

Heptanoic Acid (15).—To [1-^{3}H]heptan-1-ol (14) (10 mg) was added unlabelled heptanol (150 mg). An aliquot portion of the heptanol (50 mg) was converted into the heptyl phenylurethane. Another portion of the heptanol (110 mg) was oxidized with RuO_4.[28] The RuO_4 was prepared from RuO_2 (0·109 g) suspended in acetone (12·5 ml) containing $NaIO_4$ (0·75 g) and H_2O (3·5 ml). The mixture was worked up as described above to yield (15) which was converted into heptanamide [36] and purified, m.p. 93—94°.

3-*Oxo*[25-^{3}H]-5α-*cholestan*-26-*al* (21).—To a stirred solution of DMSO (1·6 g), benzene (3·2 ml), pyridine (0·06 ml), and TFA was added [25-^{3}H]-5α-cholestane-3β,26-diol [6] (20) (0·143 g, 0·354 mmol) and DCC (0·600 g, 2·26 mmol). The mixture was stirred for 21 h at 23°, then the solid was filtered off and washed with ethyl acetate. The filtrate was washed with water (3 × 20 ml), dried (Na_2SO_4), and evaporated to a syrup (154 mg) which was fractionated by preparative t.l.c. (ethyl acetate–hexane, 1 : 3). The fraction (R_F 0·60—0·80) yielded 3-oxo[25-^{3}H]-5α-cholestan-26-al (21) (0·110 g, 78%), m.p. 105—108° (from ethyl acetate), $\nu_{max.}$ (KBr) 2700 (aldehyde C–H), 1728 (aldehyde C=O), and 1710 (3 C=O) cm^{-1}, δ ($CDCl_3$) 9·61 (1H, d, *J* 2 Hz, 26-H), 1·10 (3H, d, *J* 8 Hz, 27-H_3), 1·00 (3H, s, 19-H_3), 0·91 (3H, d, *J* 4 Hz, 21-H), and 0·69 (3H, s, 18-H_3), *m/e* 400 (M^+), 385 (M − 15), 377 (M − 28), and 272.

[25-^{3}H]-26-*Nor*-5α-*cholestan*-3-*one* (22).—To the 26-aldehyde (21) (0·100 g, 0·25 mmol) in toluene (40 ml) under N_2 was added tris(triphenylphosphine)rhodium chloride (0·300 g, 0·324 mmol) and the mixture was refluxed for 4 h under N_2. Additional tris(triphenylphosphine)rhodium chloride (0·250 g) was added and refluxing was continued (under N_2) for 16 h. The solid obtained was removed by filtration, and the filtrate was washed (water), dried (Na_2SO_4), and evaporated. The residue was fractionated by preparative t.l.c. (ethyl acetate–hexane 1 : 4). The fraction (R_F 0·60—0·93) gave a sample (0·036 g), *m/e* 372 (M^+), which was rechromatographed on an alumina plate (acetone–hexane 1 : 4). The fraction (R_F 0·75—0·96) yielded [25-^{3}H]-26-nor-5α-cholestan-3-one (22) (0·017 g, 18%), m.p. 91—93° (from EtOAc), $\nu_{max.}$ (KBr) 1715 cm^{-1} (3 C=O), δ ($CDCl_3$) 1·00 (3H, s, 19-H_3), 0·89 (3H, d, *J* 4 Hz, 21-H_3), and 0·68 (3H, s, 18-H_3), *m/e* 372 (M^+), 357 (M − 15), 301 (M − C_4H_7O), 300 (M − C_4H_8O), and 271.

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[35] ' Dictionary of Organic Compounds,' eds. J. R. A. Pollock and R. Stevens, vol. 4, Oxford University Press, New York, 1965, p. 2561.
[36] Ref. 35, vol. 3, p. 1577.

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