Biochem. J. (1968) **106**, 623
Printed in Great Britain

The Introduction of the C-22–C-23 Ethylenic Linkage in Ergosterol Biosynthesis

BY M. AKHTAR, M. A. PARVEZ AND P. F. HUNT
Department of Physiology and Biochemistry, University of Southampton

(*Received* 14 *September* 1967)

Methods for the chemical synthesis of [23-$^3H_2$]lanosterol, [23,25-$^3H_3$]24-methyldihydrolanosterol and [24,28-$^3H_2$]24-methyldihydrolanosterol are described. It is shown that, in the biosynthesis of ergosterol from [26,27-$^{14}C_2$,23-$^3H_2$]lanosterol by the whole cells of *Saccharomyces cerevisiae*, one of the original C-23 hydrogen atoms is lost and the other is retained at C-23 of ergosterol. It is also shown that 24-methyldihydrolanosterol is converted into ergosterol in good yield and without prior conversion into a 24-methylene derivative. On the basis of these results possible pathways for the formation of the ergosterol side chain from a 24-methylene side chain are discussed.

On the basis of some past work that was misinterpreted in the original paper (Stone & Hemming, 1965), two recent reports (Stone & Hemming, 1967; Frantz & Schroepfer, 1967) have speculated on the mechanism of the alkylation step in ergosterol biosynthesis. The same mechanism, however, had been unambiguously established by us (Akhtar, Parvez & Hunt, 1966*a*; Akhtar, Hunt & Parvez, 1966*b*, 1967) before these speculations. It was proved that the *C*-alkylation step in ergosterol biosynthesis proceeds through the intermediacy of a 24-methylene compound (Akhtar *et al.* 1966*a*; Barton, Harrison & Moss, 1966) by the mechanism shown in the sequence (I)→(IV) (Scheme 1). In this paper we describe experiments that are relevant to the further conversion of the 24-methylene side chain (IV) into the ergosterol side chain (IX).

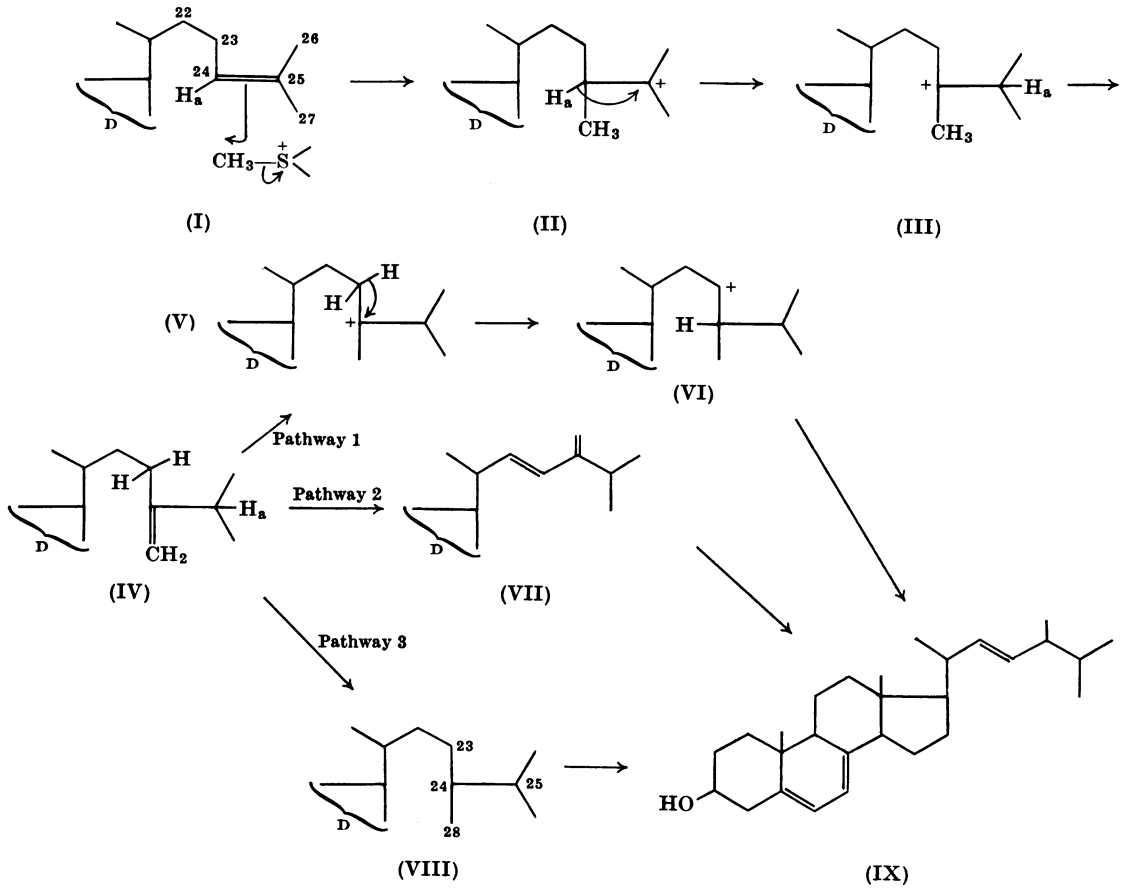
RESULTS AND DISCUSSION

A number of pathways for this conversion are feasible; however, we shall consider the three most likely possibilities, which are outlined in Scheme 1. Pathway 1 involves an intramolecular hydrogen transfer; pathway 2 shows a two-step process involving the initial formation of the C-22–C-23 double bond followed by the reduction of the C-24–C-28 double bond; pathway 3 shows the reverse of this oxidation–reduction sequence.

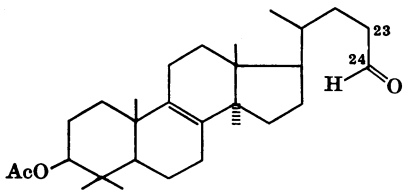
Pathway 1 was eliminated by the following experiments. When a mixture of [23-$^3H_2$]lanosterol (XII; R=H) and [26,27-$^{14}C_2$]lanosterol (XIII; R=H) ($^3H/^{14}C = 14{\cdot}0$) was incubated with whole yeast cells, the biosynthesized ergosterol had $^3H/^{14}C$ 6·9, thus showing that in this conversion one of the original C-23 hydrogen atoms was lost. Ozonolysis gave 2,3-dimethylbutyraldehyde ($^3H/^{14}C = 8{\cdot}3$) and further oxidation to 2,3-dimethylbutyric acid ($^3H/^{14}C = 0$) was accompanied by the complete removal of the tritium label, proving that the second C-23 hydrogen of the parent lanosterol remains undisturbed during the biosynthesis.

The $^3H/^{14}C$ value obtained for 2,3-dimethylbutyraldehyde was higher than expected. This we attribute to partial oxidation of the aldehyde during preparation, which preferentially removes hydrogen rather than tritium from the formyl group. It is noteworthy that in a number of independent incubations the $^3H/^{14}C$ ratio of the biosynthetic ergosterol was always found to be half that of the precursor lanosterol.

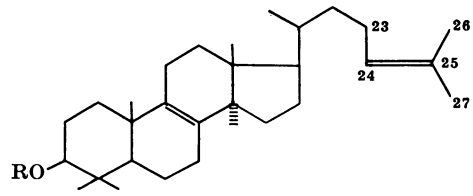
To evaluate the involvement of pathway 2 or 3 in ergosterol biosynthesis, [23,25-$^3H_3$]24-methyldihydrolanosterol (side chain as VIII) was synthesized and shown to be incorporated into ergosterol, by the whole cells, in 3–4% yield. Assuming utilization of only one of the C-24 enantiomers, the yield should be doubled. To ensure that the 24-methyldihydrolanosterol side chain (VIII) is incorporated into ergosterol without undergoing prior conversion into the 24-methylene side chain (as IV or VII) a sample of [24,28-$^3H_2$]24-methyldihydrolanosterol was converted into ergosterol. The biosynthetic ergosterol (48700 counts/min./m-mole) was ozonized and the volatile fraction divided into two parts. One part was treated with dimedone to furnish the derivative of 2,3-dimethylbutyraldehyde (47000 counts/min./m-mole). The second portion was treated with aqueous sodium hydroxide for 18hr. at room temperature under nitrogen and then converted into the dimedone



Scheme 1.



(X)
(XI) [23-$^3H_2$]



(XII) [23-$^3H_2$]
(XIII) [26,27-$^{14}C_2$]. R=H; Ac

Ac=$CH_3 \cdot CO$

derivative (33700 counts/min./m-mole). The 31% loss of radioactivity observed after base equilibration of 2,3-dimethylbutyraldehyde proves that the parent ergosterol contained tritium at C-24. Assuming that the parent lanosterol contained equal amounts of radioactivity at C-24 and C-28 and the base treatment of the aldehyde resulted in complete equilibration, the loss of activity in this experiment should be 50%. Experiments carried out under similar conditions but with non-radioactive 2,3-dimethylbutyraldehyde in a medium containing tritons showed 50–70% incorporation

in the recovered aldehyde, which was isolated as the dimedone derivative.

We therefore conclude that the 24-methyl side chain (of type VIII) is incorporated 'intact' into ergosterol *in vivo* at least to the extent of 62%. Indirect evidence in favour of a pathway of type 2 has been presented by Katsuki & Bloch (1967), who have isolated from cell-free yeast extracts a sterol having a probable 22,24(28)-diene side chain and have shown its conversion into ergosterol. Although our experiments do not throw light on the possible involvement of pathway 2 in the biosynthesis of ergosterol, they prove unambiguously that the enzyme participating in the introduction of the C-22–C-23 double bond can function without requiring the activation of an adjacent C-24–C-28 double bond. It would be interesting to see whether a mechanism of the type recently postulated for the introduction of ethylenic linkages in other biosynthetic systems (Akhtar & Marsh, 1967; Dewhurst & Akhtar, 1967) is also applicable to the present problem.

EXPERIMENTAL

Microanalyses were by Weiler and Strauss, Oxford. Infrared (i.r.) spectra were determined on a Unicam SP. 200 spectrometer. All compounds described below gave the expected i.r. spectra. Nuclear-magnetic-resonance (n.m.r.) spectra were determined in deuterochloroform on a Varian model A60 spectrometer. Optical rotations were measured with a Perkin–Elmer model 141 polarimeter, in chloroform solutions. For preparative thin-layer chromatography silica gel HF254 (E. Merck A.-G., Darmstadt, Germany) was used. All radioactivity measurements except one were made on a scintillation counter model 6012A (Isotope Developments Ltd., Beenham, Berks.) in conjunction with an Ekco model N529D scaler (E. K. Cole and Co. Ltd., Southend-on-Sea, Essex), with scintillator no. NE213 [Nuclear Enterprises (G.B.) Ltd., Edinburgh]. Both ^{14}C and ^{3}H were counted at an efficiency of 15%, corrections for quenching being made either by 'cross-addition' or by an internal standard.

Conversion of [26,27-$^{14}C_2$,23-$^{3}H_2$]*lanosterol into ergosterol.* [26,27-$^{14}C_2$]Lanosterol (XIII; R=H) (Akhtar *et al.* 1967) was mixed with [23-$^{3}H_2$]lanosterol (XII; R=H). Doubly labelled lanosterol (XII+XIII;R=H; 1mg. containing $7{\cdot}84\times10^5$ counts/100sec. of ^{3}H and $5{\cdot}6\times10^4$ counts/100sec. of ^{14}C; $^{3}H/^{14}C=14{\cdot}0$) was emulsified in Tween 80, incubated with *Saccharomyces cerevisiae* LK_2G_{12}, and the ergosterol prepared with carrier ergosterol as described by Akhtar *et al.* (1967). The biosynthesized ergosterol was diluted with ergosterol (1g.) and crystallized to constant specific radioactivity (48counts/100sec./mg., total radioactivity $48\times1000=4{\cdot}8\times10^4$counts/100sec. at the ^{3}H setting, and 7counts/100sec./mg., total radioactivity $7\times1000=7\times10^3$counts/100sec. at the ^{14}C setting; $^{3}H/^{14}C=6{\cdot}9$).

Conversion of [23,25-$^{3}H_3$]24-*methyldihydrolanosterol into ergosterol.* [23,25-$^{3}H_3$]24-Methyldihydrolanosterol (1mg.; $3{\cdot}7\times10^5$counts/100sec.) was incubated with yeast and ergosterol isolated as before. The biosynthesized ergosterol after dilution with unlabelled ergosterol (600mg.) was crystallized to constant specific radioactivity (19counts/100sec./mg., total radioactivity $19\times600=11{\cdot}4\times10^3$counts/100sec.). This represents 3% conversion of 24-methyldihydrolanosterol into ergosterol. Assuming utilization of only one C-24 enantiomer and loss of one tritium atom from C-23 during biosynthesis, the conversion is 9%.

Conversion of [24,28-$^{3}H_2$]24-*methyldihydrolanosterol into ergosterol.* [24,28-$^{3}H_2$]24-Methyldihydrolanosterol (1mg.; $2{\cdot}52\times10^5$counts/100sec.) was incubated with yeast and biosynthesized ergosterol isolated as before. After incubation of six such batches, biosynthesized ergosterol was diluted with carrier ergosterol (1100mg.) and crystallized to constant specific radioactivity (48counts/100sec./mg., total radioactivity $48\times1100=5{\cdot}28\times10^4$counts/100sec.).

Preparation of [23-$^{3}H_2$]*lanosterol* (*XII*;*R*=H). A solution of 3-acetoxytrisnorlanost-8-en-24-al (X) (200mg.) (Akhtar *et al.* 1967) in benzene (5ml.) was treated with methanolic 5% KOH (3ml.) and tritiated water (0·09ml., 5c/ml.) and was allowed to stand under N_2 overnight. Excess of water was added and the mixture was extracted with ether. The extracts were washed thoroughly with water and dried with Na_2SO_4. After removal of the solvent the residue was acetylated and thin-layer chromatography showed good recovery of the 24-aldehyde. The crude product (XI) was diluted with unlabelled 24-aldehyde (X) (100mg.) and subjected to a Wittig reaction with isopropyltriphenylphosphonium iodide as for the preparation of [24-^{3}H]-lanosterol (Akhtar *et al.* 1967). Re-acetylation and chromatography on a preparative silica-gel plate gave [23-$^{3}H_2$]-lanosteryl acetate (XII;R=Ac). The labelled lanosteryl acetate (36mg.) in dry ether was refluxed for 2hr. with $LiAlH_4$ (120mg.). Preparation as usual gave [23-$^{3}H_2$]-lanosterol (XIII;R=H) (33mg.; $1{\cdot}3\times10^6$counts/100sec./mg.).

Determination of radiochemical purity. Radiochemical purity of all labelled compounds was determined by the procedure described by Akhtar *et al.* (1967).

Synthesis of [23,25-$^{3}H_3$]24-*methyldihydrolanosterol.* [23,25-$^{3}H_3$]24-Methylenelanosterol (100mg.; $3{\cdot}7\times10^5$counts/100sec./mg.) (Akhtar *et al.* 1966*a*) in ethyl acetate (20ml.) was hydrogenated at room temperature in the presence of Adams catalyst until 1mole of hydrogen had been taken up (2·5hr.). After removal of the catalyst and solvent, [23,25-$^{3}H_3$]24-methyldihydrolanosterol was crystallized from ether–methanol. It gave yield 80mg., m.p. 149°, $[\alpha]_D^{21}+47{\cdot}6$ (Found: C, 83·58; H, 11·4; $C_{31}H_{54}O$ requires C, 84·09; H, 12·29%). The i.r. and n.m.r. spectra showed complete absence of a methylene group.

Synthesis of [24,28-$^{3}H_2$]24-*methyldihydrolanosterol.* 24-Methylenelanosterol (50mg.) (Akhtar *et al.* 1966*a*) in ethyl acetate (20ml.) was reduced in the presence of Adams catalyst with tritium gas produced by addition of lithium pellets to tritiated water (2ml., containing 50–100mc of tritium). The crystallized product had 252000counts/100sec./mg. and the i.r. and n.m.r. spectra were identical with those found above.

Degradation of the side chain of labelled ergosterol. A suspension of ergosterol (1g., $^{3}H/^{14}C=7{\cdot}2$) from the incubation of doubly labelled lanosterol (XII+XIII,R=H) ($^{3}H/^{14}C=14{\cdot}0$) in acetic acid (10ml.) was ozonized (Hanahan & Wakil, 1953). The ozonide was reduced with zinc dust (2·5g.) and 2,3-dimethylbutyraldehyde was isolated by steam-distillation. The distillate was neutralized with aq. NaOH and a portion (12ml.) was treated with

dimedone (200mg.) in ethanol (6ml.). The dimedone derivative of 2,3-dimethylbutyraldehyde (m.p. 152°) was filtered off after 1hr. and had 150counts/100sec./mg. at the ^3H setting and 18counts/100sec./mg. at the ^{14}C setting ($^3H/^{14}C=8{\cdot}3$). The remainder of the distillate was again steam-distilled and the aldehyde was oxidized with alkaline aq. $KMnO_4$, as described by Akhtar *et al.* (1967), to give 2,3-dimethylbutyric acid, which was titrated against 0·05N-NaOH. The resulting sodium salt had 30counts/100sec./mg. at the ^{14}C setting and no tritium activity ($^3H/^{14}C=0$).

Ergosterol (820mg.) from incubation of [24,28-$^3H_2$]24-methyldihydrolanosterol was degraded and prepared in the same way as above to give a solution (pH6) of 2,3-dimethylbutyraldehyde, which was divided into two portions. The first portion (40ml.) was immediately converted into the dimedone derivative. An aq. 10% NaOH soln. (3ml.) was added to the remainder (60ml.), which was allowed to stand for 18hr. under N_2. After neutralization with acetic acid the dimedone derivative was prepared as before. The parent ergosterol had a specific radioactivity of 48700counts/min./m-mole; the dimedone derivative of untreated 2,3-dimethylbutyraldehyde had a specific radioactivity of 47000counts/min./m-mole and the dimedone derivative of the equilibrated aldehyde had a specific radioactivity of 33700counts/min./m-mole, which represents a 31% loss of tritium. In this experiment radioactivity measurements were made on a Beckman model CPM200 liquid-scintillation spectrometer with scintillator Butyl-PBD [Ciba (A.R.L.) Ltd., Duxford, Cambs.] in toluene (8g./l.).

We thank Professor K. A. Munday for his kind interest and encouragement. We gratefully acknowledge a research grant from the Science Research Council for the purchase of an infrared spectrophotometer and a Beckman scintillation spectrometer. The purchase of chemicals was made possible by a grant from the Medical Research Council. We also thank the Chemistry Department of the University of Southampton for their helpful co-operation.

REFERENCES

Akhtar, M., Hunt, P. F. & Parvez, M. A. (1966*b*). *Chem. Commun.* p. 565.

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. (1966*a*). *Biochem. J.* **100**, 38c.

Barton, D. H. R., Harrison, D. M. & Moss, G. P. (1966). *Chem. Commun.* p. 595.

Dewhurst, S. & Akhtar, M. *Biochem. J.* (1967). **105**, 1187.

Frantz, I. D. & Schroepfer, G. J. (1967). *Annu. Rev. Biochemistry*, **36**, 720.

Hanahan, D. J. & Wakil, S. J. (1953). *J. Amer. chem. Soc.* **75**, 273.

Katsuki, H. & Bloch, K. (1967). *J. biol. Chem.* **242**, 222.

Stone, K. J. & Hemming, F. W. (1965). *Biochem. J.* **96**, 14c.

Stone, K. J. & Hemming, F. W. (1967). *Biochem. J.* **104**, 43.