The Location of the 4-pro-R Protons of Mevalonic Acid in Cholesterol*

(Received for publication, February 11, 1971)

L. J. MULHEIRN and ELIAHU CASPI
From The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

SUMMARY

Evidence is presented that a proton derived from the 4-pro-R position of mevalonic acid is located at C-20 of cholesterol. It is now clear that the 3 protons derived from 4-pro-R position of mevalonic acid of cholesterol are located at the 17α, 20, and 24-pro-R positions as predicted.

At present there are two recognized mechanisms of enzyme-mediated squalene (I) cyclization. The 3-deoxytriterpenes are thought to be formed by a nonoxidative proton-initiated attack on squalene (1-3). This mechanism was shown to operate in the biosynthesis of the triterpene tetrahymanol (II) in Tetrahymanea pyriformis (1-3). The over-all process of formation of tetrahymanol is equivalent to the acquisition by squalene of the elements of water. Evidence consistent with the operation of a similar mechanism in the biosynthesis of fernene (III) was also presented (4).

The more frequently encountered process is the enzymatic oxidative cyclization of squalene first formulated by Eschenmoser et al. (5). It is now recognized that this cyclization is not fully concerted as originally thought (5), but involves the initial formation of 2,3-oxido squalene (IV) (6, 7). Enzymatic opening of the oxide is presumed to generate an electron deficiency at C-2, and this precipitates the actual cyclization process. Whether the cyclization is a nonstop process (5) or involves transiently stabilized intermediates (8, 9) is unknown.

* This work was supported by Grants AM 12156, HE 10566, and CA-K3-16614 from the National Institutes of Health, Grants GB 8277 and GB 23801 from the National Science Foundation, Grants P-599-J and P-500-H from the American Cancer Society, and Grant 1377-C-1 from the Massachusetts Division of American Cancer Society.

† Present address, Milstead Laboratory of Chemical Enzymology, Shell Research, Ltd., Broad Oak Road, Sittingbourne, Kent, England.
The biosynthetic cyclization of squalene leading to lanosterol and sterols is postulated to proceed through cation Va (5) or its stabilized equivalent Vb (8). Recently, we provided experimental evidence supporting the involvement of V in the oxidative cyclization of squalene (10, 11). This evidence was obtained from an investigation of the biosynthesis of the protosterol fusidic acid (VIa) in the mold Fusidium coccineum. Results of other workers on the enzymatic cyclization of squalene analogues are also consistent with the intermediate formation of analogues of V (12, 13).

In the course of formation of lanosterol the cation (V) is supposed to undergo the indicated four concerted 1,2 migrations, resulting in another cation (VII; free or stabilized) with an electron deficiency at C-8. The loss of the C-9 hydrogen from VII and formation of the 8(9) double bond will produce lanosterol (VIIIa). The 1,2 migrations of the methyls from C-8 and C-14 of the hypothetical V to C-13 and C-14 of lanosterol (VIII) were proven (14, 15). However, because of the lack of an appropriate experimental approach, evidence for the parallel migration of protons from C-13 and 17 of V to C-20 and C-17 of VIII was lacking.

Cornforth et al. (16) have shown that the biosynthesis of squalene in a rat liver enzyme system proceeds with the stereospecific loss of six 4-pro-R protons of MVA, and the stereospecific retention of six 4-pro-S protons of MVA, and the stereospecific retention of six 4-pro-R protons of MVA, and the stereospecific retention of six 4-pro-S protons of MVA. The protons derived from the 4-pro-R hydrogens of MVA should be located at C-3, 7, 11, 14, 18, and 22 of the squalene (Ia) (the eneicarboxylic hydrogens) (16). Consideration of the mode of folding (17) and cyclization (5) of squalene-2,3-oxide by rat liver enzymes requires the presence of the protons derived from the 4-pro-R position of MVA at the indicated locations of the cation (Va). We proved that in the protosterol fusidic acid (VIIb) biosynthesized from (3RS,4R), (24C,44H)-MVA 4 atoms of tritium were retained. The tritium atoms were present at the critical 9β and 13α positions and at the 5α and 24 positions (10, 11). The 17-tritium atom of Ve was lost in the 17-20) double bond formation and the 3α-tritium was apparently removed in the course of the elimination of the 4β-methyl, which is known to proceed via a 3-ketone (18). In contrast, as mentioned earlier, formation of lanosterol requires the prior rearrangement of the protosterol cation (V) to anation VII. In this rearrangement the C-13 and C-17 protons of Va, originating from the 4-pro-R position of MVA, are supposed to migrate to C-17 and C-20 of VII, respectively. It is worthy of note that the postulated stabilization of cation VII leading to lanosterol (VIII) entails the loss of the 9β-proton which also originates from the 4-pro-R position of MVA. The over-all transformation of lanosterol to cholesterol requires the elimination of three methyls (from C-4 and 14), one of which, the 4a, originates from C-2 of MVA (19, 20); transposition of the C-8(9) double bond to C-9(8); and reduction of A. Hence, cholesterol should retain only five carbons derived from C-2 of MVA and three hydrogens derived from the 4-pro-R position of MVA. Indeed, when (3RS,4R), (24C,44H)-MVA was incubated with a rat liver enzyme system, the obtained squalene (Ib), lanosterol (VIIIb), and cholesterol (IXb) retained 6, 5, and 3 tritium atoms, respectively (21). Of the 3 tritium atoms detected in the cholesterol, only one “assumed” to be present at C-24 was still located at its original position of attachment traceable to squalene and MVA. The remaining 2 were involved in a migration. We undertook to determine the location of the 3 retained isotopic hydrogens (21, 24) and the configuration of the newly induced asymmetrical center at C-24 (21, 22).

With this in mind, biosynthetic 14C6,3H3-cholesterol (IX) (derived from (3RS,4R), (24C,44H)-MVA) was cleaved with an adrenal enzyme preparation between C-20 and 22 to yield pregnenolone (X) and isocaproic acid (XI). As expected, the pregnenolone (X) retained 3 14C atoms and 1 tritium atom at C-17 (21). The isocaproic acid which encompasses carbons 22 to 27 of cholesterol retained 2 14C atoms and 1 tritium atom (loc. cit.). Subsequently, we proved that the tritium atom was located at carbon-3 of the isocaproic acid (XI), which corresponds to C-24 of cholesterol (22, 23). We have also shown that the isocaproic acid (XI) has the 3R-3H configuration and hence the 14C6,3H3-cholesterol (IX) must have the 24-R-24H configuration (22, 23).

The above enzymatic conversion of 14C6,3H3-cholesterol (IX) to 14C6,3H3-pregnenolone (X) and 14C6,3H3-isocaproic acid (XI) proceeds with the loss of 1 atom of tritium. The unaccounted for isotopic hydrogen could have been located either at C-20 or C-22. Because of this ambiguity the location of the 3rd tritium atom in the cholesterol (IX) could not be determined by this method.

Recently, it was shown (25-28) that the protozoan Tetrahymana pyriformis converts cholesterol to cholesterol-5,7,22-trien-3β-ol (XIIa). This observation provided the means for a selective attack and search for the isotopic hydrogen at C-20 or C-22 of the 14C6,3H3-cholesterol (IX). Evidence for the C-20 location of the 3rd tritium atom in IX is presented in this paper. A preliminary account of this work was reported (24).

**EXPERIMENTAL PROCEDURE**

**Physical Methods**—Melting points were taken on a hot stage apparatus and are corrected. Infrared spectra were recorded on a Perkin-Elmer 237 spectrophotometer as KBr wafers. Mass
spectra were measured on a Varian Associates M66 instrument. Radioactive samples were counted on a Nuclear-Chicago Mark 1 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. Counting was carried out as previously described (29).

Plates for thin layer chromatography were made from Silica Gel H 234,145 and activated at 100°C for 3 hours before use. Silver nitrate-impregnated plates were made from 30 g of silver nitrate and 210 ml of water per 100 g of silica. The plates were dried, activated, and stored in the dark. Plates were scanned for radioactivity with a Vanguard model 885 instrument.

Bacto-Tryptone and Proteose-Peptone were obtained from Difco Laboratories.

$^4$H_3$^4$C_3-Cholesterol (IX)—A sample of $^4$H_3$^4$C_3-cholesterol previously obtained by incubation of (3R, 4R), (2$^14$C, 4$^3$H) -MVA with acetic anhydride-pyridine (1:1, 8 ml) at room temperature was diluted with nonradioactive cholesterol to constant $^3$H:$^14$C ratio and specific activity (specific activity 0.52 x $10^5$ dpm of $14$C per mmole; $^3$H:$^14$C ratio 4.20).

$^4$C_3$^4$H_3-Cholesterol-5,7,22-trien-3$\beta$-yl-acetate (XIIb)—The band corresponding to cholesterol-5, 7, 22-trienyl acetate from the initial chromatography (7 x $10^4$ dpm of $14$C; 90% of recovered radioactivity) was further purified by preparative TLC on silica gel-silver nitrate (ethyl acetate-hexane (1:9)). An aliquot of the recovered product (6000 dpm of $14$C) was diluted with nonradioactive cholesterol-5,7,22-trienyl acetate (10 mg) and crystallized from methanol (m.p. 135-136°C; reported m.p. 134-135°C (20); 144°C uncorrected (28)) (specific activity 1.46 x $10^5$ dpm of $14$C per mmole; $^3$H:$^14$C ratio 4.30).

$^4$C_3$^4$H_3-Cholesta-5,7,22-dien-3$\beta$-yl-acetate (XIII)—An aliquot of the purified $^4$C_3$^4$H_3-cholesta-5,7,22-trienyl acetate (XIIb) (4000 dpm of $14$C) was diluted with nonradioactive material (10 mg) and dissolved in benzene (5 ml). Trias-triphenylphosphine chlororhodium (31) (5 mg) was added and the solution was stirred magnetically. Hydrogenation at atmospheric pressure for 18 hours resulted in efficient conversion to 5alpha-cholesta-7,22-dienyl acetate (XIII) as indicated by TLC. The solvent was removed under vacuum and the crude product was dissolved in ether. The solution was passed down a small column of neutral alumina (Grade I) and the column was washed with several aliquots of ether. The product was purified by preparative TLC on silica gel-silver nitrate (ethyl acetate-hexane (1:9)) and crystallized from methanol to give $^4$C_3$^4$H_3-5alpha-cholesta-7,22-dien-3$\beta$-yl-acetate (XIII) (6 mg; m.p. 132-134°C (re- reported: 131-135°C (26)) (specific activity 0.49 x $10^5$ dpm of $14$C per mmole; $^3$H:$^14$C ratio 4.33; par. per mmole $^3$H: 1275, 1650, 1200, 1045, 980 cm$^{-1}$; mass spectrum; m/e 426 (M$^+$ base peak), 411 (M$^-$(CH$_3$), 396, 381, 342, 315, and 255.

$^4$C_3$^4$H_3-Aetoxy-23,24-dinor-5alpha-chol-7-en-22-al (XIVa)—Cholestatrienyl acetate (XIVa) (ca. 8 mg; 6.5 x $10^4$ dpm of $14$C) was diluted with purified ergosteryl acetate (1.04 g) and dissolved in benzene (70 ml). The solution was hydrogenated in the presence of Tri-triphenylphosphine chlororhodium (31) (210 mg) for 18 hours (16 ml of H$_2$ absorbed). TLC indicated approximately 90% conversion to the diene. The product was isolated by removal of the solvent and addition of ether. The solution was passed through a short column of neutral alumina (Grade I) and the product was obtained by evaporation of the solvent.

The crude mixture (1.0 g) was dissolved in dichloromethane (170 ml) and cooled to -78°C in a Dry Ice-acetone bath. The solution was then treated with ozone (1.2 eq) for 6 min (0.1 mg of O$_3$ per min). The solution was purged with nitrogen for 5 min and treated with dimethyl sulfide (2 ml) in methanol (20 ml). After stirring for 5 min at -78°C the solution was warmed to 0°C and stirred for a further 30 min, after which the solvents were removed under a stream of nitrogen and the product was recovered.
purified by preparative TLC (ethyl acetate-hexane (1:9)). The product (310 mg) was crystallized from methanol to give \[^{14}C\text{H}_2\text{H}_3\text{H}_2\text{-acetox}-23, 24\)-dinor-5a-cholesterol-7-en-22-al; m.p. 130-134\(^\circ\)C (reported 133-135\(^\circ\)C (25)) (specific activity \(2.15 \times 10^4\) dpm of \[^{14}C\] per mmol; \[^{3}H\] : \[^{14}C\] ratio 3.88 (atomic ratio 2.06). The infrared spectrum showed bands at 2970, 1735, 1725 (sh), 1650, 1245, 1160, 1140, 1090, 1030, 970, and 800 cm\(^{-1}\).

The mass spectrum contained major peaks at m/e 372 (M\(^+\), base peak), 367 (M-CH\(_3\)), 354 (M-CH\(_2\)O), 314 (M-CH\(_3\)COOH), and 297 (M-CH\(_3\)COO). The mass spectrum was dominated by peaks at m/e 297, 233, 220, 217, and 147.

Equilibration of \[^{3}R\text{-acetox}-23, 24\)-dinor-5a-cholesterol-7-en-22-al (XIVa)-A sample of the aldehyde (XIVa) (50 mg) was treated with piperidine (0.2 ml) and p-toluenesulfonic acid (10 mg) in benzene (100 ml). The solution was refluxed in a Dean-Stark apparatus for 36 hours, after which the solvent and excess piperidine were removed under vacuum.

The compound had spectroscopic and chromatographic properties similar to those of the starting aldehyde (specific activity \(2.11 \times 10^4\) dpm of \[^{14}C\] per mmol; \[^{3}H\] : \[^{14}C\] ratio 1.93).

**RESULTS AND DISCUSSION**

It was pointed out in the introductory section that cholesterol biosynthesized from mevalonic acid should retain only 3 protons derived from the 4-pro-R position of MVA (21). Indeed, cholesterol (IX) obtained by incubation of (3RS,4R), (2\(^{14}C\),4\(^3\)H)-MVA with rat liver enzymes retained only three isotopic hydrogens as expected (21). We proved that 2 of the tritium atoms were present at C-17 and C-24 and had the 17a (21) and 24R (22, 23) configurations, respectively. It was apparent from our previous work (21-23) that the 3rd tritium atom could only be located at C-20 or C-22 of (IX). To define the actual location of the remaining tritium it was necessary to gain access to C-20 and C-22. From the outset it was obvious that classical chemical degradation procedures of cholesterol could not be used since they would require excessive amounts of biosynthetically prepared radioactive cholesterol. In a search for an alternative approach we turned to the protozoan T. pyriformis which was shown to metabolize cholesterol to cholesta-5,7,22-trien-3\(\alpha\)-ol (XXII) (25-28).

The project next step was ozonolysis of the \(\Delta^2\) bond. To avoid an attack of ozone on the labile conjugated system of Ring B it was deemed advisable to reduce the \(\Delta^2\) bond. Selective hydrogenation of the 7\(\beta\) and SD protons (25, 26), 22-pro-R (25, 26), and 23-pro-S (22) protons. Clearly then the 22 double bond could provide an access to the required carbons of cholesterol.

The 31\(^4\)C, 3\(^7\)H-cholesterol (IX) biosynthesized from (3RS,4R), (2\(^{14}C\),4\(^3\)H)-MVA (22, 23) was therefore incubated with T. pyriformis, and after saponification, the neutral sterols were recovered. The products were purified as the acetates to yield unchanged cholesteryl acetate and the trienyl acetate XIVb. As seen from Table 1, the starting and the recovered cholesteryl acetate and the trienyl acetate XIVb show essentially the same \[^{3}H\] : \[^{14}C\] ratio. It can therefore be inferred that the cholesterol (IX) did not have a tritium at the 22-pro-R position.

The isolated trienyl acetate XIVb after equilibration.- I

\[\begin{align*}
\text{Specific activity: } &^{14}C \quad \text{H}^{3}C & \quad \text{Final result (}^{3}H/^{14}C\text{ ratio) } \\
\text{Specific activity: } &^{14}C \quad \text{H}^{3}C & \quad \text{Isotopic} \quad \text{Atomic} \quad \text{Calculated} \\
\text{(dpm/mmole)} \times 10^4 \\
1^a & 2^a & 3^a & 1^a & 2^a & 3^a & 1^a & 2^a & 3^a \\
\text{\(1^{14}C\text{-H}_2\text{-Cholesterol (IX)}\)} & 1.33 & 1.33 & 1.35 & 4.49 & 4.56 & 4.42 & 1.33 & 4.49 & 3.0:5 & 3.0:5 \\
\text{\(1^{14}C\text{-H}_2\text{-Cholesterol (IX) (recovered)}\)} & 0.52 & 0.50 & 0.54 & 4.23 & 4.30 & 4.08 & 0.52 & 4.20 & 2.81:5 & 3.0:5 \\
\text{\(1^{14}C\text{-H}_2\text{-Cholesteryl-5,7,22-trienyl acetate (XIVb)}\)} & 1.30 & 1.52 & 1.48 & 4.44 & 4.26 & 4.20 & 1.46 & 4.30 & 2.88:5 & 3.0:5 \\
\text{\(1^{14}C\text{-H}_2\text{-Cholesteryl-7,22-dienyl acetate (XIII)\)} & 0.49 & 4.19 & 4.46 & 0.49 & 4.33 & 2.90:5 & 3.0:5 \\
\text{\(1^{14}C\text{-H}_2\text{-20\(\beta\)-acetox-5\(\alpha\)-cholesterol-7-en-22-al (XIVa) after equilibration\}} & 2.14 & 2.15 & 2.04 & 1.05 & 1.82 & 2.00 & 2.11 & 1.93 & 1.0:3:4 & 1.0:4 \\
\end{align*}\]

\(^{1}a\) Crystallization.
showed an unchanged $^{1}H$:14C ratio 4.33 (atomic ratio 2.9:5). For the ozonization it was necessary to dilute (XIII) with non-radioactive material. Because of the lack of sufficient amounts of nonradioactive XIIa or XIII the biosynthetic XIIb (3H:14C 3.88) was used. The isotope ratio of XIIb (3H:14C 3.88) showed an unchanged $^{1}H$:14C ratio 4.33 (atomic ratio 2.9:5).

For the ozonization it was necessary to dilute (XIII) with non-radioactive material. Because of the lack of sufficient amounts of nonradioactive XIIa or XIII the biosynthetic XIIb (3H:14C 3.88) was used. The isotope ratio of XIIb (3H:14C 3.88) showed an unchanged $^{1}H$:14C ratio 4.33 (atomic ratio 2.9:5).

For the ozonization it was necessary to dilute (XIII) with non-radioactive material. Because of the lack of sufficient amounts of nonradioactive XIIa or XIII the biosynthetic XIIb (3H:14C 3.88) was used. The isotope ratio of XIIb (3H:14C 3.88) showed an unchanged $^{1}H$:14C ratio 4.33 (atomic ratio 2.9:5).

For the ozonization it was necessary to dilute (XIII) with non-radioactive material. Because of the lack of sufficient amounts of nonradioactive XIIa or XIII the biosynthetic XIIb (3H:14C 3.88) was used. The isotope ratio of XIIb (3H:14C 3.88) showed an unchanged $^{1}H$:14C ratio 4.33 (atomic ratio 2.9:5).

For the ozonization it was necessary to dilute (XIII) with non-radioactive material. Because of the lack of sufficient amounts of nonradioactive XIIa or XIII the biosynthetic XIIb (3H:14C 3.88) was used. The isotope ratio of XIIb (3H:14C 3.88) showed an unchanged $^{1}H$:14C ratio 4.33 (atomic ratio 2.9:5).

For the ozonization it was necessary to dilute (XIII) with non-radioactive material. Because of the lack of sufficient amounts of nonradioactive XIIa or XIII the biosynthetic XIIb (3H:14C 3.88) was used. The isotope ratio of XIIb (3H:14C 3.88) showed an unchanged $^{1}H$:14C ratio 4.33 (atomic ratio 2.9:5).

For the ozonization it was necessary to dilute (XIII) with non-radioactive material. Because of the lack of sufficient amounts of nonradioactive XIIa or XIII the biosynthetic XIIb (3H:14C 3.88) was used. The isotope ratio of XIIb (3H:14C 3.88) showed an unchanged $^{1}H$:14C ratio 4.33 (atomic ratio 2.9:5).
The Location of the 4-pro-R Protons of Mevalonic Acid in Cholesterol
L. J. Mulheirn and Eliahu Caspi


Access the most updated version of this article at http://www.jbc.org/content/246/12/3948

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/246/12/3948.full.html#ref-list-1