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The Conversion of Cholest-7-en-3β-ol into Cholesterol

GENERAL COMMENTS ON THE MECHANISM OF THE INTRODUCTION OF DOUBLE BONDS IN ENZYMIC REACTIONS

BY S. MARSH DEWHURST AND M. AKHTAR
Department of Physiology and Biochemistry, University of Southampton

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Convenient syntheses of 6β-tritiated Δ^7-cholestenol and 3α-tritiated Δ^7-cholestene-3β,5α-diol are described. It was shown that the conversion of 6β-tritiated Δ^7-cholestenol into cholesterol is accompanied by the complete retention of label. It was unambiguously established that the overall reaction leading to the introduction of the double bond in the 5,6-position in cholesterol occurs via a *cis*-elimination involving the 5α- and 6α-hydrogen atoms and that during this process the 6β-hydrogen atom remains completely undisturbed. Metabolic studies with 3α-tritiated Δ^7-cholestene-3β,5α-diol revealed that under anaerobic conditions the compound is not converted into cholesterol. This observation, coupled with the previous work of Slaytor & Bloch (1965), is interpreted to exclude a hydroxylation–dehydration mechanism for the origin of the 5,6-double bond in cholesterol. It was also shown that under aerobic conditions 3α-tritiated Δ^7-cholestene-3β,5α-diol is efficiently converted into cholesterol and that this conversion occurs through the intermediacy of 7-dehydrocholesterol. Cumulative experimental evidence presented in this paper and elsewhere is used to suggest that the 5,6-double bond in cholesterol originates through an oxygen-dependent dehydrogenation process and a hypothetical mechanism for this and related reactions is outlined.

We have recently described some preliminary experiments on the mechanism of the biological conversion of Δ^7-cholestenol (II) into cholesterol (VIII) (Akhtar & Marsh, 1967). This conversion (reactions 2 and 3, Scheme 1) occurs in the final stages of cholesterol biosynthesis by the sequence (I)→(II)→(VII)→(VIII), as shown in Scheme 1 (Dempsey, 1965; for a review see Clayton, 1965). Certain features of this Scheme have recently been confirmed (Wilton, Akhtar & Munday, 1966; Akhtar, Wilton & Munday, 1966).

Previous work on the mechanism of the enzymic conversion of Δ^7-[6α-^{3}H]cholestenol (IV) into cholesterol (VIII) has shown that during this conversion the 6α-^{3}H of the former is stereospecifically removed, thus suggesting that the overall conversion of Δ^7-cholestenol into cholesterol involves the *cis*-elimination of the two hydrogen atoms at C-5α and C-6α (Akhtar & Marsh, 1967).

We now describe the metabolism of Δ^7-[6β-^{3}H]-cholestenol (III) and Δ^7-[3α-^{3}H]cholestene-3β,5α-diol (V), propose a mechanism for the conversion of Δ^7-cholestenol (II) into cholesterol (VIII) and make generalized suggestions for the possible mechanisms that may be involved in the enzymic introduction of double bonds between two saturated carbon atoms.

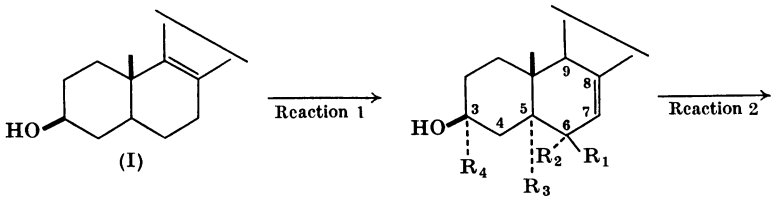
In a previous paper (Akhtar & Marsh, 1967) we considered a number of theoretically possible mechanisms for the conversion of Δ^7-cholestenol (II) into 7-dehydrocholesterol (VII); however, the results presented there eliminated all mechanisms except two: a hydroxylation–dehydration mechanism and a dehydrogenation mechanism.

RESULTS AND DISCUSSION

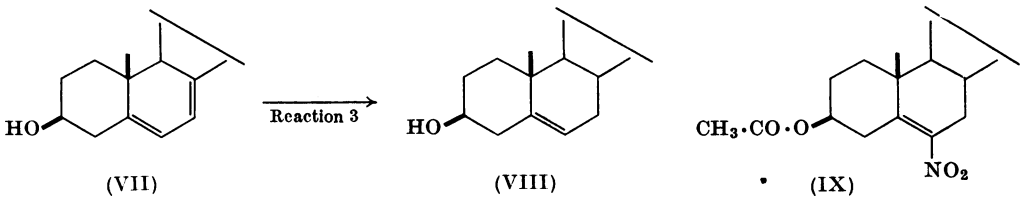
Hydroxylation–dehydration mechanism. To explain the role of oxygen in the conversion of Δ^7-cholestenol (II) into 7-dehydrocholesterol (VII), Johnston & Bloch (1957) suggested that the Δ^5-double bond in cholesterol may arise by a two-step mechanism involving an initial hydroxylation followed by dehydration.

To test this hypothesis Slaytor & Bloch (1965) studied the metabolism of two of the three possible hydroxylated intermediates, i.e. Δ^7-cholestene-3β,6α-diol and Δ^7-cholestene-3β,6β-diol, and they concluded that these compounds were not intermediates in the conversion.

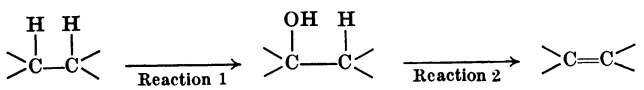
We now report that the third possible hydroxylated intermediate, Δ^7-cholestene-3β,5α-diol (VI), when incubated under anaerobic conditions with rat liver homogenates, was not converted into



(II) $R_1=R_2=R_3=R_4=H$
(III) $R_2=R_3=R_4=H$, $R_1={}^3H$
(IV) $R_1=R_3=R_4=H$, $R_2={}^3H$
(V) $R_1=R_2=H$, $R_3=OH$, $R_4={}^3H$
(VI) $R_1=R_2=R_4=H$, $R_3=OH$



Scheme 1.



Scheme 2.

Table 1. *Metabolism of* Δ^7-[3α-^{3}H]*cholestene*-3β,5α-*diol and* Δ^7-[6β-^{3}H]*cholestenol*

	Anaerobic incubation of Δ^7-[3α-^{3}H]-cholestene-3β,5α-diol			Aerobic incubation of Δ^7-[3α-^{3}H]-cholestene-3β,5α-diol			Aerobic incubation of Δ^7-[6β-^{3}H]-cholestenol		
Expt. no.	μg. of diol incubated	μg. converted into cholesterol*	% conversion into cholesterol*	μg. of diol incubated	μg. converted into cholesterol*	% conversion into cholesterol*	μg. incubated	μg. converted into cholesterol*	% conversion into cholesterol*
1	378	12·4	3·3	378	76·7	20·3			
2	189	1·55	0·85				184	90·3	49·1
3	189	0·88	0·47				184	69·4	37·7
4	193·4	2·69	1·4	193·4	59·3	29·1	207·5	91	43·8
5	193·4	2·28	1·1	193·4	94·6	49	207·5	94·9	45·7
6				193·4	72·9	37·7			
7				193·4	72·4	37·4			

* Counted as dibromide.

cholesterol (Table 1). The hydroxylation–dehydration mechanism shown in Scheme 2 requires that the dehydration step (reaction 2, Scheme 2) should operate without the involvement of oxygen. This result, showing that the diol (VI) was not an intermediate in the conversion of Δ^7-cholestenol (II) into cholesterol (VIII), was further substantiated by the fact that incubation of tritiated Δ^7-cholestenol in the presence of various amounts of non-radioactive diol (VI) (Table 2, Expts. 1–3) gave no appreciable radioactivity in the 'trap'. The failure of the three hydroxylated compounds, Δ^7-cholestene-3β,5α-diol, Δ^7-cholestene-3β,6α-diol and Δ^7-cholestene-3β,6β-diol, to serve as precursors

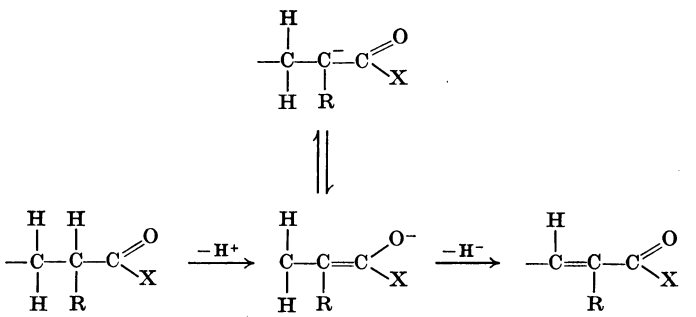
Table 2. *Aerobic incubation of labelled* Δ^7*-cholestenol* (II) *in the presence of* Δ^7*-cholestene-3*β*,5*α*-diol* (VI) *added as a 'trap' and the anaerobic incubation of* Δ^7*-[3*α*-*^{3}*H]cholestene-3*β*,5*α*-diol* (V) *in the presence of* Δ^7*-cholestenol* (II) *added as a 'trap'*

Expt. no.	Amount of substrate incubated (μg.)		Atmosphere	Amount of 'trap' added (mg.)		% incorporation of substrate radioactivity into 'trap'‡	Time of incubation (hr.)
1	(II)*	415	O_2	(VI)	2	0·6	2
2	(II)†	976	O_2	(VI)	20	0·3	½
3	(II)†	976	O_2	(VI)	20	0·1	1
4	(V)	193	CO_2	(II)	10	2·0	2
5	(V)	193	CO_2	(II)	None	0·8	2
6	(V)	193	CO_2	(II)	None	0·7	2

* Labelled Δ^7-cholestenol used was Δ^7-[6β-^{3}H]cholestenol.

† Labelled Δ^7-cholestenol used was Δ^7-[3α-^{3}H]cholestenol.

‡ Percentage incorporation of the substrate radioactivity into the 'trap' is based on the amount of substrate incubated. Since the percentage incorporation into the 'trap' was low no attempt was made to determine whether the 'trap' contained a contaminant that co-crystallized with the 'trap'.



Scheme 3.

of cholesterol under anaerobic conditions, and the inability of Δ^7-cholestenol to furnish any of the hydroxylated intermediates under the conditions of cholesterol biosynthesis, make the hydroxylation–dehydration mechanism highly improbable for this conversion.

Dehydrogenation mechanism. We therefore conclude that the Δ^5-double bond of 7-dehydrocholesterol (VII) is formed by a dehydrogenation mechanism involving the direct removal of two hydrogen atoms. An extensively studied class of dehydrogenation reactions includes the conversion of succinic acid into fumaric acid (Englard & Colowick, 1956; Tchen & van Milligan, 1960), 3-oxo steroids into Δ^1-3-oxo steroids and Δ^4-3-oxo steroids into $\Delta^{1,4}$-3-oxo steroids (Ringold, Hayano & Stefanovic, 1963; Jerussi & Ringold, 1965). These reactions have two characteristic features, the first being that they proceed via a two-step mechanism involving the initial 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 3). The second is that reactions operating through this mechanism (Scheme 3) involve a *trans*-removal of two hydrogen atoms. The two-step mechanism shown in Scheme 3 requires that the first step in the conversion may proceed under anaerobic conditions. Consistent with this requirement is the fact that succinic acid, when incubated with succinate dehydrogenase in the absence of an electron acceptor and in the presence of heavy water, incorporated one atom of deuterium (Weinmann, Morehouse & Winzler, 1947; Englard & Colowick, 1956; Gawron, Glaid, Francisco & Fondy, 1963; Gawron, Glaid & Francisco, 1964). The *trans*-removal of the two hydrogen atoms in the 'succinic acid ⇋ fumaric acid' reaction and other related reactions is also well established (Tchen & van

Milligan, 1960; Gawron, Glaid, Fondy & Bechtold, 1961). We now attempt to apply the above two criteria to the conversion of Δ^7-cholestenol (II) into 7-dehydrocholesterol (VII).

Stereochemistry of hydrogen elimination in the conversion of Δ^7-cholestenol into 7-dehydrocholesterol. It has already been shown that in the conversion of Δ^7-[6α-^{3}H]cholestenol (IV) into cholesterol (VIII) the 6α-^{3}H is lost (Akhtar & Marsh, 1967). To demonstrate that this loss of the 6α-^{3}H is not due to total oxidation of the 6-position, i.e. formation of the 6-oxo compound, Δ^7-[4-^{14}C,6β-^{3}H]cholestenol (^{3}H/^{14}C ratio 27) was converted into cholesterol (VIII) (^{3}H/^{14}C ratio 27·8). The ^{3}H/^{14}C ratios obtained for the starting material and the product showed that this conversion was accompanied by complete retention of the tritium label. The chemical conversion of the biosynthesized cholesterol into the nitro compound (IX) then resulted in complete removal of the labelled hydrogen. The above evidence therefore establishes that in the conversion of Δ^7-cholestenol (II) into 7-dehydrocholesterol (VII) the 6α-hydrogen of (II) is stereospecifically lost and that during this conversion the 6β-hydrogen remains completely undisturbed. It can thus be concluded that, unlike the 'succinic acid $\leftrightharpoons$ fumaric acid' type of dehydrogenation, dehydrogenation of Δ^7-cholesterol into 7-dehydrocholesterol is accompanied by a *cis*-elimination.

Aerobic metabolism of Δ^7-[3α-^{3}H]cholestene-3β,5α-diol. Although under anaerobic conditions the diol (V), when incubated with rat liver homogenates, was stable, we observed that in the presence of oxygen this diol (V) was efficiently converted into cholesterol (Table 1). One theoretically feasible pathway for the metabolism of the diol involves an anaerobic dehydroxylation to yield Δ^7-cholestenol (II), which through the sequence shown in Scheme 1 is then converted into cholesterol. A pathway of this type has previously been established for the metabolism of Δ^7-cholestene-3β,6β-diol into cholesterol by rat liver enzymes (Slaytor & Bloch, 1965). All attempts to demonstrate convincingly the presence of Δ^7-cholestenol in the metabolism of the diol (V), however, proved fruitless (Table 2, Expts. 4–6), and we considered alternative explanations. When the diol (V) was incubated aerobically with the liver enzyme preparation in the presence of non-radioactive 7-dehydrocholesterol (VII) about 4% of the original radioactivity was incorporated into the 'trap'; in the same experiment about 12% of the radioactivity was also found in cholesterol (Table 3, Expt. 2). As far as we are aware the aerobic conversion of the diol (V) into 7-dehydrocholesterol (VII) represents the first example of an enzymic 'oxidative–dehydration' process. Although the precise nature of the enzyme that catalyses the reaction has, as yet, not been established, the fact that Δ^7-cholestene-3β,5α-diol (VI) is metabolized to 7-dehydrocholesterol (VII) and also that the conversion requires oxygen may suggest that the conversions of Δ^7-cholestenol (II) into 7-dehydrocholesterol (VII) and of Δ^7-cholestene-3β,5α-diol (VI) into 7-dehydrocholesterol (VII) are catalysed by the same enzyme system. The indication that the diol (VI) can act as an analogue of the physiological substrate, Δ^7-cholestenol (II), has an important bearing on the mechanism of the dehydrogenation of Δ^7-cholestenol (II) to 7-dehydrocholesterol (VII).

Mechanism of the conversion of Δ^7-cholestenol into 7-dehydrocholesterol. It is postulated that in the enzymic conversion of Δ^7-cholestenol (II) into 7-dehydrocholesterol (VII) the function of the

Table 3. *Aerobic incubation of Δ^7-[3α-^{3}H]cholestene-3β,5α-diol in the presence of 7-dehydrocholesterol as a 'trap' for ½ hr.*

Expt. no.	Amount of substrate incubated (mg.)	Amount of 'trap' added (mg.)	% incorporation of substrate radioactivity into 'trap'*†	% incorporation of substrate activity into cholesterol*
1	1·33	2	3·9	—
2	1·05	2	3·8	11·9
3	1·05	—	—	12·1
4	1·05	2	4·6	—
5	1·05 (denatured enzyme)	2	0·06	—

* Percentage incorporation of the substrate radioactivity into the 'trap' or into cholesterol is based on the amount of substrate added.

† 7-Dehydrocholesterol was isolated and counted as 3β-acetoxy-5α,8α-epidioxycholest-6-ene, which was prepared as described in the Materials and Methods section.

oxygen is twofold: (*a*) to provide a basic group on the enzyme–substrate complex that could abstract a proton; (*b*) to provide an electron acceptor.

A hypothetical mechanism that may fulfil this requirement is shown in Scheme 4. It is postulated that structure (ii) arises through the interaction of oxygen with a highly reduced group –X: of the enzyme. The types of groups that may fulfil the above requirement include unusual reduced forms of metals, e.g. Fe^+, Fe^0. The formation of the reduced species –X: may, in turn, occur as:

$$-X + 2e = -X:$$

It is suggested that the 'active oxygen' in structure (ii) may then be directly involved in the *cis*-removal of the two hydrogen atoms.

The *cis*-elimination of hydrogen atoms noted in the conversions of Δ^7-cholestenol into 7-dehydrocholesterol and of cholestanol into Δ^7-cholestenol (Clayton & Edwards, 1963) is readily explicable by this mechanism (Scheme 4). Also consistent with a concerted mechanism is the fact that when doubly labelled Δ^7-cholestenol is incubated with rat liver homogenates the 6α-^{3}H in the recovered Δ^7-cholestenol remains undisturbed. Non-activated C–H bonds are also involved in the biosynthesis of the ethylenic linkage in oleic acid, arachidonic acid and other fatty acids, and in the introduction of certain double bonds in the side chains of a number of sterols. The experimental data presented by Schroepfer & Bloch (1965) on the conversion of stearic acid into oleic acid and by Stoffel & Schiefer (1966) on the conversion of homo-γ-linolenic acid into arachidonic acid are consistent with the mechanism presented in Scheme 4.

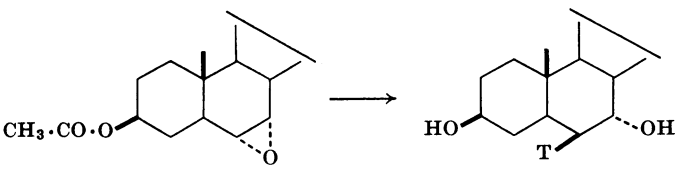
$$\underset{\text{(i)}}{\text{Enz–X:}} \xrightarrow{O=O} \underset{\text{(ii)}}{\text{Enz–}\overset{+}{\text{X}}\text{–O–O}^-}$$

Scheme 4.

Syntheses of Δ^7-[6β-^{3}H]*cholestenol* (III) *and* Δ^7-[3α-^{3}H]*cholestene*-3β,5α-*diol* (V). Δ^7-[6β-^{3}H]-Cholestenol (III) was synthesized from the epoxide (X).

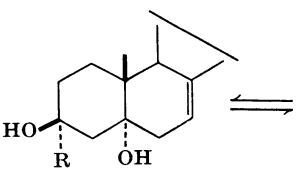
The unavailability of $LiAl^3H_4$ necessitated the development of an alternative method for the opening of the oxide ring in the epoxide (X). Treatment of the epoxide (X) with a mixture of NaB^3H_4 and anhydrous aluminium chloride in diglyme at about 75° gave the diol (XI), which was then converted into Δ^7-[6β-^{3}H]cholestenol (III) by the method previously described for the synthesis of Δ^7-[6α-^{3}H]cholestenol (IV) (Akhtar & Marsh, 1967).

Δ^7-[3α-^{3}H]Cholestene-3β,5α-diol (V) was synthesized from the non-radioactive diol (VI), which was prepared by the method described in the Addendum (Dewhurst, Kelly, Hudec & Akhtar, 1967). This diol (VI), on treatment with Jones reagent (Bowden, Heilbron, Jones & Weedon, 1946), gave the ketone (XII), which was then reduced with NaB^3H_4 to yield the 3α-tritiated diol (V). The stereochemistry of the metal hydride reduction of β-hydroxy ketones of the type (XII) into diols of the type (VI) has been established and is discussed for model compounds in a previous paper (Akhtar & Marsh, 1966).

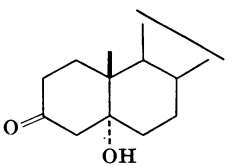


(X)

(XI)



(VI) R = H
(V) R = ^{3}H



(XII)

MATERIALS AND METHODS

Materials

[4-^{14}C]Cholest-7-en-3β-ol (Δ^7-[4-^{14}C]cholestenol), [6α-^{3}H]cholest-7-en-3β-ol (Δ^7-[6α-^{3}H]cholestenol) (IV) and 3β-acetoxy-6α,7α-epoxycholestane (X) were synthesized as previously described (Akhtar & Marsh, 1967).

Chemical syntheses of [6β-^{3}H]*cholest-7-en-3β-ol* (Δ^7-[6β-^{3}H]*cholestenol*) (III) *and* [3α-^{3}H]*cholest-7-ene-3β,5α-diol* (Δ^7-[3α-^{3}H]*cholestene-3β,5α-diol*) (V)

[6β-^{3}H]*Cholestane-3β,7α-diol* (XI). Diglyme (40ml.) was distilled over $LiAlH_4$ into a flame-dried flask containing 3β-acetoxy-6α,7α-epoxycholestane (355mg.), and a solution of anhydrous $AlCl_3$ (87·5mg.) in freshly distilled diglyme (10ml.) was added. NaB^3H_4 (75mg., 12–20mc) was added to the flask and the resulting solution was vigorously stirred at 75° for 1hr. The product was extracted with ether, the ethereal solution was evaporated to dryness and the residue in chloroform was placed on two silica-gel plates (20cm.× 9cm., 2mm. layer), which were developed in methanol–chloroform (1:49, v/v) three times and the bands then located with iodine. The main band was extracted with ether–methanol (9:1, v/v), and non-radioactive cholestane-3β,7α-diol (250mg.) was added to the organic solution. This solution was washed with $Na_2S_2O_3$ solution, dried and evaporated to dryness. Crystallization from ether–methanol gave [6β-^{3}H]cholestane-3β,7α-diol (380mg.), m.p. 149° [Fieser & Heymann (1952) quote m.p. 152°], specific activity 835000counts/min./mg.

[6β-^{3}H]*Cholest-7-en-3β-ol* (Δ^7-[6β-^{3}H]*cholestenol*) (III). [6β-^{3}H]Cholest-7-en-3β-ol was prepared from [6β-^{3}H]-cholestane-3β,7α-diol (XI) as previously described (Akhtar & Marsh, 1967).

Cholest-7-ene-3β,5α-diol (VI). Cholest-7-ene-3β,5α-diol was prepared from 3β-acetoxycholesta-5,7-diene as described in the Addendum (Dewhurst *et al.* 1967).

5α-*Hydroxycholest-7-en-3-one* (XII). Cholest-7-ene-3β,5α-diol (50mg.) in acetone (5ml.) was oxidized with Jones reagent (0·06ml.) (Bowden *et al.* 1946). Crystallization from acetone gave 5α-hydroxycholest-7-en-3-one (25mg.), m.p. 220°.

[3α-^{3}H]*Cholest-7-ene-3β,5α-diol* (Δ^7-[3α-^{3}H]*cholestene-3β,5α-diol*) (V). NaB^3H_4 (5mg., 1–2mc) was added to a solution of 5α-hydroxycholest-7-en-3-one (10mg.) in methanol (5ml.). The reaction mixture was kept at room temperature for 1hr. and then worked up as described above. Non-radioactive cholest-7-ene-3β,5α-diol (150mg.) was added to the ether extract, which was then evaporated to dryness. The solid in chloroform was placed on a preparative silica-gel layer, which was developed in methanol–chloroform (1:99, v/v) twice and the bands were then located with iodine. The main band was extracted with ether–methanol (9:1, v/v), the organic layer was washed with $Na_2S_2O_3$ solution and water and evaporated to dryness. Crystallization from acetone gave [3α-^{3}H]-cholest-7-ene-3β,5α-diol (60mg.), m.p. 202°, specific activity 410000counts/min./mg. Thin-layer chromatography (methanol–chloroform, 1:99, v/v) indicated that the [3α-^{3}H]cholest-7-ene-3β,5α-diol, prepared as described above, was slightly contaminated with a compound of greater polarity than the diol. To demonstrate that all the radioactivity was associated with the [3α-^{3}H]cholest-7-ene-3β,5α-diol this compound (200μg.) was spotted on a thin silica-gel layer (20cm.×4·5cm., 0·2mm. layer), which was developed in methanol–chloroform (1:99, v/v). Location with iodine showed two distinct bands, which when counted confirmed that all the radioactivity was associated with [3α-^{3}H]cholest-7-ene-3β,5α-diol.

Incubation procedure

The rat liver homogenates were prepared as described previously (Akhtar & Marsh, 1967). In all experiments the steroid in acetone was added to the homogenate (0·2ml. of acetone solution/3ml. of homogenate). Nitrogen was bubbled through to remove the acetone and then homogenate was added (10ml. of homogenate/3ml. of deactivated homogenate).

Aerobic incubation of Δ^7-[4-^{14}C,6β-^{3}H]*cholestenol to cholesterol*

Δ^7-[4-^{14}C,6β-^{3}H]Cholestenol (608μg., ^{3}H/^{14}C ratio 27) was incubated aerobically with rat liver homogenate (60ml.) and the cholesterol, which was isolated and purified as described previously (Akhtar & Marsh, 1967), was found to have a ^{3}H/^{14}C ratio 27·8, showing that the retention of the 6β-^{3}H in the cholesterol produced was 100%. On the basis of the recovered ^{14}C radioactivity it was found that the conversion was 25%. In a second experiment Δ^7-[4-^{14}C,6β-^{3}H]cholestenol (304μg., ^{3}H/^{14}C ratio 39·4) was incubated as described above and the conversion into cholesterol (^{3}H/^{14}C ratio 37·1) was 48%, the retention of the 6β-^{3}H being 94·5%.

Aerobic incubation of Δ^7-[6β-^{3}H]*cholestenol to cholesterol and degradation of the cholesterol formed*

Radioactive cholesterol (total radioactivity 108750 counts/min.), biosynthesized from Δ^7-[6β-^{3}H]cholestenol (1·08mg., specific activity 740000counts/min./mg.), was acetylated. The cholesterol acetate produced (specific activity 174counts/min./mg.) was nitrated to give 3β-acetoxy-6-nitrocholest-5-ene (XI) (specific activity 1count/min./mg.) (Akhtar & Marsh, 1967).

Anaerobic incubation of Δ^7-[4-^{14}C,6α-^{3}H]*cholestenol and recovery of the starting material*

Δ^7-[4-^{14}C,6α-^{3}H]Cholestenol (650μg., ^{3}H/^{14}C ratio 27·3) was incubated anaerobically with rat liver homogenate (20ml.). After 2hr. non-radioactive Δ^7-cholestenol (100mg.) in acetone was added to the incubation medium, its enzymic activity being destroyed as previously described (Akhtar & Marsh, 1967). The steroid was extracted and spotted on a preparative silica-gel layer, which was developed in acetone–light petroleum (b.p. 60–80°) (1:3, v/v) and the bands were then located by viewing in ordinary light. The main band was extracted with methanol–ether (1:9, v/v), and the organic layer was washed with water and evaporated to dryness under reduced pressure. The product was recrystallized from ether–methanol four times and it was found to have a ^{3}H/^{14}C ratio 26, showing that the retention of the 6α-^{3}H was 95% and the recovery of Δ^7-[4-^{14}C,6α-^{3}H]-cholestenol was 81%.

In a parallel experiment Δ^7-[4-^{14}C,6α-^{3}H]cholestenol

(650μg., $^3H/^{14}C$ ratio 29·2) was incubated aerobically. The cholesterol isolated from this incubation had a $^3H/^{14}C$ ratio 0·1, showing that the loss of the 6α-^3H was 99·7%, the conversion into cholesterol being 33%.

Anaerobic incubation of Δ^7-[3α-^{3}H]cholestene-3β,5α-diol and isolation of Δ^7-cholestenol

Δ^7-[3α-^{3}H]Cholestene-3β,5α-diol was incubated anaerobically in the presence of Δ^7-cholestenol (except in Expts. 5 and 6, Table 2). At the end of the incubation non-radioactive Δ^7-cholestenol (100mg. in Expts. 5 and 6, 90mg. in Expt. 4, Table 2) in acetone was added and the steroid was then extracted and purified as described above.

Aerobic incubation of radioactive Δ^7-cholestenol in the presence of Δ^7-cholestene-3β,5α-diol and isolation of Δ^7-cholestene-3β,5α-diol

Radioactive Δ^7-cholestenol was incubated in the presence of Δ^7-cholestene-3β,5α-diol as a 'trap'. At the end of the incubation non-radioactive Δ^7-cholestene-3β,5α-diol (100mg. in Expt. 1, 80mg. in Expts. 2 and 3, Table 2) in acetone was added. The steroid was extracted as described above and purified by spotting on a preparative silica-gel layer, which was developed in methanol–chloroform (1:49, v/v) and the bands were then located by viewing in ordinary light. The solid was extracted as described above and recrystallized from methanol four times.

Aerobic incubation of Δ^7-[3α-^{3}H]cholestene-3β,5α-diol and isolation of 7-dehydrocholesterol

Δ^7-[3α-^{3}H]Cholestene-3β,5α-diol was incubated in the presence of 7-dehydrocholesterol (except in Expt. 3, Table 3). At the end of the incubation non-radioactive 7-dehydrocholesterol (100mg.) in acetone was added to the incubation medium in a darkened flask. Its enzymic activity was destroyed by the addition of methanolic 2N-KOH (10ml.). It was then heated on a steam bath for 5min. under an atmosphere of nitrogen. The steroid was extracted as described above and purified by spotting on a fluorescent preparative silica-gel layer, which was developed in acetone–light petroleum (b.p. 60–80°) (1:3, v/v), and the bands were then located by viewing with u.v. light. After extraction the solid in dry pyridine (1ml.) was treated with acetic anhydride (0·1ml.) and the reaction mixture was kept under an atmosphere of nitrogen at 5° for 2 days. It was then worked up as described above. The 7-dehydrocholesterol acetate obtained was dissolved in ethanol (10ml.) and a trace of eosin was added. The solution was irradiated from beneath with a 200w frosted bulb placed in close proximity to the reaction vessel. The whole system was surrounded by aluminium foil to act as a reflector. Oxygen was bubbled through the refluxing solution. After 30min., when the u.v. spectrum indicated that the reaction had gone to completion, the solution was evaporated to dryness under reduced pressure. Crystallization from ether–methanol four times gave 3β-acetoxy-5α,8α-epidioxycholest-6-ene (20mg.), m.p. 151° (see Table 3).

A mixture of this product (10mg.) and a trace of non-radioactive 7-dehydrocholesterol acetate was spotted on a thin silica-gel plate (20cm.×4·5cm., 0·2mm. layer), which was developed in benzene–chloroform (1:1, v/v). Viewing in ordinary light showed two distinct bands, which when counted showed that all the radioactivity was associated with 3β-acetoxy-5α,8α-epidioxycholest-6-ene; there was no radioactivity in the 7-dehydrocholesterol acetate band (cholesterol acetate and related compounds in the above solvent system would run with 7-dehydrocholesterol acetate).

Measurement of radioactivity

All radioactive samples were dried in a vacuum oven for 1hr. at 45° and then counted as previously described (Akhtar & Marsh, 1967).

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ADDENDUM

Chemical Synthesis of Cholest-7-ene-3β,5α-diol

By S. MARSH DEWHURST, R. S. A. KELLY, J. HUDEC AND M. AKHTAR
Department of Physiology and Biochemistry, University of Southampton

(*Received* 18 *April* 1967)

Δ^7-Cholestene-3β,5α-diol was synthesized from 7-dehydrocholesterol acetate by the method described in the Materials and Methods section.

Since the introduction of the double bond into 3β-acetoxycholest-7-en-5α-ol was achieved by acid dehydration (Clayton, Henbest & Jones, 1953), it was necessary to establish that the dehydration had occurred between C-7 and C-8 and also that the double bond so formed did not migrate to some other position under acid conditions. Acid dehydration of 3β-acetoxycholestane-5α,8α-diol could result in the formation of a double bond in one of three positions, i.e. $\Delta^{7,8}$, $\Delta^{8,9}$ or $\Delta^{8,14}$, in the product. The nuclear-magnetic-resonance (n.m.r.) spectra of the latter two compounds would have no signal due to an olefinic proton. Examination of the n.m.r. spectrum of 3β-acetoxycholest-7-en-5α-ol showed a broad singlet at δ 5·13p.p.m. (two protons), which could be assigned to the $C_{(3)}$–H and $C_{(7)}$–H protons. Evidence that this assignment was correct was obtained by studying the n.m.r. spectra of several compounds closely related to 3β-acetoxycholest-7-en-5α-ol shown in Table 1. We observed that in compounds containing a 5α-hydroxyl group the signal due to the $C_{(3)}$–H proton was shifted to a lower field.

Table 1. *Nuclear-magnetic-resonance spectral characteristics of 3β-acetoxycholest-7-en-5α-ol and related compounds*

Compound	Position of of $C_{(3)}$–H (p.p.m.)	Position of of $C_{(7)}$–H (p.p.m.)
Cholest-7-ene-3β,5α-diol	3·99	5·09
3β-Acetoxycholest-7-en-5α-ol	5·13	5·13
Cholestane-3β,5α-diol	4·04	—
3β-Acetoxycholestane-5α-ol	5·15	—
Cholest-7-en-3β-ol	3·68	5·15
3β-Acetoxycholest-7-ene	4·69	5·13

MATERIALS AND METHODS

Materials

3β-Acetoxycholesta-5,7-diene (7-dehydrocholesterol acetate) was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., and was found by u.v. spectroscopy to be over 90% pure.

Chemical synthesis of cholest-7-ene-3β,5α-diol (Δ^7-cholestene-3β,5α-diol)

3β-Acetoxy-5α,8α-epidioxycholest-6-ene. Irradiation of 3β-acetoxycholesta-5,7-diene (2·8g.) by the method described in the main paper (Dewhurst & Akhtar, 1967) gave 3β-acetoxy-5α,8α-epidioxycholest-6-ene (1·9g.), m.p. 153°, $[\alpha]_D$ +18·8° [Tsuda (1963) quotes m.p. 155–156°].

3β-Acetoxycholestane-5α,8α-diol. A solution of 3β-acetoxy-5α,8α-epidioxycholest-6-ene (2g.) in A.R. ethyl acetate (40ml.) was hydrogenated with pre-reduced Adams catalyst (80mg.) in A.R. ethyl acetate (10ml.) for 3hr. It was then filtered over kieselguhr and evaporated to dryness. Crystallization from ether–light petroleum (b.p. 40–60°) gave 3β-acetoxycholestane-5α,8α-diol (1·45g.), m.p. 140° (decomp.), $[\alpha]_D$ −42·8° (Found: C, 75·1; H, 10·9. $C_{29}H_{50}O_4$ requires C, 75·3; H, 10·9%).

3β-Acetoxycholest-7-en-5α-ol. A warm solution of 3β-acetoxycholestane-5α,8α-diol (500mg.) in methanol (20ml.) was treated with a small drop of a mixture of methanol–conc. HCl (2:1, v/v). The reaction mixture was left at room temperature for 10min. Filtration of the suspension formed gave 3β-acetoxycholest-7-en-5α-ol (180mg.), m.p. 184° (Found: C, 78·4; H, 10·8. $C_{29}H_{48}O_3$ requires C, 78·3; H, 10·9%).

Cholest-7-ene-3β,5α-diol (Δ^7-cholestene-3β,5α-diol). $LiAlH_4$ (200mg.) was added slowly to 3β-acetoxycholest-7-en-5α-ol (1g.) in dry ether (50ml.). The reaction mixture was left at room temperature for 30min. and then the product was extracted with ether. The ethereal solution was evaporated to dryness and crystallization from methanol gave cholest-7-ene-3β,5α-diol (660mg.), m.p. 201°.

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