The Mechanism of the Elaboration of Ring B in Ergosterol Biosynthesis

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(Received 13 February 1968)

Methods for the preparation of [3α - ^3H]ergosta-7,22-dien- 3β -ol (5,6-dihydroergosterol), [$5,6$ - $^3\text{H}_2$]ergosta-7,22-dien- 3β -ol and [3α - ^3H]ergosta-7,22-diene- $3\beta,5\alpha$ -diol are described. It is shown that 5,6-dihydro[3α - ^3H]ergosterol on incubation under aerobic conditions with whole cells of *Saccharomyces cerevisiae* LK₂G₁₂ is efficiently converted into ergosterol. Studies carried out with dihydro[$5\alpha,6\alpha$ - $^3\text{H}_2$]ergosterol demonstrate that the introduction of the 5,6-double bond in ergosterol biosynthesis is attended by an overall *cis*-elimination of two hydrogen atoms. To differentiate between a hydroxylation-dehydration mechanism and a dehydrogenation mechanism, the metabolism of [3α - ^3H]ergosta-7,22-diene- $3\beta,5\alpha$ -diol was studied. It was shown that this diol is converted into ergosterol only under aerobic conditions. It is therefore suggested that the introduction of the 5,6-double bond of ergosterol does not occur through a hydroxylation-dehydration mechanism.

Continuing studies on the enzymic aspect of biosynthesis of yeast sterols (Akhtar, Parvez & Hunt, 1966a) we now report on the mechanism of introduction of the 5,6-double bond in the elaboration of ring B of ergosterol (VIIb). It is now well established that the final stages in cholesterol (VIII) biosynthesis involve the migration of 8,9-double bond (partial structure I) to the 7,8-position to give Δ^7 -cholestenol (IIa), an oxidative reaction then results in the introduction of a new double bond between C-5 and C-6 to give a 5,7-diene system (VIIa) and finally an NADPH-linked reaction (Akhtar, Wilton & Munday, 1966b) completes the conversion of the diene system (VIIa) into cholesterol. We have previously studied the mechanism of the introduction of the 5,6-double bond in the hepatic biosynthesis of cholesterol (VIII). Two basic mechanisms were considered for the origin of this double bond: a hydroxylation-dehydration mechanism and a dehydrogenation mechanism (Akhtar & Marsh, 1967). The hydroxylation-dehydration mechanism outlined in Scheme 1 requires the first step of the sequence to occur under aerobic conditions and the second step under anaerobic conditions.

The failure of the three hydroxylated compounds (cholest-7-ene- $3\beta,6\alpha$ -diol, cholest-7-ene- $3\beta,6\beta$ -diol and cholest-7-ene- $3\beta,5\alpha$ -diol) to serve as precursors of cholesterol under anaerobic conditions and the inability of Δ^7 -cholestenol (IIa) to furnish any of the hydroxylated intermediates under the conditions of cholesterol biosynthesis led us to discard the hydroxylation-dehydration mechanism as an explanation of the introduction of the 5,6-double

bond in the biosynthesis. We therefore favoured an oxygen-dependent dehydrogenation mechanism for the conversion of structure (IIa) into structure (VIIa) in the cholesterol series (Dewhurst & Akhtar, 1967).

Recently, however, Topham & Gaylor (1967) have studied the parallel reaction in the biosynthesis of ergosterol and have provided evidence in support of a hydroxylation-dehydration mechanism. It has been shown by these workers that in accordance with the requirements of the hydroxylation-dehydration mechanism a hydroxylated intermediate, ergosta-7,22-diene- $3\beta,5\alpha$ -diol (IIIb), is smoothly converted into ergosterol under anaerobic conditions with a cell-free system obtained from homogenates of dry baker's yeast (*Saccharomyces cerevisiae* Fleishmann). Our own results reported in this paper show that the main features of the introduction of the 5,6-double bond in ergosterol biosynthesis by whole cells of the yeast *Saccharomyces cerevisiae* LK₂G₁₂ are in remarkable harmony with those of the parallel reaction involved in the hepatic biosynthesis of cholesterol (Dewhurst & Akhtar, 1967). Our observations as well as conclusions are therefore at variance with the work of Topham & Gaylor (1967).

RESULTS AND DISCUSSION

Metabolism of 5,6-dihydroergosterol. By analogy with observations made in the biosynthesis of cholesterol (Dewhurst & Akhtar, 1967), we considered that the 5,6-dihydroergosterol nucleus may be the immediate precursor of ring B of ergosterol.

Scheme 1.

a, Cholesterol series; b, ergosterol series.

5,6-Dihydroergosterol (IVb) labelled with tritium at the 3α -position was synthesized by the method described in the Experimental section and shown to be converted into ergosterol under aerobic conditions with whole yeast cells in satisfactory yield (Table 1; Expts. 1 and 3B). No significant conversion occurred when the incubation was carried out under anaerobic conditions (Table 1, compare Expts. 3B and 3A). The oxygen requirement for the introduction of the 5,6-double bond in ergosterol is the same as already established for the parallel reaction in cholesterol biosynthesis (Dewhurst & Akhtar, 1967; Dempsey, 1965).

Stereochemistry of hydrogen elimination in the formation of the 5,6-double bond in ergosterol biosynthesis. We next attended to the determination of the stereochemistry of hydrogen elimination in the conversion of structure (IIb) into structure (VIIb). Ergosterol was hydrogenated with tritium gas in the presence of Raney nickel (Laubach & Brunings, 1952). The knowledge that the catalytic hydrogenation involves a *cis*-addition of hydrogen

to a double bond and the fact that in this particular case the hydrogen at the C-5 position is added from the α -side of the steroid molecule allows one to formulate the hydrogenated product as 5,6-dihydro[5 α ,6 α - $^3\text{H}_2$]ergosterol (Vb). The biosynthesis of ergosterol from 5,6-dihydro[5 α ,6 α - $^3\text{H}_2$]ergosterol (Vb) resulted (Table 2) in the complete removal of tritium from both the 5 α - and the 6 α -position. We therefore conclude that the introduction of the 5,6-double bond in ergosterol (VIIb) biosynthesis is accompanied by an overall *cis*-removal of the 5 α - and 6 α -hydrogen atoms. This result is in agreement with the stereochemistry of hydrogen elimination already established in the biosynthesis of cholesterol (Akhtar & Marsh, 1967).

Metabolism of ergosta-7,22-diene-3 β ,5 α -diol (VIb). To evaluate the possible involvement of a hydroxylation-dehydration mechanism in the biosynthesis of the 5,6-double bond of ergosterol, we synthesized ergosta-7,22-diene-3 β ,5 α -diol (VIb) (Clayton, Henbest & Jones, 1953) labelled with tritium at the 3α -position. When the diol (VIb) was incubated

Table 1. *Biological conversion of [3 α -³H]ergosta-7,22-dien-3 β -ol (IVb) and [3 α -³H]ergosta-7,22-diene-3 β ,5 α -diol (VIb) into ergosterol*

In all the experiments 1 mg. of the substrate was used and incubations were carried out at 30°. The specific radioactivities of compounds (IVb) and (VIb) were 2×10^6 and 7×10^5 counts/min./mg. respectively. In Expts. 1 and 2 the substrate was incubated in 4l. of medium (Klein, Eaton & Murphy, 1954) with an inoculum (1–3 mg.) of *Saccharomyces cerevisiae* LK₂G₁₂. Cells were harvested and total sterols were isolated and processed (Akhtar *et al.* 1966a). In Expts. 3 and 4 yeast cells were grown anaerobically in 8l. of medium (as above) for 48 hr. and the cells were harvested and reincubated in the above medium (1.5l.). In Expt. 5 cells were grown anaerobically for 48 hr. in 8l. of medium but aerated for 30 min. before harvesting and then reincubated as in Expts. 3 and 4. In Expt. 6, yeast cells were grown aerobically in medium (4l.) for 20 hr. and the cells were then harvested and reincubated with sterol as in Expts. 3 and 4.

Expt. no.	Substrate	Incubation (hr.)		Incorporation of radioactivity into cells (%)	Conversion into ergosterol (%)	
		First under N ₂	Then under O ₂		Based on substrate incubated	Based on substrate incorporated into cells
1	(IVb)	48	24	88	31.5	36.2
2	(VIb)	48	24	82	17.5	21.35
3A	(IVb)	72	0	64.2	2.2	3.5
3B	(IVb)	48	24	40.3	15.2	37.3
4A	(VIb)	40	0	73	0.6	0.8
4B	(VIb)	25	15	38.4	3.5	9.1
5A	(VIb)	72	0	59.8	1.2	2.1
5B	(VIb)	48	24	44	12.8	28.8
6A	(VIb)	72	0	50.8	0.08	0.16
6B	(VIb)	48	24	39.7	4.7	11.8

Table 2. *Stereochemistry of hydrogen elimination in the enzymic conversion of [5 α ,6 α -³H₂]ergosta-7,22-dien-3 β -ol (Vb) into ergosterol*

Expt. no.	Substrate	Conversion into ergosterol (%)	Radioactivity found in ergosterol† (counts/min.)	Radioactivity required in ergosterol (counts/min.) for loss of		
				No tritium atom	One tritium atom	Both tritium atoms
1	(Vb) (1 mg.; 3.2×10^6 counts/min.)	16*	4.7×10^4	51.2×10^4	25.6×10^4	0
2	(Vb) (1 mg.; 3.2×10^6 counts/min.)	38*	7.1×10^4	121.6×10^4	55.8×10^4	0

* The amount converted in this experiment was determined from a parallel experiment with [3 α -³H]ergosta-7,22-dien-3 β -ol (IVb).

† The bioynthesized ergosterol was degraded by the method of Barton & Robinson (1954) into 3 β ,5 α -dihydroxyergosta-7,22-dien-6-one 3-acetate and it was shown that this radioactivity was not located at C-5 or C-6, but somewhere else (side chain?) (see the Experimental section).

under anaerobic conditions with whole cells of *Saccharomyces cerevisiae* LK₂G₁₂ that had been grown either aerobically or anaerobically (Table 1; Expts. 4A, 5A and 6A), no conversion into ergosterol occurred. It is to be noted that Topham & Gaylor (1967) have provided spectroscopic evidence for the conversion of ergosta-7,22-diene-3 β ,5 α -diol (VIb) into ergosterol under anaerobic conditions with a partially purified enzyme system from dry baker's yeast (*Saccharomyces cerevisiae* Fleishmann). The disagreement between the present work done

in vivo and the previous work with a semi-purified system is not readily explicable. We were, however, able to demonstrate quite convincingly that, in parallel experiments carried out in the presence of oxygen, ergosta-7,22-diene-3 β ,5 α -diol (VIb) was readily converted into ergosterol (Table 1; Expts. 2, 4B, 5B and 6B). It is gratifying to note that the behaviour of a steroid of the type (VIb) as a precursor of the 5,7-diene system is the same in the hepatic biosynthesis of cholesterol and in the biosynthesis of ergosterol with whole yeast cells

(Dewhurst & Akhtar, 1967). The evidence presented in this paper therefore suggests that the mechanism of introduction of the 5,6-double bond in ergosterol biosynthesis closely resembles the mechanism proposed for the parallel reaction in cholesterol biosynthesis (Dewhurst & Akhtar, 1967).

EXPERIMENTAL

Infrared (i.r.) spectra were determined on a Unicam SP.200 spectrometer. Nuclear-magnetic-resonance (n.m.r.) spectra were determined in deuteriochloroform on a Varian model A 60 spectrometer. Optical rotations were measured in chloroform solutions with a Perkin-Elmer model 141 polarimeter. All compounds described below gave satisfactory i.r. spectra and n.m.r. spectra. For preparative thin-layer chromatography silica gel HF254 (E. Merck A.-G., Darmstadt, Germany) was used. All radioactivity measurements were made on a Beckman liquid-scintillation system 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 5 α ,8 α -epidioxyergosta-6,22-dien-3 β -ol 3-acetate. Ergosterol acetate (5g.) dissolved in ethanol (625ml.) together with eosin (30–40mg.) was irradiated under reflux by means of a 150 w lamp placed beneath the flask, while oxygen was bubbled through the solution. This preparation was covered with aluminium foil. The reaction was stopped when the solution ceased to show the ergosterol absorption peak at 280m μ (about 5hr.). The product was crystallized from ether-methanol (yield 2.5g.), m.p. 196–198°.

Preparation of ergosta-22-ene-3 β ,5 α ,8 α -triol 3-acetate. 5 α ,8 α -Epidioxyergosta-6,22-dien-3 β -ol 3-acetate (2g.) was dissolved in ethyl acetate (30ml.) and shaken in an atmosphere of hydrogen in the presence of Adams catalyst (100mg.) at 30°. A dense white precipitate separated during the reaction. After 3hr. the uptake of hydrogen ceased when about 2mol.prop. of hydrogen was used. The precipitated material was removed and crystallized from chloroform-methanol (yield 1.2g.).

Preparation of ergosta-7,22-diene-3 β ,5 α -diol 3-acetate. A solution of ergosta-22-ene-3 β ,5 α ,8 α -triol 3-acetate (1.2g.) in methanol (150ml.) was treated with a trace of HCl and the reaction mixture was left at room temperature for 30min. The resulting solid was removed by filtration and crystallized from chloroform-methanol (yield 640mg.), m.p. 224°, $[\alpha]_D^{25} +1.5^\circ$. The above two experiments are based on the original work of Clayton *et al.* (1953)

Preparation of ergosta-7,22-diene-3 β ,5 α -diol (IIIb). A solution of ergosta-7,22-diene-3 β ,5 α -diol 3-acetate (640mg.) in sodium-dried ether (40ml.) was treated with LiAlH₄ (200mg.) and the reaction mixture refluxed for 1hr. The product was extracted with ether and crystallized from ether-methanol to give compound (IIIb) (yield 405mg.), m.p. 227°, $[\alpha]_D^{25} +0.98^\circ$; Clayton *et al.* (1953) quote $[\alpha]_D +1^\circ$.

Preparation of 5 α -hydroxyergosta-7,22-dien-3-one. Ergosta-7,22-diene-3 β ,5 α -diol (100mg.) was dissolved in acetone (20ml.) and treated with Jones reagent (0.1ml.). The reaction mixture was stirred for 15min. and diluted with water, and the product was extracted with ether and

after crystallization from ether-methanol was used in the next experiment without any further purification.

Preparation of [3 α -³H]ergosta-7,22-diene-3 β ,5 α -diol (VIb). NaB³H₄ (2–3mg.; specific radioactivity 1–2mc/mg.) was added to a solution of 5 α -hydroxyergosta-7,22-dien-3-one in methanol (20ml.) and the reaction mixture was kept at room temperature overnight. NaBH₄ (8–10mg.) was then added to the same reaction mixture and the product was extracted with ether. Crystallization from ether-methanol gave a highly radioactive product, which was diluted with carrier to give compound (VIb) (specific radioactivity 234000 counts/min./mg.).

Preparation of ergosta-7,22-dien-3 β -ol (IIb) (5,6-dihydro-ergosterol). Ergosterol (1g.) in dioxan (30ml.) was reduced in the presence of Raney nickel (2–3g.) at room temperature. The uptake of hydrogen ceased after 5hr., when approx. 1mol.prop. of hydrogen gas was used. The catalyst was filtered over kieselguhr and the filtrate crystallized from ether-methanol to give compound (IIb) (yield 600mg.), m.p. 170°; Laubach & Brunings (1952) quote m.p. 173–176°, $[\alpha]_D^{25} -19.3^\circ$.

Preparation of ergosta-7,22-dien-3-one. A solution of ergosta-7,22-dien-3 β -ol (IIb) (150mg.) in acetone (25ml.) was treated with Jones reagent (0.12ml.). The reaction mixture was stirred for 10min. at 15°, diluted with water and extracted with ether. Crystallization from ether-methanol gave ergosta-7,22-dien-3-one (yield 60mg.).

Preparation of [3 α -³H]ergosta-7,22-dien-3 β -ol (IVb). To a solution of ergosta-7,22-dien-3-one (50mg.) in ether (5ml.) and methanol (25ml.) was added NaB³H₄ (1–2mg.) and the reaction mixture was left at room temperature for 1hr. The reaction mixture was then treated with a further quantity of non-radioactive NaBH₄ (10–15mg.) and left at room temperature for 2hr. Dilution with water and extraction of the product with ether gave, after crystallization from ether-methanol, compound (IVb) (yield 20mg.; specific radioactivity 2072000 counts/min./mg.).

Preparation of [5,6-³H₂]ergosta-7,22-dien-3 β -ol (Vb). Ergosterol (100mg.) dissolved in dioxan (10ml.) was reduced in the presence of Raney nickel (1g.) with tritium gas, produced by dropping lithium metal into tritiated water (2ml. containing 50–100mc). The catalyst was removed by filtration and the filtrate crystallized from ether-methanol to give compound (Vb) (specific radioactivity 1358000 counts/min./mg.).

Determination of chemical purity. Radiochemical purity of all labelled compounds was determined by the procedure described by Akhtar, Hunt & Parvez (1967).

Degradation of ergosterol biosynthesized from [5,6-³H₂]ergosta-7,22-dien-3 β -ol (Vb). Ergosterol acetate (320mg.; specific radioactivity 63 counts/min./mg.) biosynthesized from [5,6-³H₂]ergosta-7,22-dien-3 β -ol (Vb) by the method of Akhtar *et al.* (1967) was suspended in acetic acid (7ml.) and treated with chromium trioxide (0.7ml.; 175mg. of CrO₃ dissolved in 0.7ml. of 80% acetic acid). The reaction mixture was stirred at room temperature for 1hr. and then left at the same temperature overnight. Addition of methanol (a few drops) and dilution with water gave a solid that was crystallized from ethyl acetate to give 3 β ,5 α -dihydroxyergosta-7,22-dien-6-one 3-acetate (specific radioactivity 57 counts/min./mg.). The complete retention of radioactivity in this conversion proves that the label is not located at C-5 or C-6, but somewhere else in the molecule (side chain?).

Biological conversion of [3 α -³H]ergosta-7,22-dien-3 β -ol (IV) into ergosterol (VII). [3 α -³H]Ergosta-7,22-dien-3 β -ol (IVb) (1 mg.; specific radioactivity 2072000 counts/min./mg.) was converted into ergosterol (VIIb) as described in Table 1 (Expt. 3B). Sterols were isolated and chromatographed by the method of Akhtar *et al.* (1967). The ergosterol band, which contained 836500 counts/min., was eluted and crystallized with carrier ergosterol (VIIb) (1 g.) to yield a solid with specific radioactivity 836 counts/min./mg. Since in a control experiment it was shown that the starting material, ergosta-7,22-dien-3 β -ol (IVb), cannot be separated from ergosterol (VIIb), an alternative method for its purification was devised involving the conversion of biosynthetic ergosterol (VIIb) into 5 α ,8 α -epidioxyergosta-6,22-dien-3 β -ol 3-acetate.

The biosynthetic ergosterol (VIIb) (specific radioactivity 836 counts/min./mg.) was converted into 5 α ,8 α -epidioxyergosta-6,22-dien-3 β -ol 3-acetate (specific radioactivity 596 counts/min./mg.). 5 α ,8 α -Epidioxyergosta-6,22-dien-3 β -ol 3-acetate (1 mg.) was chromatographed on a preparative thick-layer plate (chloroform) into a 5 α ,8 α -epidioxyergosta-6,22-dien-3 β -ol 3-acetate band (R_F 6.5 cm.), an ergosterol acetate band (R_F 8.5 cm.) and an ergosta-7,22-dien-3 β -ol 3-acetate band (R_F 8.5 cm.). The 5 α ,8 α -epidioxyergosta-6,22-dien-3 β -ol 3-acetate band contained 263 counts/min. on dilution with carrier 5 α ,8 α -epidioxyergosta-6,22-dien-3 β -ol 3-acetate (20 mg.) and crystallization to constant specific radioactivity gave 13 counts/min./mg. Therefore the specific radioactivity in the original epidioxide is 13×20 counts/min./mg. and the total radioactivity in ergosterol (VIIb) is:

$$260 \times \frac{\text{Mol. wt. of epidioxide}}{\text{Mol. wt. of ergosterol}} \times 1000 = 260 \times \frac{470}{396} \times 1000 \\ = 308000 \text{ counts/min.}$$

This corresponds to a 14.86% conversion into ergosterol.

Biological conversion of ergosta-7,22-diene-3 β ,5 α -diol (VIb) into ergosterol (VIIb). [3 α -³H]Ergosta-7,22-diene-3 β ,5 α -diol (VIb) (1 mg.; specific radioactivity 700000 counts/min./mg.) was converted into ergosterol (VIIb), and addition of carrier (1 g.) and crystallization as described by Akhtar *et al.* (1967) gave ergosterol (specific radioactivity 124 counts/min./mg.; total radioactivity $124 \times 1000 = 124000$ counts/min.; this corresponds to a 17.85% conversion). That the entire radioactivity was associated with ergosterol was proved by the degradation method described in the above experiment.

REFERENCES

- Akhtar, M., Hunt, P. F. & Parvez, M. A. (1967). *Biochem. J.* **103**, 616.
 Akhtar, M. & Marsh, S. (1967). *Biochem. J.* **102**, 462.
 Akhtar, M., Parvez, M. A. & Hunt, P. F. (1966a). *Biochem. J.* **100**, 38c.
 Akhtar, M., Wilton, D. C. & Munday, K. A. (1966b). *Biochem. J.* **101**, 23c.
 Barton, D. H. R. & Robinson, C. H. (1954). *J. chem. Soc.* p. 3045.
 Clayton, R. B., Henbest, H. B. & Jones, E. R. H. (1953). *J. chem. Soc.* p. 2015.
 Dempsey, M. E. (1965). *J. biol. Chem.* **240**, 4176.
 Dewhurst, S. M. & Akhtar, M. (1967). *Biochem. J.* **105**, 1187.
 Klein, H. P., Eaton, N. R. & Murphy, J. C. (1954). *Biochim. biophys. Acta*, **13**, 591.
 Laubach, G. D. & Brunings, K. J. (1952). *J. Amer. chem. Soc.* **74**, 705.
 Topham, R. W. & Gaylor, J. L. (1967). *Biochem. biophys. Res. Commun.* **27**, 644.