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Studies on the Biosynthesis of the Ergosterol Side Chain

By M. AKHTAR, M. A. PARVEZ AND P. F. HUNT
Department of Physiology and Biochemistry, University of Southampton, SO9 5NH

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1. A convenient synthesis of 24-methylene[23,25-$^3H_3$]dihydrolanosterol is described. 2. A general anaerobic–aerobic method for the incorporation of sterols into whole yeast cells is also described and illustrated by experiments with ^3H-labelled lanosterol. 3. The method was used to convert labelled 24-methylenedihydrolanosterol into ergosterol, in good yield, by *Saccharomyces cerevisiae*. 4. Degradation of the biosynthetic ergosterol provided confirmation of the conversion, which supports the proposed mechanism for the biosynthesis of the ergosterol side chain. 5. Mechanisms for the further conversion of the 24-methylene side chain into the ergosterol side chain are discussed and it was shown that a compound, [3α-$^3H_1$]-ergost-7-en-3β-ol, with a fully saturated side chain, can also be efficiently incorporated into ergosterol. 6. This result was confirmed by a procedure involving formation of the 5,8-epidioxide and subsequently the 5,8-epidioxy-22,23-epoxide of the biosynthetic ergosterol.

The side chain of ergosterol (I) contains two unique features that are not found in the side chain of cholesterol. The first is the presence of an 'extra' carbon atom at position C-24 and the second is the existence of a double bond between C-22 and C-23. Early work by Parks (1958) established that the extra carbon atom at C-24 is derived from *S*-adenosylmethionine. Later work by Lederer (1964) revealed that only two of the three original hydrogen atoms from the methyl group of *S*-adenosylmethionine were incorporated into the C-28 methyl group of ergosterol. This observation led us and others (Akhtar, Parvez & Hunt, 1966*a*; Castle, Blondin & Nes, 1963; Lederer, 1964; Clayton, 1965; Goad, Hammam, Dennis & Goodwin, 1966) to consider a mechanism of the type outlined in Scheme 1 for the *C*-alkylation step in ergosterol biosynthesis. Evidence in favour of this mechanism was provided by our work (Akhtar, Hunt & Parvez, 1966*b*) and independent observations on the mechanism were subsequently reported by a number of workers (Goulston, Goad & Goodwin, 1967; Castle, Blondin & Nes, 1967; Russell, van Aller & Nes, 1967; Raab, de Souza & Nes, 1968). The mechanism presented in Scheme 1 should predict the intermediary role of the side chain of type (V) in the biosynthesis of ergosterol, and indeed a sterol with this side chain has been isolated from yeast (Barton, Harrison & Widdowson, 1968).

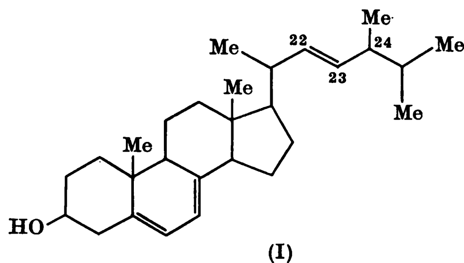
In this paper we report the synthesis of tritiated 24-methylenedihydrolanosterol (XI) and its conversion into ergosterol by whole cells of the yeast *Saccharomyces cerevisiae*. We also describe experiments that demonstrate the further conversion of the 24-methylene side chain of type (V) into the ergosterol side chain. Preliminary accounts of a part of this work, from this laboratory and elsewhere, have been published (Akhtar *et al.* 1966*a*; Barton, Harrison & Moss, 1966).

RESULTS AND DISCUSSION

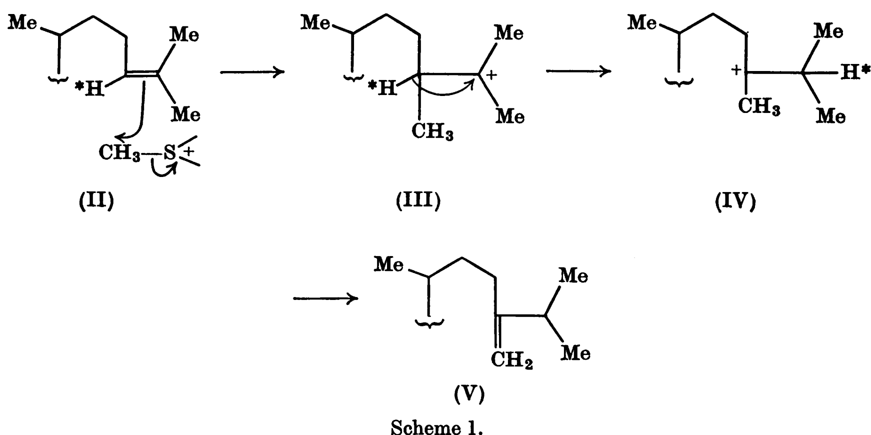
Incorporation of sterols into yeast cells. The pathway leading to the biosynthesis of ergosterol in yeast takes place without a requirement for oxygen up to the stage of squalene. The conversion of squalene into ergosterol involves a number of oxygen-dependent reactions. We have observed that anaerobically growing yeast cells, deficient in endogenous sterols, can incorporate efficiently a variety of sterols from the medium. This has enabled us to develop an 'anaerobic–aerobic' method for the conversion of precursor sterols into ergosterol in good yield. This method is a modification of the method of Kodicek (1959).

Synthesis of tritiated 24-methylenedihydrolanosterol and its conversion into ergosterol. 24-Methylenedihydrolanosterol (XI), tritiated at positions C-23 and C-25, was synthesized by the sequence of reactions shown in Scheme 2. 24-Methylenedihydrolanosterol was converted by using the anaerobic–aerobic method into ergosterol in 6·2% yield: evidence for the conversion is described in the Experimental section.

Observations on the mechanism of the introduction of the C-22–C-23 *double bond.* Akhtar, Parvez &



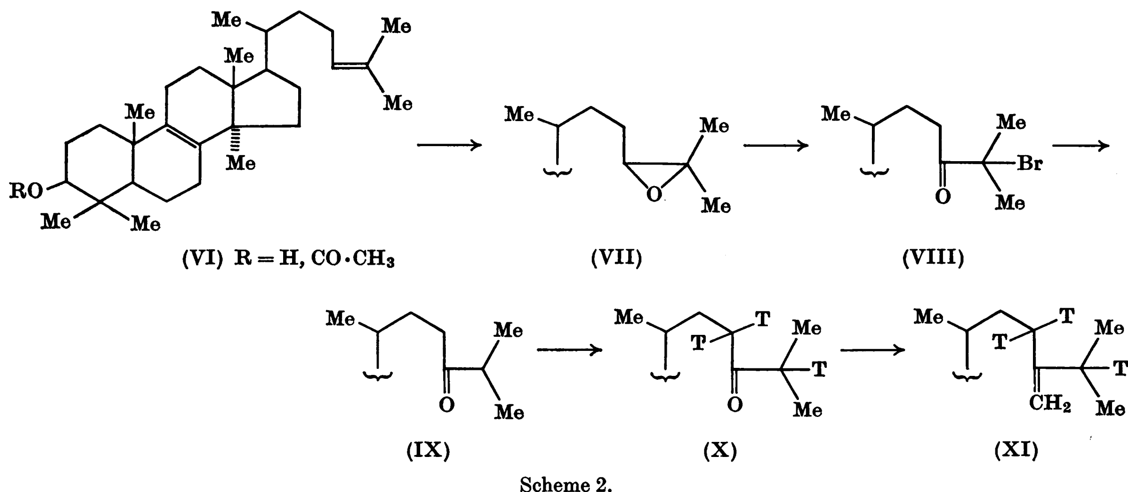
(I)



(II) (III) (IV)

(V)

Scheme 1.



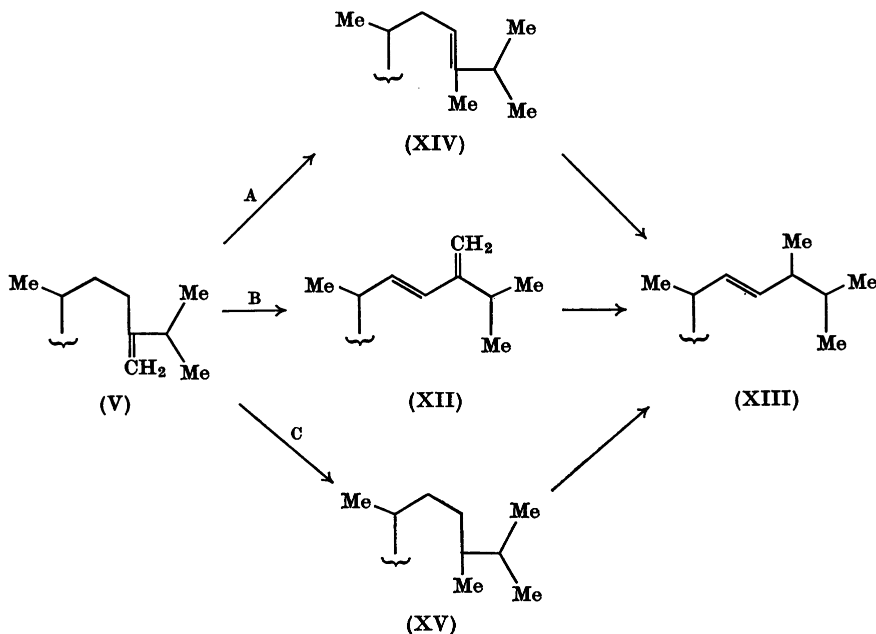
(VI) R = H, CO·CH_3 (VII) (VIII)

(IX) (X) (XI)

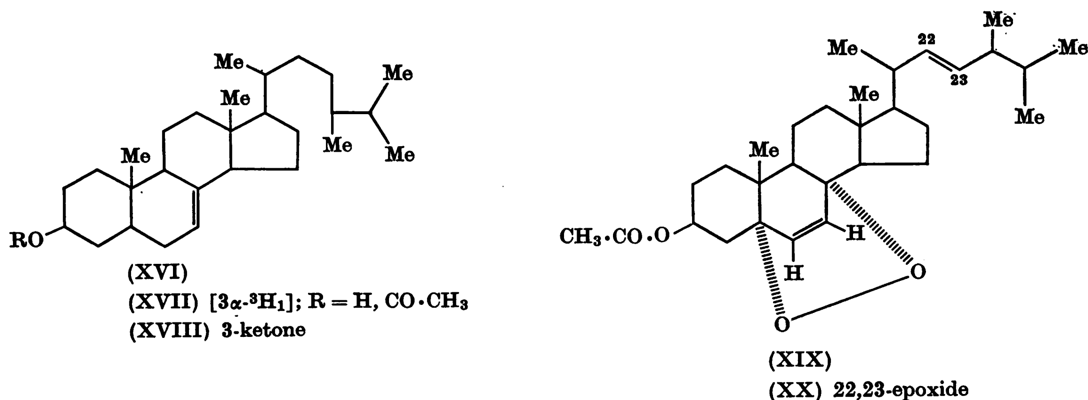
Scheme 2.

Hunt (1968) discussed a number of general pathways for the conversion of structure (V) into structure (XIII) (Scheme 3).

The three most likely possibilities are displayed in Scheme 3. Pathway A involves a series of 1,2-shifts of the ethylenic linkage; pathway B represents 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 C shows the reverse of the oxidation–reduction sequence. Indirect evidence provided by Katsuki & Bloch (1967) suggested the involvement of the pathway B for the elaboration of the ergosterol side chain. We have recently studied the



Scheme 3.



Scheme 4.

metabolism of 24-methyldihydrolanosterol (XV), which contains the fully saturated side chain, and have shown that this compound is converted into ergosterol. Although the 22,24(28)-diene is the most probable biological intermediate (Katsuki & Bloch, 1967), this observation indicates that the enzymes participating in the formation of the C-22–C-23 double bond do not require the activation of a neighbouring double bond. In support of this conclusion we now report that another related compound, ergost-7-en-3β-ol (XVI; R = H), is converted into ergosterol in 14·9% yield.

Conversion of [3α-^{3}H]*ergost-7-en-3β-ol (XVII; R = H) into ergosterol.* Ergosterol acetate was hydrogenated and hydrolysed to give ergost-7-en-3β-ol (XVI; R = H); this on oxidation followed by reduction with tritiated sodium borohydride gave [3α-^{3}H]ergost-7-en-3β-ol (XVII; R = H). When the later compound was incubated with whole cells of yeast by the anaerobic–aerobic method, 14·9% of the original radioactivity crystallized with ergosterol. Since the conversion of compound (XVI) into ergosterol (I) involves modification at two positions in the molecule, namely formation of

the 5,7-diene system and insertion of the C-22–C-23 double bond, it was necessary to establish that the radioactivity was associated with the compound having both these features. The conversion of the biosynthetic ergosterol acetate into the epidioxide (XIX) occurred with complete retention of radioactivity, thus showing the presence of the diene system in the radioactive compound. The presence of the C-22–C-23 double bond in the radioactive compound was established by a new method that involves conversion of the epidioxide (XIX) into the epoxy-epidioxide (XX). This conversion was once again accompanied by the retention of radioactivity. In the epoxidation of the C-22–C-23 double bond two epoxides were formed and were readily separated by chromatography. Professor D. H. R. Barton (personal communication) has noted a similar phenomenon with a related compound.

The conversion of compound (XVI) into ergosterol is in agreement with the previous conclusion (Akhtar *et al.* 1968) that the enzymes participating in the formation of the C-22–C-23 double bond in ergosterol biosynthesis do not require the activation of the C-24–C-28 double bond.

The observation (Smith, Goad & Goodwin, 1968) that formation of the C-22–C-23 double bond of poriferasterol (and probably ergosterol also) is accompanied by the elimination of two *cis*-oriented hydrogens, supports the proposal (Dewhurst & Akhtar, 1967) that the mechanism is probably analogous to that involved in the introduction of the C-5–C-6 double bond of cholesterol (Dewhurst & Akhtar, 1967) and the double bonds of certain long-chain fatty acids (Schroepfer & Bloch, 1965; Morris, Harris, Kelly & James, 1968).

EXPERIMENTAL

The i.r. spectra were determined with a Unicam SP. 2000 spectrometer and all compounds described below gave the expected i.r. spectra. The n.m.r. spectra were determined in deuterochloroform with a Varian model A60 spectrometer. Optical rotations were measured in chloroform solutions with a Perkin–Elmer model 141 polarimeter. For preparative t.l.c. silica gel H (E. Merck A.-G., Darmstadt, Germany) was used. Radioactivity measurements were made on a Beckman liquid-scintillation system with 2-(4-*tert.*-butylphenyl)-5-(4-biphenylyl)-1-oxa-3,4-diazole [CIBA (A.R.L.) Ltd., Duxford, Cambs.] (8g./l.) in toluene as scintillator. Corrections for quenching were made with an internal standard. Melting points were determined in capillary tubes on a Gallenkamp melting-point apparatus.

Preparation of 3β-*acetoxy*-24,25-*epoxylanost*-8-*ene* (*VII*; *R*=$CH_3\cdot CO$). *m*-Chloroperbenzoic acid (5g., 1·15 moles, 85% of per-acid) was added to a stirred solution of lanosteryl acetate (VI, R=$CH_3\cdot CO$) (10g.) in chloroform (200ml.) at $-10°$. The solution was vigorously stirred for 2½hr. at the same temperature, followed by successive washing with aq. 10% Na_2SO_3, $NaHCO_3$, NaCl and finally distilled water. The organic layer was dried with Na_2SO_4 and the solvent was removed under vacuum. The resulting solid was crystallized from acetone–methanol to give the monoepoxide (VII; R=$CH_3\cdot CO$), m.p. 181–182°, $[\alpha]_D^{21}$ +55° (Found: C, 78·6; H, 10·7. $C_{32}H_{52}O_3$ requires C, 79·3; H, 10·8%).

Preparation of 3β-*acetoxy*-25-*bromolanost*-8-*en*-24-*one* (*VIII*; *R*=$CH_3\cdot CO$). Conc. HBr (0·5ml.) was added to a suspension of 3β-acetoxy-24,25-epoxylanost-8-ene (VII; R=$CH_3\cdot CO$) (500mg.) in acetic acid (12·5ml.) at room temperature and the reaction mixture was left standing until clear (5min.). The reaction was stopped by addition of cold water and the precipitated bromohydrin was extracted with ether. The organic layer was dried with Na_2SO_4 and the solvent was removed under vacuum. The resulting solid was oxidized in acetone with Jones reagent (0·4ml.) (Bowden, Heilbron, Jones & Weedon, 1946). The excess of reagent was decomposed with methanol. The product was extracted with ether, washed with aq. $NaHCO_3$ and dried with Na_2SO_4. The ethereal solution was evaporated to dryness and the residue was chromatographed on silica gel (20g.) with benzene–light petroleum (b.p. 80–100°) (3:2, v/v) as eluent. This ratio was finally increased to 9:1 (v/v). The compound (VIII; R=$CH_3\cdot CO$) was obtained in pure form (170mg.), m.p. 135° (Found: C, 68·0; H, 9·0; Br, 13·8. $C_{32}H_{51}BrO_3$ requires C, 68·0; H, 9·0; Br, 14·0%).

Preparation of 3β-*acetoxylanost*-8-*en*-24-*one* (IX; *R*=$CH_3\cdot CO$). Zinc dust (2g.) was added to a solution of 3β-acetoxy-25-bromolanost-8-en-24-one (VIII; R=$CH_3\cdot CO$) (600mg.) in acetic acid (120ml.) The reaction mixture was stirred vigorously at room temperature for 3½hr. Zinc dust was filtered off and the filtrate was poured into water (200ml.). The precipitate was extracted with ether and the extracts were washed successively with aq. $NaHCO_3$ and water. The ethereal extracts were dried with Na_2SO_4 and the product was crystallized from acetone–methanol (yield 400mg.), m.p. 128° (Found: C, 79·5; H, 10·8. $C_{32}H_{52}O_3$ requires C, 79·2; H, 10·8%).

Preparation of 3β-*acetoxy*[23,25-$^3H_3$]*lanost*-8-*en*-24-*one* (*X*; *R*=$CH_3\cdot CO$). Ethanolic 5% (w/v) KOH (3ml.), tetrahydrofuran (10ml.) and tritiated water (0·08ml., 450mc) were added to a solution of 3β-acetoxylanost-8-en-24-one (IX; R=$CH_3\cdot CO$) (300mg.) in benzene (5ml.) and the mixture was left at room temperature for 25hr. Addition of water (200ml.) gave a white precipitate, which was extracted with ether. The extracts were washed thoroughly with water and dried with Na_2SO_4. The solvent was removed under vacuum and the remaining solid was acetylated to give 3β-acetoxy[23,25-$^3H_3$]lanost-8-en-24-one (X; R=$CH_3\cdot CO$) (230mg.; sp. radioactivity $6·6\times10^6$c.p.m./mg.).

Preparation of 24-*methylene*[23,25-$^3H_3$]*dihydrolanosterol* (*XI*; R=H). Wittig's triphenylphosphinemethylene reagent (2·96g.) was dissolved in dry ether under an atmosphere of N_2. A 20% solution of butyl-lithium (560mg.) in light petroleum was added and the mixture refluxed with constant stirring. After 15min. 3β-acetoxy[23,25-$^3H_3$]-lanost-8-en-24-one (X; R=$CH_3\cdot CO$) (400mg.) was added and refluxing was continued for 3hr. Acetone was added dropwise until the orange colour disappeared. The solution was filtered and the residue washed with ether. The filtrate and washings were washed with water–methanol (3:1, v/v) and distilled water. The extracts were dried with Na_2SO_4 and the solvent was removed under vacuum. Crystallization from acetone–methanol gave 24-methylene[23,25-$^3H_3$]-dihydrolanosterol (XI; R=H) (188mg.), m.p. 148–150°.

The crystallized product, when tested for radiochemical purity on a t.l.c. plate, showed a polar radioactive impurity (starting material). Column chromatography on silica gel (10g.) and elution with benzene–light petroleum (b.p. 60–80°) (3:2, v/v) gave the pure compound (XI; R=H) (90mg.; sp. radioactivity $5{\cdot}4\times10^6$ c.p.m./mg.), m.p. 154° (Found: C, 84·1; H, 11·5. $C_{31}H_{52}O_2$ requires C, 84·5; H, 11·9%).

Conversion of 24-methylene[23,25-$^3H_3$]dihydrolanosterol (XI; R=H) into ergosterol. 24-Methylene[23,25-$^3H_3$]-dihydrolanosterol (XI; R=H) (1mg.; $5{\cdot}4\times10^6$ c.p.m.) was converted into ergosterol by whole cells of *Saccharomyces cerevisiae* LK_2G_{12} by the method described below. The non-saponifiable fraction, which contained 28% ($1{\cdot}38\times10^6$ c.p.m.) of the original radioactivity when chromatographed on a preparative t.l.c. plate (with chloroform), showed 17% (9×10^5 c.p.m.) of the original radioactivity in the ergosterol band, which was eluted, diluted with non-radioactive ergosterol (1g.) and crystallized to constant specific radioactivity (338 c.p.m./mg.; total radioactivity $338\times1000=3{\cdot}38\times10^5$ c.p.m.). This represents 6·2% conversion of 24-methylene[23,25-$^3H_3$]dihydrolanosterol (XI; R=H) into ergosterol (taking into account the loss of one tritium atom from C-23, the conversion is 9·3%).

Degradation of biosynthetic ergosterol derived from 24-methylene[23,25-$^3H_3$]dihydrolanosterol. Biosynthetic ergosterol (500mg.; 98 c.p.m./mg.) was suspended in acetic acid (5ml.) and ozone was bubbled until all the solid had dissolved (2hr.). The solution was cooled to 10°, zinc dust (1·25g.) was added and the reaction mixture stirred for 1hr. Zinc was filtered off and washed with acetic acid. 2,3-Dimethylbutyraldehyde was isolated and converted into the dimedone derivative as described by Akhtar *et al.* (1966*b*). The derivative (100mg.) was recrystallized from ethanol–water and had m.p. 150–152° and specific radioactivity 107 c.p.m./mg.

Preparation of ergost-7-en-3β-ol (Δ^7-ergostenol) (XVI; R=H). Ergosterol acetate (1·5g.), dissolved in ethyl acetate (50ml.), was reduced at room temperature in the presence of pre-reduced Adams catalyst (200mg.) in ethyl acetate (5ml.) until 2mol.prop. of hydrogen had been taken up (Wieland & Benend, 1943). After filtering off of the catalyst, the solvent was removed and the solid crystallized from ether–methanol to yield ergost-7-en-3β-ol acetate (XVI; R=CH_3·CO) (1·3g.). The acetate (1g.) was dissolved in dry ether, treated with $LiAlH_4$ (300mg.) and refluxed for 1hr. Excess of $LiAlH_4$ was decomposed with methanol and the product was extracted with ether. Crystallization from ether–methanol gave ergost-7-en-3β-ol (XVI; R=H) (600mg.), m.p. 140°; Wieland & Benend (1943) quote 148°.

Preparation of ergost-7-en-3-one (XVIII). Ergost-7-en-3β-ol (XVI; R=H) (200mg.) in acetone (20ml.) was cooled to 15° and treated with Jones reagent (0·2ml.). The reaction mixture was stirred for 10min. Excess of reagent was decomposed with methanol and the product was extracted with ether. The extracts were washed with aq. $NaHCO_3$ and water and dried with Na_2SO_4. Crystallization from ether–methanol gave the ketone (XVIII) (160mg.).

Preparation of [3α-$^3H_1$]ergost-7-en-3β-ol (XVII; R=H). Ergost-7-en-3-one (XVIII) (158mg.), dissolved in methanol (20ml.), was treated at room temperature with tritiated $NaBH_4$ (2–3mg.). After 30min. non-radioactive $NaBH_4$ (10–15mg.) was added and the mixture allowed to stand for a further 60min. The product was precipitated by addition of excess of water and extracted with ether. Crystallization from ether–methanol gave compound (XVII; R=H) (60mg.), specific radioactivity $1{\cdot}44\times10^7$ c.p.m./mg.

Conversion of [3α-$^3H_1$]ergost-7-en-3β-ol (XVII; R=H) into ergosterol. [3α-$^3H_1$]Ergost-7-en-3β-ol (XVII; R=H) (1mg.; $1{\cdot}42\times10^7$ c.p.m.) was incubated with *Saccharomyces cerevisiae* LK_2G_{12} as described below and ergosterol was isolated by the method of Akhtar *et al.* (1966*a*). The biosynthetic ergosterol was diluted with carrier ergosterol (2g.) and crystallized to constant specific radioactivity (1058 c.p.m./mg.; total radioactivity $1058\times2000=2{\cdot}12\times10^6$ c.p.m.; 14·9% conversion).

Preparation of 5α,8α-epidioxyergosta-6,22-dien-3β-ol acetate (XIX) from biosynthetic ergosterol. Ergosterol acetate (200mg.) in ethanol (30ml.) containing eosin (2–3mg.) was irradiated under reflux with a 150w incandescent lamp while O_2 was bubbled through the solution. The reaction was stopped when the solution ceased to show an absorption maximum at 282nm. The product was crystallized from ether–methanol to give compound (XIX) (100mg.), m.p. 196–198°; Clayton, Henbest & Jones (1953) quote m.p. 195–200°. The parent ergosterol had a specific radioactivity of $4{\cdot}19\times10^5$ c.p.m./m-mole and the epoxide had the same specific radioactivity.

Preparation of 5α,8α-epidioxy-22,23-epoxyergost-6-en-3β-ol acetate (XX). *m*-Chloroperbenzoic acid (80mg.) was added to a solution of 5α,8α-epidioxyergosta-6,22-dien-3β-ol acetate (XIX) (50mg.; $4{\cdot}19\times10^5$ c.p.m./m-mole) in chloroform (5ml.) at 0° and the reaction was allowed to proceed for 14hr. The excess of per-acid was decomposed by washing with aq. Na_2SO_3, $NaHCO_3$, NaCl and finally water. The solution was dried and the solvent removed. Crystallization of the residue from ether–methanol gave a solid (30mg.), which was chromatographed on a preparative t.l.c. plate (silica gel H) into two bands *A* (mobility 8cm.) and *B* (mobility 6·5cm.), which were eluted and crystallized separately. Analysis of the compound *A* obtained from band *A* showed C, 73·06; H, 9·56; $C_{30}H_{46}O_5$ (monoepoxide) requires C, 74·04; H, 9·53%. Compound *B* from band *B* showed C, 73·33; H, 9·84; $C_{30}H_{46}O_5$ (monoepoxide) requires C, 74·04; H, 9·53%. The n.m.r. spectra for both the compounds *A* and *B* showed the presence of the C-6–C-7

Table 1. *Position of protons at* C-6 *and* C-7 *and at* C-22 *and* C-23 *in the n.m.r. spectra of various related steroid derivatives*

Compound	Position of C-6 and C-7 protons (p.p.m.)	Position of C-22 and C-23 protons (p.p.m.)
5α,8α-Epidioxyergosta-6,22-dien-3β-ol acetate	6·1–6·6	5·1–5·3
Ergost-22-ene-5α,8α,3β-triol 3-acetate	—	5·1–5·3
5α,8α-Epidioxycholest-6-en-3β-ol acetate	6·1–6·6	—
5α,8α-Epidioxy-22,23-epoxyergost-6-en-3β-ol acetate (isomer *A*)	6·1–6·6	—
5α,8α-Epidioxy-22,23-epoxyergost-6-en-3β-ol acetate (isomer *B*)	6·1–6·6	—

double bond and the absence of the C-22–C-23 double bond. This was confirmed by the n.m.r. spectra of related compounds as shown in Table 1. Thus compounds *A* and *B* were isomers of 5α,8α-epidioxy-22,23-epoxyergost-6-en-3β-ol acetate (XX). Isomers *A* and *B* had specific radioactivities of 408100 c.p.m./m-mole and 419100 c.p.m./m-mole respectively.

Radiochemical purity of all the above compounds was established by t.l.c.

Incorporation of lanosterol and its conversion into ergosterol by the whole cells of the yeast Saccharomyces cerevisiae *LK_2G_{12} grown under anaerobic and aerobic conditions.* [24-$^3H_1$]Lanosterol (Akhtar *et al.* 1966*b*) (1 mg.; $8{\cdot}3\times10^5$ c.p.m.) emulsified in Tween 80 (15 mg.) (Tchen, 1963) was added to a sterilized medium (Klein, Eaton & Murphy, 1954) separately to two flasks (4 l. in each). After the addition of an inoculum (4–6 mg.) of yeast *Saccharomyces cerevisiae* LK_2G_{12}, both the flasks were incubated at 30°, one under an atmosphere of O_2 and the other under an atmosphere of N_2. After 48 hr. incubation, 1 l. of medium was taken from each flask; the cells were harvested and saponified and the non-saponifiable fraction was extracted. The anaerobic cells showed a total incorporation of $5{\cdot}68\times10^5$ c.p.m. (=68·4%), and the aerobically grown cells showed a total incorporation of $1{\cdot}92\times10^5$ c.p.m. (=23·1%). At this stage the N_2 supply of the anaerobic incubation vessel was replaced by O_2 and the two flasks were incubated for a further 24 hr. The cells from the anaerobic–aerobic incubation and from the totally aerobic incubation were harvested and saponified and the sterols were extracted separately. At this stage (end of 72 hr. incubation), the anaerobic–aerobic cells showed a total incorporation of 93·3% of lanosterol, whereas the aerobically grown cells showed a total incorporation of 28% of lanosterol. In each case the non-saponifiable fraction was diluted with ergosterol (1 g.) and crystallized to constant specific radioactivity. The anaerobic–aerobic experiment gave ergosterol having 290 c.p.m./mg. (total radioactivity $2{\cdot}9\times10^5$ c.p.m.), which represents 34·9% conversion of lanosterol into ergosterol, whereas the aerobic experiment gave ergosterol having 62 c.p.m./mg. (total radioactivity $0{\cdot}62\times10^5$ c.p.m.), which represents 7·4% conversion.

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