The Role of a Cholesta-8,14-dien-3 β -ol System in Cholesterol Biosynthesis

By M. AKHTAR, I. A. WATKINSON, A. D. RAHIMTULA, D. C. WILTON AND K. A. MUNDAY
Department of Physiology and Biochemistry, University of Southampton, SO9 5NH

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The biosynthesis of cholesterol from squalene and tritiated water is described. Degradation of the cholesterol indicated that C-15 may be involved in cholesterol biosynthesis. In accordance with this view it is shown that in the conversion of [2*RS*-³H₂]mevalonic acid into cholesterol one of the hydrogen atoms at C-15 is removed. A mechanism for the removal of the 14 α -methyl group in steroid biosynthesis that involves the labilization of a C-15 hydrogen atom is outlined. In accordance with the requirement of this scheme it is shown that 4,4'-dimethylcholesta-8,14-dien-3 β -ol is converted into cholesterol.

An important reaction in the conversion of lanosterol (Ia) into cholesterol is the removal of the 14-methyl group. The work of Olson, Lindberg & Bloch (1957) showed that this methyl group is lost as carbon dioxide after being oxidized to a carboxyl group (compound IIa), and that 4,4'-dimethylcholesta-8,24-dien-3 β -ol (IVa) is the intermediate product of the reaction. In the past it has been assumed that the activation for this decarboxylation is provided by the presence of the C-8-C-9 double bond and occurs through a mechanism of the type shown in the sequence (Ia) $\rightarrow$ (IIa) $\rightarrow$ (IIIa) $\rightarrow$ (IVa). We now show that the removal of the 14-methyl group may proceed through the oxidation of the 15-position. Although a number of mechanisms involving position 15 for the removal of the 14-methyl group may be envisaged one possible sequence is (Ia) $\rightarrow$ (Va) $\rightarrow$ (VIa) $\rightarrow$ (IVa). This latter sequence predicts that in the conversion of lanosterol into cholesterol one of the two C-15 hydrogen atoms would be exchanged, and that 4,4'-dimethylcholesta-8,14,24-trien-3 β -ol (VIa) would be an intermediate. Experimental evidence fulfilling these predictions is presented below.

RESULTS AND DISCUSSION

Early experiments with squalene and tritiated water indicating the involvement of position 15 in cholesterol biosynthesis. Current information on the biosynthesis of sterols suggests that, in the biological conversion of squalene into cholesterol, new C-H bonds at positions 3, 4, 7, 8, 9, 14, 24 and 25 may be formed (Akhtar, Wilton & Munday, 1966; Wilton, 1967). Therefore the conversion of squalene into cholesterol by a 500g supernatant of rat liver homogenate was studied in the presence of tritiated water. When the biosynthetic cholesterol was degraded, the approximate distribution of radioactivity outlined in Table 1 was found.

However, this analysis leaves about 37% of the radioactivity unaccounted for and suggests the existence of some hitherto unobserved reaction(s) that results in the incorporation of additional radioactivity into the cholesterol molecule. To rationalize these results we predicted that some of the additional radioactivity may be located at C-15. The reasons for this prediction were originally discussed by Wilton (1967) and the salient features of the argument are summarized below.

In the lanosterol nucleus the elimination of carbon dioxide may depend on the presence of the C-8-C-9 double bond. However, the process could be greatly assisted if, before decarboxylation, the 15-position was oxidized to a hydroxyl (structure of type Va) or a carbonyl group. The regeneration of the methylene group at C-15 would then occur by a reductive process, resulting in the incorporation of radioactivity from the medium at this position. Our belief in the involvement of position 15 in

Table 1. *Distribution of radioactivity in biosynthetic cholesterol*

Position in cholesterol	Percentage of total radioactivity
C-3	3
C-4 (2H)	9
C-7	15
C-8	5
C-9	1.1, 4
C-14	12
C-20 to C-27 (side chain)	15
Total	63

Cholesterol was biosynthesized from squalene in the presence of tritiated water with a 500g supernatant of rat liver homogenate. Values for positions 9 and 14 were the results of two determinations, and the other results were the results of four or more determinations.

cholesterol biosynthesis was further strengthened by the observation that 14 α -methylcholest-7-en-3 β -ol is efficiently converted into cholesterol (Knight, Klein & Szezepanik, 1966). If the removal of the 14 α -methyl group as carbon dioxide occurs through the activation provided by an adjacent double bond by a mechanism of the type shown in the conversion (II) $\rightarrow$ (III), then the enzyme aids the decarboxylation process by protonating C-9 (IIa). However, with 14 α -methylcholest-7-en-3 β -ol as the substrate, the same acidic group on the enzyme would have to be so located as to be able to protonate C-7. This seems unlikely. We therefore argued that the only common feature that could be involved in the removal of a 14 α -methyl group in a Δ^7 as well as in a Δ^8 compound may be the presence of an electron-withdrawing or a leaving group located at C-15. A mechanism of the type illustrated by the conversion (V) $\rightarrow$ (VI) or an equivalent process such as:

(where X=O or N but not C) has abundant analogies in biological chemistry. Indeed, we are unaware of a decarboxylation process in biochemistry that operates via a mechanism of the type (II) $\rightarrow$ (III).

Cholesterol biosynthesized from squalene in the presence of tritiated water was not available in sufficient quantity to enable the determination of radioactivity at C-15 to be carried out. We therefore tested the hypothesis for the involvement of a C-15 hydrogen atom by an alternative approach.

Loss of a C-15 hydrogen atom in the biosynthesis of cholesterol from mevalonic acid. Cholesterol was biosynthesized from [2RS- $^3\text{H}_2$]mevalonic acid, positions 1, 7, 15, 22 and 26 of the biosynthetic cholesterol having arisen from C-2 of the mevalonic acid. When the biosynthetic cholesterol was converted into the ketone (VII) (Akhtar *et al.* 1966) about 12% of the original radioactivity was lost, thus showing, as expected (Akhtar & Rahimtula,

1968; Canonica *et al.* 1968a), the presence of one tritium atom at C-7. The conversion of the ketone (VII) into the conjugated ketone (VIII) (Woodward, Patchett, Barton, Ives & Kelly, 1957) was attended by a further loss of 12–13% of the radioactivity, thus showing that this amount of radioactivity was present at C-15. These results prove that only one tritium atom is located at C-15; the second tritium atom at this position therefore must have been lost during the biosynthesis. If no loss of a tritium atom from C-15 during the biosynthesis had occurred, then the loss of radioactivity in the conversion (VII) $\rightarrow$ (VIII) would have been 25%. The distribution of label in cholesterol biosynthesized from [2*RS*- $^3\text{H}_2$]mevalonic acid is therefore: C-1 (2 ^3H), C-7 (1 ^3H), C-15 (1 ^3H), C-22 (2 ^3H), C-26 (2 ^3H).

The loss of a C-15 hydrogen atom in cholesterol biosynthesis may be rationalized by the hydroxylation of this position, followed by a concomitant loss of carbon dioxide from C-14 and the hydroxyl group from C-15, resulting in the formation of a C-14–C-15 double bond [conversion (Va) $\rightarrow$ (VIa)]. A similar loss of a C-15 hydrogen atom in the biosynthesis of cholesterol was reported independently by Canonica *et al.* (1968b).

Conversion of 4,4'-dimethylcholesta-8,14-dien-3 β -ol into cholesterol. The sequence (Ia) $\rightarrow$ (Va) $\rightarrow$ (VIa) $\rightarrow$ (IVa), postulated above for the initial stages in the conversion of lanosterol into cholesterol, would require the intermediacy of 4,4'-dimethylcholesta-8,14,24-trien-3 β -ol (VIa) in the biosynthesis. We tested this hypothesis by studying the metabolism of a related compound, 4,4'-dimethylcholesta-8,14-dien-3 β -ol (VI), which lacks the side-chain double bond. It has been our experience that enzymes participating in reactions involving the steroid nucleus are relatively insensitive to the saturation status of the side chain (Wilton, Munday, Skinner & Akhtar, 1968; Akhtar *et al.* 1966; Akhtar & Rahimtula, 1968; cf. Dempsey, 1965). The tritiated diene (VI) was synthesized by the acid rearrangement of 4,4'-dimethylcholesta-5,7-dien-3 β -ol essentially by the method of Gautschi & Bloch (1958), except that the reaction was carried out in the presence of tritiated hydrochloric acid. The diene prepared by this method should contain radioactivity at positions 5, 6, 7 and 15. When a sample of this diene (VI) (100 μg .; 10^6 counts/min.) was incubated under aerobic conditions with a 10000g supernatant of rat liver homogenate as described by Wilton *et al.* (1968), about 4% of the original radioactivity was incorporated into cholesterol, which was isolated as its dibromide derivative. In an identical experiment performed under anaerobic conditions no significant radioactivity (less than 0.1%) was found in cholesterol. It is well known that, in the biosynthesis of cholesterol, hydrogen atoms from

positions 5, 6 and 7 are removed (Dewhurst & Akhtar, 1967; Akhtar & Rahimtula, 1968); the 4% conversion observed above is therefore a minimal value. Under conditions identical with those used for the incubation of the diene (VI), a related compound, lanosterol, was converted into cholesterol in about 8% yield.

The above results endorse the view that in the biosynthesis of cholesterol one of the C-15 hydrogen atoms is removed, probably via the formation of an 8(9),14(15)-diene system of the type (VI). The further conversion of this diene system into cholesterol may occur either through a 1,4-addition of hydrogen atoms resulting in the formation of a compound of the type (III) or through a 1,2-addition giving rise to a compound of the type (IV).

EXPERIMENTAL

Materials were obtained, radioactivity was measured, thin-layer chromatography was performed, enzymic systems (500g supernatant and 10000g supernatant of rat liver homogenate) were prepared, incubations were carried out and processed, and cholesterol was isolated and purified as described by Wilton *et al.* (1968). Solid steroids (about 1mg.) for incubation were dissolved in a minimal amount of acetone, and Tween 80 (50mg.) was added to form an emulsion. Acetone was removed with a stream of N_2 , and the resultant oil emulsified with 1 ml. of buffer.

Biosynthesis of cholesterol from squalene and tritiated water. Samples (5 ml.) of a 500g supernatant of rat liver homogenate were incubated (Wilton *et al.* 1968) at 37° under aerobic conditions with glucose 6-phosphate (10 μmoles), GSH (10 μmoles), NAD^+ (4.5 μmoles), NADP^+ (4 μmoles) and squalene (0.01 ml.) for 1 hr. in the presence of tritiated water (2.2×10^{10} counts/min.). In this experiment about 15800 counts/min. were incorporated into cholesterol; which was isolated and purified by the method of Wilton *et al.* (1968). In a parallel experiment carried out without squalene about 1180 counts/min. were incorporated into cholesterol. The proportion of radioactivity found in the control incubation (without squalene) varied from experiment to experiment. In some incubations the control had up to 40% of the radioactivity of the experimental. Although no definite solution to overcome this variation could be found, we noted that in general male Wistar albino rats weighing less than 175 g. and killed after a 12 hr. starvation period gave low incorporations of radioactivity in the control incubations. Cholesterol was used for

degradations only from those batches where the incorporation of radioactivity in the control incubation was less than 17%. The specific radioactivity of the biosynthetic cholesterol used for determining the radioactivity at various positions was always in the range 60–100 counts/min./mg.

Degradation of the biosynthetic cholesterol. The radioactivity at C-3 of the cholesterol biosynthesized from squalene and tritiated water was determined by converting cholesterol into cholest-5-en-3-one by the method described below. The radioactivity at C-4 was determined by converting cholest-5-en-3-one into 4,4'-dimethylcholest-5-en-3 β -ol (Woodward *et al.* 1957). The radioactivity associated with C-6, C-7 and C-8 was determined as described by Akhtar *et al.* (1966). For the determination of radioactivity at C-9 and C-14, biosynthetic cholesterol was first converted into cholest-7-en-3 β -ol acetate (Akhtar & Marsh, 1967); the latter compound was then degraded for the above-mentioned positions by the method described by Akhtar & Rahimtula (1968). The conversion of cholesterol into dehydroepiandrosterone by the method of Hershberg (1948) gave the radioactivity in the side chain. The percentage radioactivity quoted in Table 1 for positions 9 and 14 was the result of only two degradations in each case; all other positions were analysed independently at least four times, and reproducible values were always obtained.

In view of the fact that in the squalene-tritiated water experiment the radioactive cholesterol arose not only from squalene but also from other unknown precursors present in the rat liver homogenate, the result of such an experiment should be treated with caution. Our object in including this section in this paper is, not to emphasize the amount of radioactivity present at each position, but to highlight the fact that, after the removal of all those hydrogen atoms that should contain the entire radioactivity on the basis of previously held views, a considerable amount of radioactivity remained unaccounted for.

Conversion of cholesterol into cholest-5-en-3-one. To a vigorously stirred solution of cholesterol (100 mg.) in tetrahydrofuran (1 ml.) and acetone (9 ml.) at 7° under an atmosphere of CO₂, was added dropwise Jones reagent (Bowden, Heilbron, Jones & Weedon, 1946) (0.11 ml.). The mixture was further stirred at 7° for 3 min. After the mixture had been filtered through cotton-wool, crystallization was induced by the addition of water to give cholest-5-en-3-one (60 mg.), which was recrystallized from ether-methanol.

Biosynthesis of cholesterol from [2RS-³H₂]mevalonic acid. Samples (10 ml.) of a 10000g supernatant of rat liver homogenate were incubated (Wilton *et al.* 1968) at 37° with constant shaking under aerobic conditions with [2RS-³H₂]mevalonic acid (0.15 μ mole; 1.0 $\times$ 10⁷ counts/min.), glucose 6-phosphate (10 μ moles), GSH (10 μ moles) and ATP (14 μ moles) for 90 min. Ten such incubations when combined gave cholesterol (1.5 $\times$ 10⁷ counts/min.), which was isolated and purified as described by Wilton *et al.* (1968).

Determination of radioactivity at C-7 and C-15 of cholesterol biosynthesized from [2RS-³H₂]mevalonic acid. Cholesterol (1.1 g., 379000 counts/min./m-mole) was converted into cholest-5-en-3 β -ol-7-one acetate (330 mg., 332000 counts/min./m-mole) by the method of Wilton *et al.* (1968). This resulted in a loss of 12.65% of the original radioactivity. Conversion of this ketone (300 mg.) into cholesta-5,7-dien-3 β -ol benzoate (95 mg.; 331000 counts/min./m-mole) by the method of Wilton *et al.* (1968) resulted in no further loss of radioactivity. At this stage the cholesta-5,7-dien-3 β -ol

benzoate was diluted with carrier to bring the specific radioactivity to 53090 counts/min./m-mole. Cholesta-5,7-dien-3 β -ol benzoate (300 mg.; 53090 counts/min./m-mole) was converted into cholest-8(14)-en-3 β -ol-15-one benzoate by the method of Knight *et al.* (1966). The crude ketone so obtained was put on a Kieselgel GF254 thick-layer plate (20 cm.; 1 mm. thick) and developed three times in benzene. The compound that had moved 7 cm. from the origin was extracted with ether and crystallized three times from methylene chloride-methanol to give pure cholest-8(14)-en-3 β -ol-15-one benzoate (45 mg.; 46188 counts/min./m-mole). This degradation therefore proves that the cholesta-5,7-dien-3 β -ol benzoate contains 13% of its radioactivity at C-15.

Preparation of radioactive 4,4'-dimethylcholesta-8,14-dien-3 β -ol. 4,4'-Dimethylcholesta-5,7-dien-3 β -ol acetate (200 mg.) was dissolved in a mixture of acetic acid (3 ml.), benzene (5 ml.) and acetic anhydride (3 ml.) and was heated on a steam bath for 40 min. with a mixture of 36% (w/v) HCl (0.09 ml.) and tritiated water (0.2 ml.; about 200 mc). Acetic anhydride (2 ml.) and 36% (w/v) HCl (0.05 ml.) were then added and the mixture was heated under reflux for 1 hr. Sodium acetate (0.1 g.) was added, and the mixture was diluted with water and extracted with ether, to give on evaporation a yellow oil. The crude diene so obtained was spotted on a Kieselgel HF254-10% AgNO₃ thick-layer plate (20 cm. $\times$ 10 cm.; 1.5 mm. thick) and developed in benzene-light petroleum (b.p. 40–60°) (7:3, v/v). The band (R_F 0.45) visible under u.v. light was eluted with ether and crystallized once from ether-methanol to give pure 4,4'-dimethylcholesta-8,14-dien-3 β -ol acetate. On hydrolysis (Akhtar & Marsh, 1967) this gave 4,4'-dimethylcholesta-8,14-dien-3 β -ol (10 mg.; 4.12 $\times$ 10⁶ counts/min./m-mole), ϵ 17600 [Gautschi & Bloch (1958) quote ϵ 18100].

Biosynthesis of cholesterol from 4,4'-dimethylcholesta-8,14-dien-3 β -ol. Samples (10 ml.) of a 10000g supernatant of rat liver homogenate were incubated (Wilton *et al.* 1968) at 37° with constant shaking under aerobic conditions with glucose 6-phosphate (10 μ moles), GSH (10 μ moles) and 4,4'-dimethylcholesta-8,14-dien-3 β -ol (100 μ g.; 10⁶ counts/min.) emulsified with Tween 80 (50 mg.). The cholesterol (4.26 $\times$ 10⁴ counts/min.) was isolated and purified as described by Wilton *et al.* (1968).

Degradation of cholesterol biosynthesized from 4,4'-dimethylcholesta-8,14-dien-3 β -ol. Since in the above biosynthesis the substrate contains radioactivity at several positions (see the text) it was of passing interest to determine the positions of radioactivity in the biosynthetic cholesterol. The degradation of this cholesterol by the method of Wilton *et al.* (1968) showed that 6% of the radioactivity was at C-6 and 7% was at C-7, and consequently 87% was at C-15.

Measurement of radioactivity. All samples were counted in a Beckman model CPM 200 scintillation system and were corrected for quenching by internal standardization with respect to a sample of tritiated cholesterol. The efficiency of the counting system as assessed by the standard sample provided by the manufacturer was about 40–50%.

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