The Stereochemistry of Hydrogen Elimination from C-7 in Cholesterol and Ergosterol Biosynthesis

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The synthesis of [7α - ^3H]lanosterol is described. It is shown that in the conversion of [7α - ^3H , 26, 27- $^{14}\text{C}_2$]lanosterol into cholesterol by a rat liver system, it is the 7β -hydrogen atom that is predominantly removed. On the other hand, the conversion of doubly labelled lanosterol into ergosterol by whole yeast cells results in the loss of the 7α -hydrogen atom. These results therefore suggest that the C-7 hydrogen atoms with opposite stereochemistry are labilized by the rat liver and the yeast Δ^8 - Δ^7 steroid isomerases.

During the biosynthesis of cholesterol (II) or ergosterol (III) the $\Delta^{8,9}$ -double bond originally present in lanosterol (I) should in principle be isomerized to the Δ^7 -position. It has recently been demonstrated that during the conversion of specifically labelled mevalonic acid into cholesterol by rat liver it is the 7β -hydrogen atom that is lost (Caspi, Grieg, Ramm & Varma, 1968; Canonica *et al.* 1968; Gibbons, Goad & Goodwin, 1968; Akhtar, Rahimtula, Watkinson, Wilton & Munday, 1969).

The stereochemistry of hydrogen elimination from C-7 in the biosynthesis of the C_{28} steroid ergosterol (III) has not been directly studied, though the work of Caspi & Ramm (1969) may be extended to suggest that in this case it is the 7α -hydrogen atom that is eliminated. Caspi & Ramm (1969) studied the metabolism of 2*R* and 2*S* tritiated mevalonic acids with yeast homogenate; in such a cell-free system mevalonic acid is converted into steroid intermediates and no ergosterol is formed (Katsuki & Bloch, 1967). Degradation studies on a C_{27} sterol thus obtained, which was related to Δ^7 -cholestenol, led these authors to conclude that the 7α -hydrogen atom is removed by the yeast homogenate in the formation of the Δ^7 -bond of the C_{27} steroid.

This difference in stereospecificity thus suggested could be due either to a different mechanism of squalene cyclization in liver and yeast [for example, various modes of cyclization of squalene giving intermediates which can act as precursors of ergosterol (III) and cholesterol (II) have been considered by Rees, Goad & Goodwin (1968)] or to a difference in the stereospecificity of the Δ^8 - Δ^7 steroid isomerase from these two preparations. In

order to distinguish between these two hypotheses it was necessary to synthesize lanosterol (I) stereospecifically labelled with tritium at C-7 and to incubate this labelled steroid with the liver and yeast systems. Since the resulting transformations will be subsequent to the squalene cyclization process any difference in the stereospecificity observed must be the result of the Δ^8 - Δ^7 steroid isomerase.

RESULTS AND DISCUSSION

Preparation of radioactive lanosterol. In order to study the Δ^8 - Δ^7 isomerisation reaction both [^{14}C]- and [7 - ^3H]-lanosterol (I) were required. [26, 27- ^{14}C]Lanosterol was prepared by the method of Akhtar, Hunt & Parvez (1967). [7 - ^3H]Lanosterol was synthesized by the following procedure. Lanosteryl acetate dibromide, prepared from crude lanosteryl acetate by the method of Lewis & McGhie (1956), was equilibrated in dry chloroform saturated with hydrogen chloride gas and containing one drop of tritiated water (5 Ci/ml). The resulting mixture of Δ^8 - and Δ^7 -lanosteryl acetate dibromides was debrominated and the two isomeric acetates were separated by t.l.c. [7 - ^3H]Lanosterol was obtained by lithium aluminium hydride reduction of the corresponding acetate and was mixed with [26, 27- ^{14}C]lanosterol ($^3\text{H}/^{14}\text{C}$ ratio 18.5).

Incubation of radioactive lanosterol with a rat liver homogenate. The [7 - ^3H , 26, 27- ^{14}C]lanosterol (50 μg ; 220 000 c.p.m. of ^3H ; $^3\text{H}/^{14}\text{C}$ ratio 18.5) was incubated aerobically with a 10 000 g supernatant of rat liver homogenate and the cholesterol (II) was isolated and purified by the method of Wilton, Munday, Skinner & Akhtar (1968). The cholesterol

Scheme 1.

of radioactive lanosterol (I) into cholesterol (II) or ergosterol (III). These results therefore show that the C-7 hydrogen atoms with opposite stereochemistry are removed by the rat liver enzyme and the yeast enzyme.

To establish the absolute stereochemistry of the tritium labelling at C-7 of the lanosterol it is necessary only to establish the stereochemistry of the hydrogen atom at C-7 lost for one of the systems described above. In another experiment $[2S\text{-}^3H]$ -mevalonic acid was converted into cholesterol by the rat liver system. The isolated cholesterol (II) (52 000 c.p.m./mmol) was converted into 3β -acetoxycholest-5-en-7-one (IV) (51 600 c.p.m./mmol). It has been established that $[2S\text{-}^3H]$ -mevalonic acid will result in tritium being located at the 7β -position of lanosterol (Cornforth, Cornforth, Popjak & Yengoyan, 1966) and hence the lack of radioactivity at C-7 of cholesterol biosynthesized from $[2S\text{-}^3H]$ -mevalonic acid confirms that under the experimental conditions used for cholesterol biosynthesis it is the 7β -hydrogen atom that is lost. Since the chemically prepared $[7\text{-}^3H]$ -lanosterol retained most of the radioactivity during its conversion into cholesterol, it follows that the sample of lanosterol had the tritium atom located predominantly at the 7α -position.

These results therefore establish that the difference in stereospecificity of hydrogen elimination from C-7 during the biosynthesis of cholesterol (II) and ergosterol (III) is due to a difference in the stereospecificity of the Δ^8 - Δ^7 steroid isomerase. Thus in the case of cholesterol biosynthesis it is a *trans* addition-elimination of hydrogen atoms and a *cis*-directed process is observed in ergosterol biosynthesis as shown in Scheme 1.

Other aspects of the mechanism of isomerization of the Δ^8 -double bond in sterol biosynthesis have previously been described (Akhtar & Rahimtula, 1968; Lee, Kammereck, Lutsky, McCloskey & Schroepfer, 1969; Wilton, Rahimtula & Akhtar, 1969).

EXPERIMENTAL

Preparation of $[7\text{-}^3H]$ -lanosterol. To a saturated solution of HCl gas in dry chloroform (3 ml; prepared by passing HCl gas through chloroform for 10 min) was added one drop of tritiated water (5 Ci/ml) and lanosteryl acetate dibromide (30 mg) prepared by the method of Lewis & McGhie (1956). The flask was stoppered and allowed to stand at room temperature for 2.5 h; the chloroform was then evaporated *in vacuo* and the last traces of tritiated water were removed azeotropically with benzene and methanol. The resulting mixture of Δ^8 - and Δ^7 -lanosteryl acetate dibromides was refluxed with vigorous stirring in benzene-methanol [2 ml; 6:1, v/v; with zinc dust (30 mg)] for 1.5 h. Another 30 mg of zinc dust was then added and the refluxing was continued for a further 2 h. The zinc was filtered off and the filtrate, after dilution with ether

(10 ml), was washed with water, dried and evaporated to dryness. The residue was spotted on to six silica gel plates impregnated with 20% (w/w) $AgNO_3$ (20 cm $\times$ 10 cm $\times$ 0.3 mm thick), which were run five times in light petroleum (b.p. 40–60°C)-benzene (3:1, v/v). Development with iodine showed two bands, with R_F values of 0.5 and 0.6, which overlapped slightly. The upper band, corresponding to lanosteryl acetate, was removed, eluted with ether and the residue was rechromatographed on two silica gel plates as above to free it completely from Δ^7 -lanosteryl acetate. Lithium aluminium hydride reduction of the product in ether for 30 min gave pure $[7\text{-}^3H]$ -lanosterol (2 mg; 2×10^{10} c.p.m./mmol).

Preparation of $[26,27\text{-}^{14}C]$ -lanosterol. This was prepared by the method of Akhtar *et al.* (1967).

Preparation of 3β -acetoxycholest-5-en-7-one (IV). This was prepared by the method of Wilton *et al.* (1968).

Preparation of 3β -acetoxy- $5\alpha,8\alpha$ -epidioxyergosta-6,22-diene (V). This was prepared by the method of Akhtar, Parvez & Hunt (1969).

Incubations with rat liver homogenate. $[7\text{-}^3H, 26,27\text{-}^{14}C]$ -lanosterol (I) (50 μ g; $^3H/^{14}C$ ratio 18.5) was dissolved in 25 mg of Tween 80 and incubated under aerobic conditions with 10 ml of a 10 000g supernatant of rat liver homogenate according to the method of Akhtar, Watkinson, Rahimtula, Wilton & Munday (1969). After termination of the incubation the cholesterol was isolated and purified according to the method of Wilton *et al.* (1968). The method for the conversion of radioactive mevalonic acid into cholesterol has already been described (Akhtar *et al.* 1969).

Incubations with whole cells of *Saccharomyces cerevisiae*. $[7\text{-}^3H, 26,27\text{-}^{14}C]$ -lanosterol (I) (300 μ g; $^3H/^{14}C$ ratio 18.5) was dissolved in 15 mg of Tween 80 and incubated with whole cells of *Saccharomyces cerevisiae* in 8 litres of medium as described by Akhtar *et al.* (1967). The ergosterol was isolated as described by Akhtar *et al.* (1967).

Measurement of radioactivity (see Wilton *et al.* 1969). The radioactivity of all samples was measured in an Intertechnique SL 40 liquid-scintillation counter. Corrected $^3H/^{14}C$ ratios were obtained by using the external standard and the appropriate computer programme.

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