The Stereospecific Addition of a 24-*pro*-*S*-Hydrogen in the Biosynthesis of Cholesterol from Lanosterol

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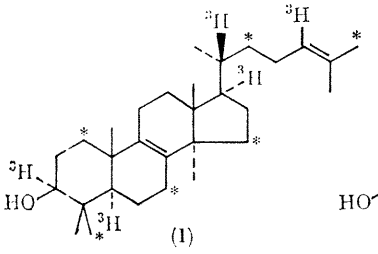
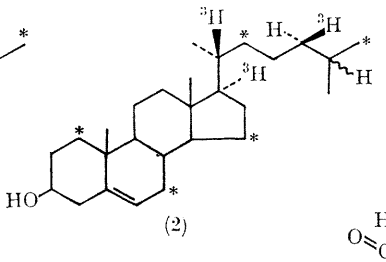
IN the course of the biosynthetic conversion of lanosterol into cholesterol the C-24 double bond of the former is reduced. The enzyme involved in this reduction is apparently not substrate specific[1,2] but requires NADPH as a cofactor.[2] Berseus,[3] and Mitropoulos and Myant[4] have demonstrated that this reduction is stereospecific with regard to the introduction of hydrogen at C-25.

Lanosterol biosynthesized from (4*R*)-[4-$^3H_1$]mevalonic acid (MVA) should be labelled at the 3α-, 5α-, 17α-, 20*R*-, and 24-positions (1).[5] Further metabolic transformations of this intermediate yield cholesterol (2), which is expected to have tritium at the 17α-, 20*R*-, and 24ξ-positions.[5] During this sequence a stereospecific reduction of the C-24 double bond will give rise to an isotopically induced asymmetric centre at C-24. From the configuration at this carbon atom the side of addition of the hydrogen atom to the C-24 double bond can be determined.

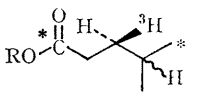


(1)

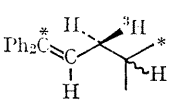


(2)

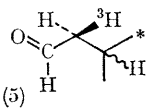
* = carbon originating from C-2 of mevalonic acid



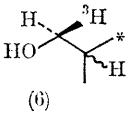
(3a) R=H
(3b) R=Me



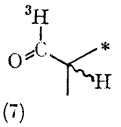
(4)



(5)



(6)



(7)

The incubation of cholesterol, biosynthesized from (4*R*)-[4-^{3}H,2-^{14}C]MVA, with a preparation of adrenal enzymes affords 4-methyl[3-^{3}H,1,5-$^{14}C_2$]pentanoic acid (3a), comprising C(22)—C(27) of the side chain.[5] This acid can be degraded,[6] without alteration of the stereochemistry of the tritium at C-3 (corresponding to C-24 of cholesterol) to [1-^{3}H,3-^{14}C]isobutyl alcohol. We have proved that the oxidation of isobutyl alcohol to 2-methylpropanal by yeast alcohol dehydrogenase proceeds with the loss of the 1-*pro*-*R*-hydrogen.[6] Consequently, the loss or retention of tritium during such an oxidation of the [1-^{3}H,3-^{14}C]isobutyl alcohol defines the configuration of the C-24 tritium atom in the original cholesterol.

The dibenzylethylenediamine salt of (4*R*)-[4-^{3}H]MVA was prepared[7] and mixed with the corresponding salt of [2-^{14}C]MVA, (^{3}H:^{14}C ratio 8·03). The mixture (100 μc of ^{14}C) was incubated with the microsomal and soluble enzyme fraction (42 ml.) of a rat-liver homogenate.[8] After 3 hr. the incubation was terminated and the non-saponifiable lipids were extracted. Squalene and cholesterol were isolated by preparative t.l.c. on silica gel (development with ethyl acetate–hexane, 3 : 7 v/v). After elution the squalene band was purified by t.l.c. and *via* the thiourea adduct[9] (^{3}H:^{14}C ratio 8·48). The cholesterol zone was eluted and twice repurified by t.l.c. on silica gel (twofold development with methanol–benzene, 1 : 9 v/v). This afforded cholesterol (7·8 mg.; 9·2 μc of ^{14}C; ^{3}H : ^{14}C ratio 5·02) which showed a single, symmetrical peak on radiochromatography A sample of this cholesterol was mixed with non-radioactive material and purified *via* the dibromide;[10] after two recrystallizations the cholesterol had a constant ^{3}H : ^{14}C ratio of 4·93.

The biosynthetic [^{3}H,^{14}C]cholesterol (8·0 μc of ^{14}C; 6·8 mg.) was suspended in water (6·0 ml.) with Triton X-100 (150 mg.) and added to a suspension of an acetone powder (5·0 g.) of bovine adrenal mitochondria[11] in 0·02M-phosphate buffer (233 ml; pH 7·4). After addition of 3β-hydroxychol-5- enoic acid (11·22 mg.) dissolved in dimethylformamide (1·0 ml.)[11a] and an NADPH generating system, the mixture was incubated aerobically (37°; 4 hr.). The incubate was then acidified and, after addition of non-radioactive 4-methylpentanoic acid (254 mg.), was steam distilled.[12] The distillate (1·5 l.) was saturated with sodium chloride and continuously extracted with ether for 48 hr. The extract (5·05 × 10^5 d.p.m. of ^{14}C) was dried and treated with diazomethane. This solution of methyl 4-methylpentanate (3b) was converted into the diphenylalkene (4) (^{3}H : ^{14}C ratio 4·31) by reaction with phenylmagnesium bromide and subsequent dehydration. Ozonolysis of (4) yielded 3-methylbutanal (5), ^{3}H : ^{14}C ratio 8·46. Baeyer–Villiger oxidation of this aldehyde (5), and subsequent cleavage of the ester (lithium aluminium hydride), afforded isobutyl alcohol (6), ^{3}H : ^{14}C ratio 7·90. Oxidation of a sample of this [^{3}H,^{14}C]isobutyl alcohol gave isobutyric acid which was devoid of tritium. As predicted,[5] biosynthetic cholesterol retains a 4-*pro*-*R*-hydrogen of MVA at C-24.

Incubation of the [1-^{3}H, 3-^{14}C]isobutyl alcohol (6) with yeast alcohol dehydrogenase and NAD^+ yielded 2-methylpropanal (7) which was isolated as its dimedone derivative and found to have a ^{3}H : ^{14}C ratio of 6·31. A duplicate experiment gave 2-methylpropanal (7) with a ^{3}H : ^{14}C ratio of 8·26 (Table). Since yeast alcohol dehydrogenase removes

	^3H : ^{14}C ratio (d.p.m.)	^3H : ^{14}C ratio (atomic) Found	Theoretical
Mevalonic acid	8·03		1 : 1
Squalene	8·48	6·34 : 6	6 : 6
Cholesterol (3)	4·93	3·07 : 5	3 : 5
4-Methyl-1,1-diphenylpent-1-ene (4)	4·31	1·07 : 2	1 : 2
3-Methylbutanal (5) ..	8·46	1·05 : 1	1 : 1
Isobutyl alcohol (6) ..	7·90	0·92 : 1	1 : 1
2-Methylpropionic acid ..	0·019	0·002 : 1	0 : 1
2-Methylpropanal (7) ..	6·31	0·79 : 1	1 : 1
	8·26	1·03 : 1	

the 1-*pro-R*-hydrogen of isobutyl alcohol,[6] these results indicate that the [1-^3H, 3-^{14}C]isobutyl alcohol has the (1*S*)-configuration. It follows that in cholesterol biosynthesized from (4*R*-[4-$^3H_1$]MVA the 24-tritium occupies the 24-*pro-R*-position and the newly introduced hydrogen assumes the 24-*pro-S*-position. Clearly the enzymatic reduction is stereospecific at C-24, as it is at C-25.[3,4]

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[1] DeW. S. Goodman, J. Avignan, and D. Steinberg, *J. Biol. Chem.*, 1963, **238**, 1287.
[2] J. Avignan, DeW. S. Goodman, and D. Steinberg, *J. Biol. Chem.*, 1963, **238**, 1283; J. Avignan and D. Steinberg, *ibid.*, 1961, **236**, 2898.
[3] O. Berseus, *Acta Chem. Scand.*, 1965, **19**, 325.
[4] K. A. Mitropoulos and N. B. Myant, *Biochem. J.*, 1965, **97**, 26C.
[5] 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.
[6] K. R. Varma and E. Caspi, in the press.
[7] J. W. Cornforth, R. H. Cornforth, C. Donninger, and G. Popjak, *Proc. Roy. Soc.*, 1966, *B*, **163**, 492.
[8] N. L. R. Bucher and K. McGarrahan, *J. Biol. Chem.*, 1956, **222**, 1; J. W. Cornforth, R. H. Cornforth, A. Pelter, M. G. Horning, and G. Popjak, *Tetrahedron*, 1959, **5**, 311.
[9] L. J. Goad and T. W. Goodwin, *Biochem. J.*, 1966, **99**, 735.
[10] L. F. Fieser, *Org. Synth.*, 1963, Coll. Vol. 4, 196.
[11] (a) P. R. Raggatt and M. W. Whitehouse, *Biochem. J.*, 1966, **101**, 819; (b) T. Kimura, P. S. Satoh, and T. T. Tchen, *Analyt. Biochem.*, 1966, **16**, 355.
[12] *Cf.* E. Staple, W. S. Lynn, and S. Gurin, *J. Biol. Chem.*, 1956, **219**, 845.