ENZYMATIC STEREOSPECIFICITY IN THE CONVERSION OF Δ^7-CHOLESTEN-3β-OL TO 7-DEHYDROCHOLESTEROL[1]

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The enzyme-catalyzed formation of cholesterol (I) from Δ^7-cholesten-3β-ol (II) involves introduction of a Δ^5-double bond, yielding 7-dehydrocholesterol (III), and subsequent reduction of the Δ^7-bond of 7-dehydrocholesterol, (Figure 1) (Schroepfer and Frantz, 1961; Kandutsch, 1962; Dempsey et al., 1964). The precise

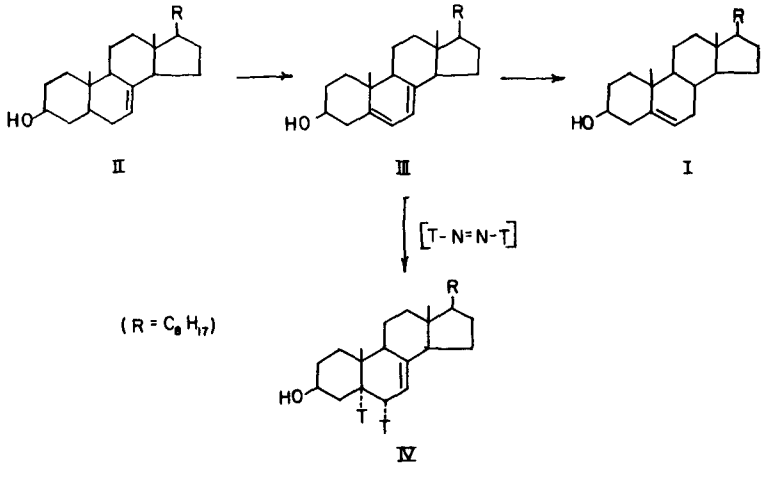

Figure 1. Enzymatic synthesis of cholesterol (I) from Δ^7-cholesten-3β-ol (II) and chemical synthesis of Δ^7-cholesten-3β-ol-5α,6α-$^3H_2$ (IV) from 7-dehydrocholesterol (III) and tritium-labeled diimide.

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mechanism of the introduction of the Δ^5-bond of 7-dehydrocholesterol is not known. Previous studies (Frantz et al., 1959; Dempsey et al., 1964) have established a requirement for molecular oxygen. The conversion of Δ^7-cholestenol to 7-dehydrocholesterol does not appear to involve Δ^7-cholesten-3β,6β-diol (Harvey and Bloch, 1961; Slaytor and Bloch, 1965), Δ^7-cholesten-3β,6α-diol, Δ^7-cholesten-3β-ol-6-one (Slaytor and Bloch, 1965), or Δ^6-cholesten-5α,8α-epiperoxy-3β-ol (Paliokas and Schroepfer, 1967) as intermediates.

The purpose of the present study was to determine whether or not the enzymatic conversion of Δ^7-cholesten-3β-ol to 7-dehydrocholesterol is stereospecific with respect to the hydrogen removal from carbon atom 6. With this goal in mind, we prepared samples of Δ^7-cholesten-3β-ol labeled stereospecifically with isotopic hydrogen in the axial (β) and the equatorial (α) configurations. Using these substrates we have found that the enzymatic introduction of the Δ^5-bond involves stereospecific removal of the equatorial (α) hydrogen from carbon atom 6.

This investigation was facilitated by our finding that upon reduction of 7-dehydrocholesterol by diimide (generated from hydrazine or by the action of acetic acid on potassium azodicarboxylate) Δ^7-cholesten-3β-ol is obtained in yields varying from 10 to 52%. The product was characterized by melting point (123.5-124.0°), the absence of specific ultraviolet absorption above 200mμ, precipitability with digitonin, colorimetric behavior with the Liebermann-Burchard reagent, the identity of its infrared spectrum with that obtained from an authentic sample of II, molecular weight determination of the 3β-methoxy derivative (400; mass spectrometry), behavior on silicic acid column (Schroepfer and Frantz, 1961), and thin-layer (Kammereck et al., 1967) chromatographic analysis, and the behavior of the 3β-methoxy derivative on gas-liquid chromatographic analysis. Further direct evidence that the reaction involves reduction of the Δ^5-double bond of III was obtained by reduction of III with deuterium-labeled diimide (generated from deuterated hydrazine) under conditions previously utilized in the preparation of 9,10-dideuteriostearic acid from oleic acid (Schroepfer and Bloch, 1965). The dideuterated Δ^7-cholesten-3β-ol was characterized by melting point (122-124°),

infrared spectroscopy (λ 2125 cm^{-1}; C-D stretch), and its behavior on thin-layer chromatographic analysis. The retention time of the 3β-methoxy derivative of the deuterated compound on gas-liquid chromatographic analysis was identical with that of the methyl ether of II. The molecular weight of the 3β-methoxy derivative was 402 (mass spectrometry), indicating the uptake of two deuterium atoms.

Since the reduction of olefins by diimide occurs by cis-addition of hydrogen (Corey et al., 1961), the application of diimide, labeled with isotopic hydrogen, to the reduction of 7-dehydrocholesterol should yield Δ^7-cholestenol labeled stereo specifically in the 5α- and 6α-configurations. Accordingly, Δ^7-cholesten-3β-ol-5α, 6α-$^3H_2$ (IV) was synthesized from III and tritium-labeled diimide (generated from CH_3COO^3H, and potassium azodicarboxylate).

Δ^7-cholesten-3β-ol-6β-$^3H_1$ was prepared as outlined in Figure 2. The product was characterized by melting point (122.6-123.5°), thin-layer chromatographic analy mass spectrometry, and gas-liqid chromatographic analysis. The radiopurity, as judged by chromatographic analysis was in excess of 98%. (The corresponding unlabeled and monodeuterated compounds have also been prepared).

The tritium-labeled substrates were mixed with Δ^7-cholesten-3β-ol-4-^{14}C, prepared previously (Schroepfer and Frantz, 1961), and incubated with a 10,000 x g supernatant fraction of rat liver homogenates for 3 hours at 37° in an atmosphere

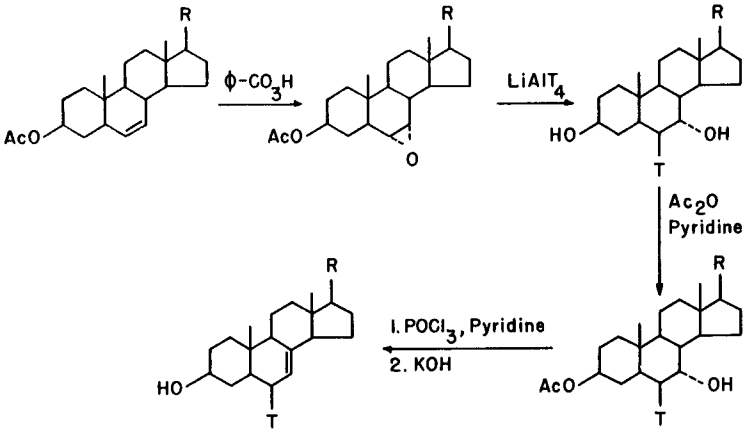

Figure 2. Chemical synthesis of Δ^7-cholesten-3β-ol-6β-^3H

of air. Δ^7-cholesten-3β-ol, 7-dehydrocholesterol, and cholesterol were isolated by a combination of column and/or thin-layer chromatographic techniques. Cholesterol was further purified via the dibromide. In the case of the incubation of the Δ^7-cholesten-3β-ol-5α, 6α-$^3H_2$-4-^{14}C, the total recovery of added ^{14}C was 63% (after silicic acid column chromatography). Approximate percentages of the recovered ^{14}C associated with Δ^7-cholesten-3β-ol, 7-dehydrocholesterol, and cholesterol were 13, 34, and 53, respectively. Recovery of added tritium was only 28% that of the ^{14}C, indicating extensive removal of labeled hydrogen.

In the case of the incubation of the Δ^7-cholesten-3β-ol-6β-^3H, the total recovery of added ^{14}C and ^3H was 85% and 86%, respectively. Approximate percentages of the recovered ^{14}C associated with Δ^7-cholesten-3β-ol, 7-dehydrocholesterol, and cholesterol were 10, 18, and 70, respectively.

The tritium to ^{14}C ratios found in the three sterols isolated from the incubation mixture are listed in Table 1 along with those ratios expected for stereospecific removal of the (1), 6β-hydrogen, (2), the 6α-hydrogen, and (3), non-stereospecific removal of hydrogen from carbon atom 6. The ratios of tritium to ^{14}C are expressed relative to an assigned value of unity in the incubated substrate. In the calculation of the expected ratios, the absence of isotope effects in removal of labeled hydrogen was assumed.

The results indicate that the hydrogen removal from carbon atom 6 is indeed stereospecific, the equatorial (α) hydrogen being specifically removed during the course of the introduction of the Δ^5-double bond of 7-dehydrocholesterol.

The ratio of tritium to ^{14}C in the Δ^7-cholesten-3β-ol-5β,6β-$^3H_2$-4-^{14}C substrate recovered after incubation is significantly higher than that of the substrate before incubation, a finding which is compatible with the presence of an isotope effect on removal of one or both of the tritium atoms. In the case of the Δ^7-cholesten-3β-ol-6β-^3H-4-^{14}C, the tritium to ^{14}C ratio in the recovered substrate was significantly lower than that in the incubated substrate, a finding which is compatible with the presence of an inverse secondary isotope effect.

The enzymatic introduction of the Δ^5-bond and of 7-dehydrocholesterol therefore

Table 1. $^{3}H/^{14}C$ Ratios in Products Formed by a Rat Liver Homogenate from Δ^{7}-cholesten-3β-ol-5α, 6α-$^{3}H_{2}$-4-^{14}C (A) and Δ^{7}-cholesten-3β-ol-6β-$^{3}H_{1}$-4-^{14}C (B)

Substrate	Compound	Expected Ratio for Removal of:			Found
		6β-hydrogen	6α-hydrogen	6α-or 6β-hydrogen	
A	Δ^{7}-cholestenol				
	Incubated	1.00	1.00	1.00	
	Recovered	1.00	1.00	1.00	1.80
	7-dehydrocholesterol	0.50	0.00	0.25	0.01
	cholesterol	0.50	0.00	0.25	0.00
B	Δ^{7}-cholestenol				
	Incubated	1.00	1.00	1.00	
	Recovered	1.00	1.00	1.00	0.70
	7-dehydrocholesterol	0.00	1.00	0.50	0.92
	cholesterol	0.00	1.00	0.50	0.99

involves removal of the 5α (axial) and 6α (equatorial) hydrogens. This constitutes one of the few clear examples that an enzymatic reaction involving the introduction of a carbon-carbon double bond can proceed by removal of two cis-hydrogens. The enzymatic conversion of stearate to oleate is characterized by stereospecific removal of the 9D- and 10D-hydrogens of stearic acid (Schroepfer and Bloch, 1965). While it is quite possible that this reaction also proceeds by stereospecific removal of two cis-hydrogens, lack of information regarding the specific conformation of the substrate at the enzyme surface and the possibility of rotation about the bond between carbon atoms 9 and 10 during the course of the enzymatic reaction precludes a more definitive statement on this point. In the present case, rotation about the bond between carbon atoms 5 and 6 is not possible due to the geometry of the sterol molecule. Another example in the area of sterol metabolism is the conversion, by the intact cockroach, of cholestan-3β-ol to Δ^7-cholesten-3β-ol, a reaction which involves the stereospecific removal of the 7β- and 8β-hydrogen atoms (Clayton and Edwards, 1963).

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