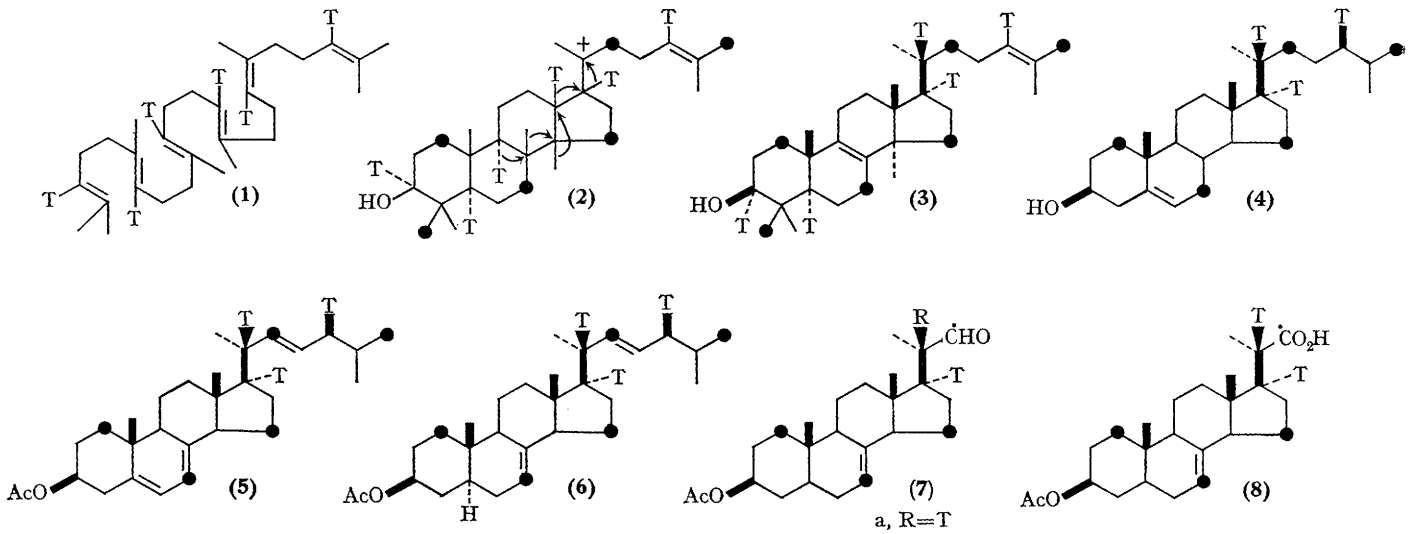
The Biosynthetic Origin of the C-20 Proton of Cholesterol

By ELIAHU CASPI* and LAWRENCE J. MULHEIRN

(*The Worcester Foundation for Experimental Biology, Inc., Shrewsbury, Massachusetts* 01545)

Summary The presence of tritium at C-20 of cholesterol biosynthesized from (4*R*)-[4-^{3}H, 2-^{14}C]mevalonic acid is confirmed.

THE biosynthesis of squalene from mevalonic acid (MVA) was shown to proceed with the stereospecific elimination of the 4-*pro-S* protons and retention of the 4-*pro-R* protons.[1] Thus incubation of (4*R*)-[4-^{3}H]-MVA with a rat-liver preparation yields [$^3H_6$]squalene labelled as indicated in (**1**). Oxidative cyclisation of squalene,[2] *via* squalene 2,3-oxide,[3] is presumed to proceed through the cation (**2**), which then undergoes the indicated four concerted rearrangements to yield, after extrusion of the 9α-tritium atom, [$^3H_5$]lanosterol (**3**). Biosynthetic transformation of lanosterol to cholesterol involves the loss of two tritium atoms from C-3 and C-5 and three methyl groups[4] from C-4 and C-14, one of which (the 4α[5]) is derived from C-2 of MVA. Indeed, when (4*R*)-[4-^{3}H, 2-^{14}C]-MVA, having an atomic ratio (a.r.) of ^{3}H : ^{14}C (1 : 1), was incubated with a rat-liver preparation of ^{14}C) and cholesta-5,7,22-trien-3β-yl acetate (**5**) (7 × 10^5 d.p.m. of ^{14}C). The triene (**5**) was further purified on t.l.c. and then a portion was diluted with inactive material and co-crystallized (Table). A second portion was hydrogenated in the presence of tristriphenylphosphinerhodium chloride[10] to yield 5α-cholesta-7,22-dien-3β-yl acetate (**6**), which showed the same ^{3}H : ^{14}C ratio as (**5**) (Table). The remainder of the triene (**5**) was diluted with inactive ergosteryl acetate (1·04 g.) and hydrogenated as above. The recovered mixture of products (which showed only end-absorption in the u.v.) was dissolved in dichloromethane



Carbon atoms indicated by a heavy dot originate from C-2 of mevalonic acid. T ≡ ^{3}H.

the squalene, lanosterol, and cholesterol (**4**) obtained had the predicted atomic ratios ^{3}H : ^{14}C (1 : 1), (5 : 6), and (3 : 5), respectively.[6]

The location of the three remaining tritium atoms in cholesterol, which are expected to be present at C-17, -20, and -24, is of considerable interest. While the tritium atom at C-24 should be located at its "original" position, traceable directly to squalene and MVA, those at C-17 and C-20 are thought to have been involved in migration.[2] Earlier we proved that, in cholesterol biosynthesised from (4*R*)-[4-^{3}H, 2-^{14}C]-MVA, tritium atoms were present at the 17α-[6] and 24-*pro-R*-[7] positions. Because of the experimental approach previously employed, involving the enzymatic conversion of cholesterol into pregnenolone with a bovine adrenal preparation,[6,7] the precise location of the third tritium atom could not be defined. The proof for the C-20 location of the remaining tritium atom is now presented. Our experimental approach was based on the observation that the protozoan *Tetrahymena pyriformis* converts cholesterol into cholesta-5,7,22-trien-3β-ol.[8,9]

Cholesterol[7] (**4**), prepared from (4*R*)-[4-^{3}H, 2-^{14}C]-MVA with a rat-liver enzyme system, was re-purified by t.l.c. to radiochemical purity. The sample (1·2 × 10^6 d.p.m. of ^{14}C, ^{3}H : ^{14}C ratio 4·5) was diluted with 15 mg. of inactive cholesterol and distributed equally (as an ethanol solution) between six Fernbach flasks containing growing cultures of *T. pyriformis* in 1 l. of medium.[9] The cultures were shaken at 28° for 48 hr., and the sterols were recovered as previously described.[9] The recovered mixture of products (75% of the added ^{14}C radioactivity) was acetylated and purified by t.l.c.[9] to yield cholesteryl acetate (3 × 10^4 d.p.m.

$^3H:^{14}C$ *Ratios for cholesterol {from* (4R)-[4-^{3}H, 2-^{14}C]-MVA} *and its transformation products*

Compound	$^3H:^{14}C$ (d.p.m.)	$^3H:^{14}C$ (atomic) Exp.	Theor.
Cholesterol (**4**)	4·5[a]	3·0 : 5	3 : 5
Recovered cholesterol[b] (**4**)	4·2	2·8 : 5	3 : 5
Cholesta-5,7,22-trien-3β-yl acetate(**5**)	4·3	2·9 : 5	3 : 5
5α-Cholesta-7,22-dien-3β-yl acetate (**6**)	4·3	2·9 : 5	3 : 5
3β-Acetoxy-23,24-bisnor-5α-chol-7-en-22-al (**7a**)	3·9	2·1 : 4	2 : 4
3β-Acetoxy-23,24-bisnor-5α-chol-7-en-22-oic acid (**8**)	3·8	2·0 : 4	2 : 4
(**7a**) Equilibrated *via* enamine to (**7b**)	1·9	1·0 : 4	1 : 4

[a] The sample of cholesterol was obtained from the same incubation as that used in ref. 7. The 8% decrease in observed $^3H:^{14}C$ ratio is accounted for by the natural decay of tritium.
[b] Recovery from incubation with *T. pyriformis*.

and treated with 1·2 moles of ozone at −70° to yield (dimethyl sulphide–methanol work-up[11]) 3β-acetoxy-23,24-bisnor-5α-chol-7-en-22-al (**7a**) ($^3H:^{14}C$ ratio 3·9). Oxidation of the aldehyde (**7a**) (Jones' reagent) to the acid (**8**) ($^3H:^{14}C$ 3·8) proceeded without loss of tritium, revealing the absence of isotopic hydrogen at C-22. Treatment of the aldehyde (**7a**) with piperidine and a trace of toluene-*p*-sulphonic acid gave an enamine, which was not isolated but hydrolysed to aldehyde (**7b**) (stereochemistry at C-20 unspecified). The purified (t.l.c.) and crystallized (**7b**) retained only 50% of the tritium present in the aldehyde (**7a**) (Table).

The results demonstrate that in cholesterol biosynthesized from (4*R*)-[4-^{3}H, 2-^{14}C]-MVA the third tritium atom is located at the 20-position as expected on the basis of the accepted mode of formation of lanosterol from squalene.[2] In addition, it can be concluded that the introduction of the C-22(23) olefinic bond in cholesterol by *Tetrahymena pyriformis* proceeds without the loss of hydrogen (tritium) from the 20 and 24-*pro-R*-positions.

The support of this work by grants from the American Cancer Society, National Institute of Health, and NIH Career Award is gratefully acknowledged.

(*Received, September 22nd,* 1969; *Com.* 1433.)

[1] J. W. Cornforth, R. H. Cornforth, C. Donninger, and G. Popják, *Proc. Roy. Soc.*, 1966, *B*, **163**, 492.
[2] A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, 1955, **38**, 1890.
[3] E. J. Corey, W. E. Russey, and P. R. O. de Montellano, *J. Amer. Chem. Soc.*, 1966, **88**, 4750; E. E. van Tamelen, J. D. Willett, R. B. Clayton and K. E. Lord, *ibid.*, p. 4752.
[4] K. Bloch, *Science*, 1965, **150**, 19.
[5] K. J. Stone, W. R. Roeske, R. B. Clayton, and E. E. van Tamelen, *Chem. Comm.*, 1969, 530; G. P. Moss and S. A. Nicolaidis, *Chem. Comm.*, 1969, 1072.
[6] 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.
[7] E. Caspi, K. R. Varma, and J. B. Greig, *Chem. Comm.*, 1969, 45.
[8] R. L. Conner, F. B. Mallory, J. R. Landrey, and C. W. L. Iyengar, *J. Biol. Chem.*, 1969, **244**, 2325.
[9] E. Caspi and J. M. Zander, *Chem. Comm.*, 1969, 1141.
[10] A. J. Birch and K. A. M. Walker, *J. Chem. Soc.* (*C*), 1966, 1894.
[11] J. A. Pappas, W. P. Keaveney, E. Gancher, and M. Berger, *Tetrahedron Letters*, 1966, 4273.