The Stereochemistry of the Hydrogen Elimination in the Biological Conversion of Cholest-7-en-3 β -ol into Cholesterol

By M. AKHTAR AND S. MARSH

Department of Physiology and Biochemistry, University of Southampton

(Received 11 July 1966)

1. The syntheses of Δ^7 -[4- ^{14}C]cholestenol (XVI, Scheme 3) and Δ^7 -[6 α - ^3H]-cholestenol (XII, Scheme 2) are described. 2. The metabolism of doubly labelled Δ^7 -cholestenol (II, Scheme 1) by rat-liver homogenates was studied. 3. During the enzymic conversion of Δ^7 -cholestenol into cholesterol (IV, Scheme 1) the 6 α -hydrogen atom of the former is lost and the overall reaction corresponds to a *cis*-elimination. 4. In the light of these results various mechanisms for the conversion of Δ^7 -cholestenol into cholesterol are discussed.

The enzymic conversion of lanosterol into cholesterol involves the loss of methyl groups from positions 4, 4' and 14, saturation of the side-chain double bond between positions 24 and 25, and replacement of the double bond between C-8 and C-9 by that between C-5 and C-6 (for a review see Clayton, 1965). It has been suggested that the 5,6-double bond of cholesterol (IV, Scheme 1) originates through a sequence of three enzymic reactions (Dempsey, 1965, and references cited therein). The first reaction (reaction 1, Scheme 1) involves the migration of the double bond from between C-8 and C-9 to C-7 and C-8 (Dempsey, 1965, and references cited therein). Enzymic requirements for this reaction have hitherto not been investigated. The second reaction (reaction 2, Scheme 1) requires oxygen and results in the introduction of a new double bond between C-5 and C-6 with the formation of a $\Delta^{5,7}$ -diene system (III, Scheme 1) (Dempsey, 1965). The reduction of the double bond between C-7 and C-8 in 7-dehydrocholesterol (III, Scheme 1), which requires NADPH as a cofactor, constitutes the final event in the biosynthesis of cholesterol (reaction 3, Scheme 1). We have described the mechanism of the enzymic conversion of 7-dehydrocholesterol into cholesterol (reaction 3, Scheme 1) (Wilton, Akhtar & Munday, 1966), and the present paper describes observations pertinent to the mechanism of the conversion of Δ^7 -cholestenol (II, Scheme 1) into cholesterol (IV, Scheme 1).

METHODS

Chemical synthesis of [6 α - ^3H]cholest-7-en-3 β -ol (Δ^7 -[6 α - ^3H]cholestenol; XII, Scheme 2)

and [4- ^{14}C]cholest-7-en-3 β -ol (Δ^7 -[4- ^{14}C]cholestenol; XVI, Scheme 3)

3 β -Acetoxy-6-nitrocholest-5-ene. A suspension of cholesteryl acetate (40g.) in conc. HNO_3 (1.2l.) was stirred vigorously. NaNO_2 (3 $\times$ 10g.) was added at 0, 5 and 10 min. The suspension was stirred for a further 12 min. and it was then diluted with ice-water (1.2l.). The oil formed was filtered, dissolved in ether-light petroleum (b.p. 40–60°) (1:1, v/v) washed extensively with conc. NaHCO_3 soln. and water, and finally crystallized from methanol to give 3 β -acetoxy-6-nitrocholest-5-ene. (31g., 70%), m.p. 98° [Mauthner & Suida (1903) quote m.p. 104°].

3 β -Acetoxycholestan-6-one. Zinc (50g.) was added slowly to a vigorously stirred refluxing solution of 3 β -acetoxy-6-nitrocholest-5-ene (30g.) in acetic acid (750ml.) and water (84ml.). More zinc (50g.) was added over a period of 2 hr. and the reaction was continued for a further 1 hr. The suspension was filtered and the filtrate was cooled at -10° until the appearance of crystals, whereupon water (300ml.) was added slowly. The solid was filtered, washed with conc. NaHCO_3 soln. (1.5l.) and water and finally dried in air. Crystallization from methanol gave 3 β -acetoxycholestan-6-one (20g., 71%), m.p. 124° [Mauthner & Suida (1903) quote m.p. 128°].

3 β -Acetoxy-7 α -bromocholestan-6-one (V). To a solution of 3 β -acetoxycholestan-6-one (11.7g.) in ether (150ml.) and acetic acid (30ml.) was added dropwise bromine (1.425ml.) in acetic acid (50ml.). The resulting solution was refluxed for 22 hr., then evaporated to a small volume under vacuum, water was added dropwise to turbidity, and the turbid solution was left at -10° for $\frac{1}{2}$ hr. The solid precipitated was filtered and dried. Since 3 β -acetoxy-7 α -bromocholestan-6-one was required in a high state of purity the crude product obtained (12g.) was recrystallized three times from acetone-methanol to give pure 3 β -acetoxy-7 α -bromocholestan-6-one (3.1g.), m.p. 143° [Heilbron, Jones & Spring (1937) quote m.p. 144–145°].

3 β -Acetoxy[6- ^3H]cholest-6-ene (VII). NaB^3H_4 (100mg., 12–20mc) was added to a solution of 3 β -acetoxy-7 α -

Scheme 1.

Scheme 2.

Scheme 3.

bromcholestan-6-one (1g.) in propan-2-ol (62.5 ml.), which was then vigorously stirred for 2 hr. The excess of hydride was decomposed by the addition of dil. H_2SO_4 , the product was extracted with ether-chloroform (10:1, v/v), and this layer was washed with water and evaporated to dryness. The residue was dissolved in acetic acid (75 ml.) and the resulting solution was vigorously stirred and refluxed with zinc (3g.) for 10 min. The suspension was filtered, the zinc was washed with ether, and the acetic acid solution was extracted three times with ether. The combined ether extracts and washings were washed with water, NaHCO_3 soln. and water, and evaporated to dryness. Thin-layer chromatography indicated that the product was impure, and hence it was diluted with non-radioactive β -acetoxycholest-6-ene (200 mg.) and chromatographed over alumina (15g.) with benzene-light petroleum (b.p. 40–60°) (1:9, v/v) as eluent. Crystallization from methanol gave β -acetoxy-[6- ^3H]cholest-6-ene (700 mg.), m.p. 102° [Corey & Snee (1956) quote m.p. 106–107°], activity 3440000 counts/min./mg.

β -Acetoxy-6 α ,7 α -epoxy[6- ^3H]cholestane (VIII). *m*-Chloroperbenzoic acid (237.5 mg.) in chloroform (7.5 ml.) chilled to 0° was added to β -acetoxy[6- ^3H]cholest-6-ene (350 mg.) in chloroform (2.5 ml.), also chilled to 0°, over a period of 30 min. The reaction solution was placed at 0° for 16 hr. and at room temperature for a further 4 hr. It was then worked up in the usual way. Addition of non-radioactive β -acetoxy-6 α ,7 α -epoxycholestane (350 mg.) and crystallization from methanol gave β -acetoxy-6 α ,7 α -epoxy[6- ^3H]cholestane (550 mg.), m.p. 175° [Corey & Snee (1956) quote m.p. 178°].

[6- ^3H]Cholestane-3 β ,7 α -diol (IX). LiAlH_4 (158 mg.) was added slowly to β -acetoxy-6 α ,7 α -epoxy[6- ^3H]cholestane (550 mg.) in dry ether (18 ml.). The reaction mixture was stirred and refluxed for 15 hr. and then worked up in the usual way. Crystallization from methanol gave [6- ^3H]cholestane-3 β ,7 α -diol (392 mg.), m.p. 148° [Fieser & Heymann (1952) quote m.p. 152°].

β -Acetoxy[6- ^3H]cholestan-7 α -ol (X). Acetic anhydride (0.15 ml.) was added to [6- ^3H]cholestane-3 β ,7 α -diol (392 mg.) in dry pyridine (3.92 ml.). The reaction mixture was kept for 15 hr. at room temperature. It was then worked up in the usual way. The product was chromatographed over alumina (10g.) with light petroleum (b.p. 40–60°) containing increasing amounts of benzene, and finally benzene-ethyl acetate (97:3, v/v) as eluents. The fractions were analysed by thin-layer chromatography (benzene-chloroform, 1:1, v/v) and contained in order of increasing polarity the diacetate, the monoacetate and finally the starting diol. The fractions containing the monoacetate gave on crystallization from methanol β -acetoxy[6- ^3H]cholestan-7 α -ol (250 mg.), m.p. 112° [Wintersteiner & Moore (1943) quote m.p. 117°].

β -Acetoxy[6- ^3H]cholest-7-ene (XI). POCl_3 (3 ml.) was added to β -acetoxy[6- ^3H]cholestan-7 α -ol (200 mg.) in dry pyridine (3 ml.). The reaction mixture was refluxed for 1 hr., cooled and extracted with ether. The organic layer was washed with ice-water, NaHCO_3 soln. and water and evaporated to dryness. The product was chromatographed over alumina (10g.), with light petroleum (b.p. 40–60°) containing increasing amounts of benzene as eluents. Crystallization from methanol three times gave β -acetoxy-[6- ^3H]cholest-7-ene (66 mg.), m.p. 110° [Buser (1947) quotes m.p. 116–119°], activity 1000000 counts/min./mg.

[6- ^3H]Cholest-7-ene-3 β -ol (Δ^7 -[6- ^3H]cholestenol; XII). Methanolic 10% KOH (0.1 ml.) was added to a solution of β -acetoxy[6- ^3H]cholest-7-ene (6 mg.) in methanol (1 ml.) and the reaction mixture was kept at room temperature for 15 hr. The reaction mixture was worked up in the usual way and the crystalline residue was used for incubation experiments without further purification.

β -Acetoxy[4- ^{14}C]cholest-5-ene ([4- ^{14}C]cholesteryl acetate; XIII). [4- ^{14}C]cholest-5-ene-3 β -ol ([4- ^{14}C]cholesterol) (300 mg., activity 50 μC) in dry pyridine (3 ml.) was acetylated with acetic anhydride (0.3 ml.) in the usual way. Crystallization from methanol gave β -acetoxy[4- ^{14}C]cholest-5-ene (276 mg.), m.p. 109°, activity 52000 counts/min./mg.

β -Acetoxy[4- ^{14}C]cholesta-5,7-diene (7-dehydrocholesterol acetate; XIV). β -Acetoxy[4- ^{14}C]cholest-5-ene (206 mg.) was treated by the method of Akhtar & Gibbons (1965) to give β -acetoxy[4- ^{14}C]cholest-5,7-diene (135 mg., after the addition of 100 mg. of non-radioactive β -acetoxycholest-5,7-diene) activity 15000 counts/min./mg.

[4- ^{14}C]Cholesta-5,7-dien-3 β -ol (7-dehydrocholesterol; XV). Hydrolysis of β -acetoxy[4- ^{14}C]cholesta-5,7-diene (75 mg.) by the method of Akhtar & Gibbons (1965) gave [4- ^{14}C]cholesta-5,7-dien-3 β -ol (70 mg., after the addition of 25 mg. of non-radioactive cholesta-5,7-dien-3 β -ol).

[4- ^{14}C]Cholest-7-ene-3 β -ol (Δ^7 -[4- ^{14}C]cholestenol; XVI). A solution of [4- ^{14}C]cholesta-5,7-dien-3 β -ol (60 mg.) in dioxan (2 ml.) was hydrogenated with Raney nickel W4 for 3 hr. It was then filtered over kieselguhr and evaporated to dryness. Crystallization from methanol three times gave [4- ^{14}C]cholest-7-ene-3 β -ol (30 mg.), m.p. 118° [Buser (1947) quotes m.p. 116–119°], activity 6000 counts/min./mg.

Incubation procedure

Livers taken from male albino rats weighing 200 g. were homogenized with 0.1 M-sodium phosphate buffer, pH 7.2 (1.25 ml. of buffer/g. of liver) (Bucher, 1953). The 500g cell-free supernatant was incubated with ^3H - and ^{14}C -labelled Δ^7 -cholestenol (10 μg . of steroid/ml. of supernatant).

In Expts. 1 and 4 the steroid was dissolved in propylene glycol (0.05 ml. of propylene glycol/100 μg . of steroid) and then added to the homogenate. In Expts. 2 and 3 the steroid in acetone (0.05 ml. of acetone/100 μg . of steroid) was added to the homogenate (5 ml.). Nitrogen was bubbled through to remove the acetone and the remaining homogenate was then added. In all cases incubations were carried out for 2 hr. at 37° under aerobic conditions.

Isolation and purification procedure

Non-radioactive cholesterol (100 mg.) in acetone (except in Expt. 3, when 200 mg. was used) was added to the incubation medium, its enzymic activity being destroyed by the addition of methanolic 2 N-KOH (30 ml.). It was then heated on a steam bath for 10 min. and extracted with 4 $\times$ 50 ml. of ether-light petroleum (b.p. 40–60°) (1:1, v/v). The residue was spotted on a silica-gel plate (20 cm. $\times$ 9 cm., 2 mm.-thick layer), which was developed in ether-light petroleum (b.p. 60–80°) (1:3, v/v) and then located by viewing in ordinary light. The main band was extracted with ether, and the organic layer was washed with water and evaporated to dryness under reduced pressure. The solid in ether (1 ml.) was treated with sodium acetate (3.3 mg.) and bromine (0.015 ml.) in acetic acid (0.6 ml.) and the reaction

Table 1. *Loss of the 6 α -hydrogen atom in the biological conversion of Δ^7 -cholestenol into cholesterol*

Expt. no.	Δ^7 -[4- ^{14}C , 6 α - ^3H]- cholestenol incubated		Cholesterol isolated $^3\text{H}/^{14}\text{C}$	Cholesterol dibromide isolated $^3\text{H}/^{14}\text{C}$	Conversion (%)	Loss of 6 α - ^3H (%)
	$^3\text{H}/^{14}\text{C}$	$\mu\text{g.}$				
1	24.6	570	—	1.47	24	94
2	47.8	650	—	0	37	100
3	34.3	1200	—	0.29	27.5	99.1
4	21.7	570	1.4	—	25.6	93.6

mixture was left at 0° for 15 min. The crystalline solid was filtered, crystallized four times from ether-methanol, to yield cholesterol 5 α ,6 β -dibromide, m.p. 114° [Barton & Miller (1950) quote m.p. 112–114°]. This dibromide was used for the measurement of radioactivity in Expts. 1, 2 and 3. In Expt. 4, after one crystallization, the dibromide was dissolved in ether (10 ml.) and reduced with zinc (2 g.) in the presence of acetic acid (2 drops). After vigorous stirring for 1 hr. at room temperature the reaction mixture was worked up in the usual way. Cholesterol was obtained by crystallization from acetone, dried and counted.

Measurement of radioactivity

The radioactive samples were dissolved in Nuclear Enterprise 213 liquid-scintillation fluid (4 ml.) and counted in an Isotope Developments Ltd. 2022 liquid-scintillation counter at 15% efficiency (both for ^3H and ^{14}C). Since cholesterol dibromide quenches ^{14}C activity, in Expts. 1, 2 and 3 the same amount of non-radioactive cholesterol dibromide as the radioactive sample counted was added to the samples of Δ^7 -[4- ^{14}C , 6 α - ^3H]cholestenol, to determine the original $^3\text{H}/^{14}\text{C}$ ratio, and to [4- ^{14}C]cholesteryl acetate, to determine the conversion factor.

RESULTS

In an appropriate blank experiment it was established that under the above-mentioned purification procedure all possible steroid contaminants were quantitatively removed only after the dibromide had been subjected to four or five recrystallizations from ether-methanol. Radioactivity measurements on the dibromide revealed that in the biological conversion of compound (II) into compound (IV) (Scheme 1) at least 94% of the ^3H radioactivity of Δ^7 -[6 α - ^3H]cholestenol was lost. These results could be reproduced in three consecutive experiments, shown in Table 1. In one experiment the dibromide was treated with zinc dust and converted into cholesterol; determination of radioactivity again showed that the cholesterol contained very little radioactivity due to ^3H .

DISCUSSION

Current information on the final stages of cholesterol biosynthesis is consistent with the sequence Δ^7 -cholestenol (II) $\rightarrow$ 7-dehydrocholes-

terol (III) $\rightarrow$ cholesterol (IV, see Scheme 1) (Dempsey, 1965; Dvornik, Kraml & Bagli, 1966). The main object of the present investigation was to determine the stereochemistry and locate the position of the 6-hydrogen that is removed in the formation of cholesterol (IV) from Δ^7 -cholestenol (II). The present results clearly show that the biosynthesis of cholesterol is accompanied by the loss of 5 α - and 6 α -hydrogen atoms and thus the overall conversion of compound (II) into compound (IV) corresponds to a *cis*-elimination. In the light of present results and those reported by other workers we shall evaluate various mechanisms for the conversion of compound (II) into compound (IV) (Scheme 1). Although more likely mechanisms for the formation of cholesterol from Δ^7 -cholestenol are those that operate via 7-dehydrocholesterol, nevertheless we shall also consider other possibilities that do not proceed through 7-dehydrocholesterol.

Mechanism 1: hydride-transfer mechanism. This mechanism involves a proton addition at C-8 to yield an electron-deficient species at C-7, which may then furnish cholesterol by an intramolecular transfer of a hydride ion from C-6 to C-7 followed by proton elimination from C-5 (Scheme 4). A mechanism of this type has recently been considered in the biosynthesis of methyl sterols (Akhtar, Hunt & Parvez, 1966). This mechanism would require the retention of both 6-hydrogen atoms in cholesterol. The loss of 6 α -hydrogen shown by the present study and the fact that molecular oxygen is essential (Dempsey, 1965) for this reaction makes mechanism 1 highly unlikely.

Mechanism 2: Mechanism involving successive prototropic shifts. The conversion may proceed through a series of prototropic shifts. This would involve the intermediacy of Δ^6 -cholestenol; however, failure by previous workers (Schroepfer & Frantz, 1961) to convert Δ^6 -cholestenol into cholesterol makes this mechanism highly improbable.

In discussing the two remaining mechanisms we shall assume that 7-dehydrocholesterol is an obligatory intermediate in the conversion of compound (II) into compound (IV) and will

Scheme 4.

Scheme 5.

Scheme 6.

consider the problem associated with the enzymic introduction of a double bond between two saturated carbon atoms.

Mechanism 3: hydroxylation-dehydration mechanism. To explain the role of oxygen in the formation of cholesterol (IV) from Δ^7 -cholestenol (II), Johnston & Bloch (1957) suggested that the Δ^5 -double bond in cholesterol arises by a two-step reaction involving an initial hydroxylation at C-6 followed by elimination of water. To test this hypothesis, Slaytor & Bloch (1965) studied the metabolism of Δ^7 -cholestene- $3\beta,6\alpha$ -diol and Δ^7 -cholestene- $3\beta,6\beta$ -diol in rat-liver homogenates and concluded that these compounds are not intermediates in the conversion. Although these workers were unable to synthesize the third possible hydroxylated intermediate, i.e. Δ^7 -cholestene- $3\beta,5\alpha$ -diol, they considered the intermediacy of this steroid unlikely.

It has been shown that enzymic elimination reactions of the type shown in Scheme 5 normally proceed through a *trans*-elimination (Levy, Talalay & Vennesland, 1962). If Δ^7 -cholestene- $3\beta,5\alpha$ -diol were an intermediate in the conversion of compound (II) into compound (IV) then results of the present study would make it necessary that the elimination of water from this compound should occur through a *cis*-elimination. This is unlikely but not

impossible. We hence suggest that until the metabolism of Δ^7 -cholestene- $3\beta,5\alpha$ -diol has been studied the hydroxylation-dehydration mechanism should not be considered as completely eliminated.

Mechanism 4: dehydrogenation mechanism. Finally, it may be considered that the Δ^5 -double bond of 7-dehydrocholesterol (III) is formed by a dehydrogenation reaction. An extensively studied class of dehydrogenation reactions includes the conversion of succinic acid into fumaric acid, 3-oxo steroids into Δ^1 -3-oxo steroids and Δ^4 -3-oxo steroids into $\Delta^1,4$ -3-oxo steroids (Jerussi & Ringold, 1965). These reactions have been shown to proceed via a two-step mechanism involving the removal of a proton from the carbon atom adjacent to an electron-withdrawing group, followed by a hydride transfer to a suitable oxidizing agent (Scheme 6). The reactions operating through this mechanism normally involve a *trans*-diaxial loss of two hydrogen atoms. If it is assumed that the conversion sequence (II) $\rightarrow$ (III) $\rightarrow$ (IV) proceeds via a dehydrogenation mechanism then our results allow only a *cis*-elimination of two hydrogen atoms. Such a dehydrogenation reaction has hitherto not been recognized to occur in mammalian tissues.

We thank Professor K. A. Munday for his kind interest and encouragement. We are indebted to the Medical Research Council for a research grant.

REFERENCES

- Akhtar, M. & Gibbons, C. J. (1965). *J. chem. Soc.* p. 5964.
Akhtar, M., Hunt, P. F. & Parvez, M. A. (1966). *Chem. Commun.* p. 565.
Barton, D. H. R. & Miller, E. (1950). *J. Amer. chem. Soc.* **72**, 1066.
Bucher, N. L. R. (1953). *J. Amer. chem. Soc.* **75**, 498.
Buser, W. (1947). *Helv. chim. Acta*, **30**, 1379.
Clayton, R. B. (1965). *Quart. Rev. chem. Soc., Lond.*, **19**, 168.
Corey, E. J. & Sneed, R. A. (1956). *J. Amer. chem. Soc.* **78**, 6269.
Dempsey, M. E. (1965). *J. biol. Chem.* **240**, 4176.
Dvornik, D., Kraml, M. & Bagli, J. F. (1966). *Biochemistry*, **5**, 1060.
Fieser, L. F. & Heymann, H. (1952). *Helv. chim. Acta*, **35**, 631.
Heilbron, I. M., Jones, E. R. H. & Spring, F. S. (1937). *J. chem. Soc.* p. 801.
Jerussi, R. & Ringold, H. J. (1965). *Biochemistry*, **4**, 2113.
Johnston, J. D. & Bloch, K. (1957). *J. Amer. chem. Soc.* **79**, 1145.
Levy, H. R., Talalay, P. & Vennesland, B. (1962). *Progr. Stereochem.* **3**, 299.
Mauthner, J. & Suida, W. (1903). *Mh. Chem.* **24**, 648.
Schroepfer, G. J., jun. & Frantz, I. D., jun. (1961). *J. biol. Chem.* **236**, 3137.
Slaytor, M. & Bloch, K. (1965). *J. biol. Chem.* **240**, 4598.
Wilton, D. C., Akhtar, M. & Munday, K. A. (1966). *Biochem. J.* **98**, 29c.
Wintersteiner, O. & Moore, M. (1943). *J. Amer. chem. Soc.* **65**, 1503.