The Intermediary Role of a Steroid 8,14-Dien-3 β -ol System in Ergosterol Biosynthesis

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1. A mechanism for the removal of the 14 α -methyl group in ergosterol biosynthesis that involves the intermediacy of an 8,14-diene system is outlined. 2. In accordance with the requirements of this scheme, it is shown that 5 α -ergosta-8,14-dien-3 β -ol is converted into ergosterol by *Saccharomyces cerevisiae*.

One of the steps in the conversion of lanosterol (structure of the type I where Y=CH₃) into ergosterol (VII) is the removal of the 14 α -methyl group. The work of Olson, Lindberg & Bloch

(1957) suggested that in sterol biosynthesis this methyl group may be lost as carbon dioxide subsequent to its oxidation to a carboxyl group. In the past it has been assumed that the activation for

Scheme 1

the decarboxylation is provided by the presence of the 8,9-double bond, and that decarboxylation occurs by a mechanism of the type shown in the sequence (I)→(II)→(III)→(IV) (Scheme 1). Recent work (Akhtar, Watkinson, Rahimtula, Wilton & Munday, 1969b; Canonica *et al.* 1968; Gibbons, Goad & Goodwin, 1968), however, has suggested that in cholesterol biosynthesis the removal of the C-14 methyl group occurs through the oxidation of C-15, involving a series of steps as in the sequence (I)→(V)→(VI)→(IV) (Scheme 1) (Akhtar, Rahimtula, Watkinson, Wilton & Munday, 1969a). In accordance with the requirements of this sequence, it was shown that an 8,14-diene system of the type (VI) can be trapped during the biosynthesis of cholesterol from dihydrolanosterol (Watkinson & Akhtar, 1969). The presence of an enzyme system in rat liver homogenate that catalyses the reduction of the 14,15-double bond in a variety of dienes has also been shown (Akhtar *et al.* 1969b; Akhtar, Watkinson, Rahimtula, Wilton & Munday, 1968, and unpublished work). It was therefore decided to investigate whether such a process could also be involved in ergosterol biosynthesis. With this aim in mind, we have now studied the biosynthesis of ergosterol with whole yeast cells, using 5 α -ergosta-8,14-dien-3 β -ol as a substrate.

RESULTS AND DISCUSSION

Preparation of [3H]5 α -ergosta-8,14-dien-3 β -ol and its conversion into ergosterol. The tritiated diene (VI) was prepared essentially by the method of Gautschi & Bloch (1958) except that the rearrangement of ergosterol (VII) was carried out in the presence of tritiated hydrochloric acid. The acetic anhydride used as solvent also acetylated the 3 β -hydroxy group, thus giving 5 α -ergosta-8,14-dien-3 β -ol acetate. The corresponding alcohol was obtained when this compound was hydrolysed with lithium aluminium hydride. The diene prepared under these conditions should contain radioactivity at positions 5, 6, 7 and 15.

When samples (1mg.; 1.7 $\times$ 10⁶ c.p.m.) of this diene were incubated by the method of Akhtar, Parvez & Hunt (1966), an average of 60% of the original radioactivity was incorporated into a fraction corresponding to ergosterol. However, in a control experiment in which the yeast was incubated anaerobically for 72 hr., the same fraction incorporated an equal amount of radioactivity, thus suggesting that a method had to be found to separate the contaminating starting substrate from ergosterol. After dilution with the carrier, the biosynthetic ergosterol was isolated and acetylated, and the product purified by thick-layer chromatography. The ergosterol acetate was then converted into the epidioxide derivative (VIII), which was readily

separable from contaminating 8,14-diene by thick-layer chromatography. After the products were crystallized to constant specific radioactivity, the experimental epidioxide contained an average of 11.5% of the original radioactivity whereas the control contained no significant radioactivity (less than 0.7% of the amount incubated). Under the conditions mentioned above, lanosterol, a related sterol, was converted into ergosterol in 14.7% yield.

These results show that yeast cells contain an enzyme system capable of reducing the 14,15-double bond of an 8,14-diene system, thus supporting the idea that the sequence (I)→(V)→(VI)→(IV) shown in Scheme 1 may also be involved in the removal of the 14 α -methyl group in ergosterol biosynthesis. Since the stage at which the removal of the 14 α -methyl group in ergosterol biosynthesis occurs is not known, the present work gives no information as to which particular diene is the true physiological substrate on the biosynthetic pathway.

EXPERIMENTAL

All incubations were carried out with *Saccharomyces cerevisiae* LK₂G₁₂. The i.r. spectra were determined on a Unicam SP.200 spectrometer, and melting points taken on a Gallenkamp melting-point apparatus. All i.r. and m.p. determinations were as expected for the compounds described below. Preparative t.l.c. plates were made with silica gel HF₂₅₄ (E. Merck A.-G., Darmstadt, Germany). All radioactivity measurements were made with a Beckman model CPM 200 liquid scintillation counter, with 2-(4-tert.-butylphenyl)-5-(4-biphenyl)-1-oxa-3,4-diazole [Ciba (A.R.L.) Ltd., Duxford, Cambs.] in toluene (8g./l.) as scintillator.

Preparation of tritiated 5 α -ergosta-8,14-dien-3 β -ol. Ergosterol (2g.) was dissolved in a mixture of acetic acid (5ml.), acetic anhydride (25ml.), benzene (5ml.), 36% (w/v) HCl (1ml.) and tritiated water (1ml., 200 mc) in a 50 ml. round-bottomed flask. The flask was fitted with an air-cooled condenser and the mixture heated on a steam bath for 90 min., the radioactive distillate being collected in an ice-cooled flask as a precautionary measure. Sodium acetate (3g.) was added to the almost black solution, and the dark-green pro-

duct diluted with diethyl ether–light petroleum (b.p. 40–60°) (1:1, v/v) (200 ml.). The resulting solution was washed three times with water and once with saturated NaCl solution, and was then dried with anhydrous Na_2SO_4 . The yellow oil obtained after evaporation to dryness crystallized on standing. This product was dissolved in benzene and applied to a column (30 cm. $\times$ 3 cm.) of aluminium oxide type H, and the column was eluted with benzene. The fractions corresponding to the 8,14-diene, detected by a single peak absorption at 249 nm., were pooled and recrystallized twice from ether–methanol to give large plate-like crystals of 5 α -ergosta-8,14-dien-3 β -ol acetate (1.16 g.), m.p. 134–135°, λ_{max} 249 nm. and ϵ 17700 [Fieser, Rosen & Fieser (1952) quote λ_{max} 249 nm. and ϵ 18000]. This was hydrolysed with LiAlH_4 and after one crystallization was used without further purification.

Incubation of [^3H]5 α -ergosta-8,14-dien-3 β -ol with yeast, and the isolation of ergosterol. Tritiated 15 α -ergosta-8,14-dien-3 β -ol from above (1 mg.; 1.7 $\times$ 10⁶ c.p.m./mg.) was dissolved in Tween 80 (25 mg.) and acetone (1 ml.). The acetone was removed by a stream of nitrogen and the resultant oil emulsified with water (5 ml.). This was incubated (Akhtar *et al.* 1966) with *Saccharomyces cerevisiae* in a liquid culture medium (Klein, Eaton & Murphy, 1954). The cells were harvested and the sterols isolated as described by Akhtar *et al.* (1966). The non-saponifiable fraction, which contained about 60% of the original radioactivity (1.0 $\times$ 10⁶ c.p.m.), was diluted with 10 mg. of non-radioactive ergosterol and spotted on to a preparative thin-layer plate (20 cm. $\times$ 10 cm., 1 mm. thick), which was then developed in chloroform. The ergosterol band (R_F 0.2), which was visible under u.v. light, was scraped off and eluted with ether, and ergosterol (390 mg.) was added to it. The solution was evaporated to dryness, and acetylated by standing overnight at room temperature in pyridine (30 ml.) and acetic anhydride (10 ml.). The product obtained by dropwise addition of water was filtered and crystallized once from ether–methanol to give ergosterol acetate (380 mg.; 1540 c.p.m.). At this stage, however, the ergosterol acetate band was contaminated with some of the original 8,14-diene, and the next step was therefore needed to separate the two.

Preparation of 5 α ,8 α -epidioxyergosta-6,22-dien-3 β -ol acetate (VIII) from biosynthetic ergosterol. A solution of ergosterol acetate (200 mg.; 1540 c.p.m./mg.) in ethanol (30 ml.) and containing eosin (2.5 mg.) was irradiated with a 150 w tungsten bulb placed at a distance of 10 cm. The solution

was cooled by a 'cold finger', and oxygen was slowly bubbled through. The reaction was terminated when the ergosterol absorption peak at 282 nm. could no longer be detected. The product was then applied to a preparative thin-layer silica-gel plate (20 cm. $\times$ 10 cm., 2 mm. thick) and developed three times in chloroform. The epidioxide band (final R_F 0.45; 5 α -ergosta-8,14-dien-3 β -ol acetate has R_F 0.6) was eluted with ether and crystallized from ether–methanol to give pure 5 α ,8 α -epidioxyergosta-6,22-dien-3 β -ol acetate (VIII), m.p. 196–198°, specific radioactivity 430 c.p.m./mg.

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