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Investigations on the Biosynthesis of Steroids and Terpenoids
XI. The 24-Methylene Sterol 24(28)-Reductase of *Saccharomyces cerevisiae*[1]

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The microsomal fraction of *Saccharomyces cerevisiae* has been shown to catalyse the NADPH-dependent reduction of ergosta-5,7,22,24(28)-tetraen-3β-ol to ergosterol. This cell-free system together with whole-cell cultures of polyene-resistant mutants has been used to compare the rates of reduction of other 24-methylene sterols. The results indicate that the enzyme involved exhibits a marked specificity for ergosta-5,7,22,24(28)-tetraen-3β-ol and support the concept of a major terminal step in ergosterol biosynthesis.

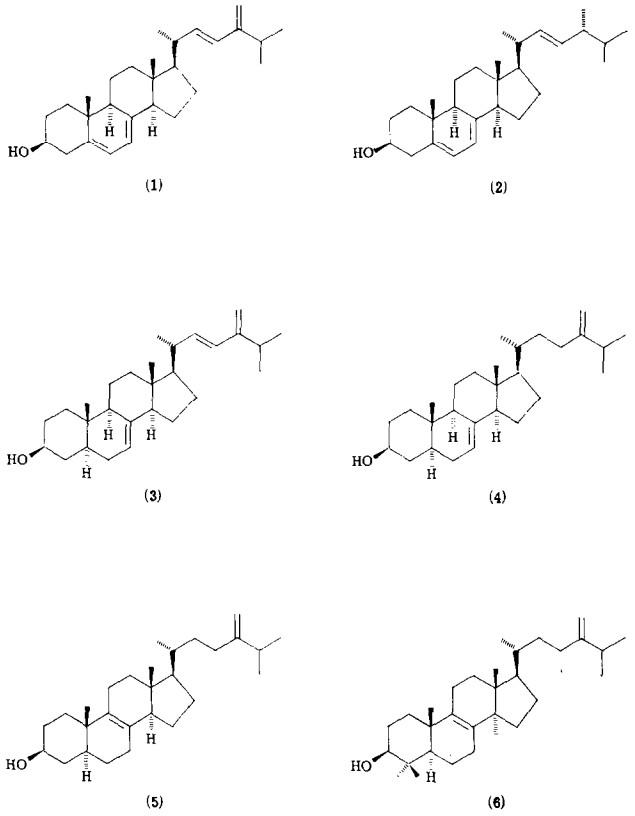
INTRODUCTION

Intermediates in the biosynthetic transformation of lanosterol into ergosterol by *Saccharomyces cerevisiae* have been recognized by their isolation from both wild-type and mutant strains (*1–3*). This was confirmed by refeeding the intermediates in radiolabelled form and demonstrating the incorporation of radioactivity into ergosterol and proposed intermediates by whole cells. Biosynthetic routes have been proposed on the basis of this information (*3, 4*). Recent studies with mutants defective in ergosterol biosynthesis indicate that although the later stages need follow no defined sequence, each step is accomplished by a single enzyme species (*2*). We are currently attempting to obtain an understanding of this biosynthetic system at the enzymic level and report here the identification of an NADPH-dependent ergosta-5,7,22,24(28)-tetraen-3β-ol (**1**) reductase activity in cell-free extracts of *S. cerevisiae*. An *S*-adenosyl methionine: Δ^{24}-sterol methyl-transferase is the only enzyme of this multistep process to have been studied in isolation (*5*). Previous studies with cell-free systems have shown the incorporation of radioactively labelled mevalonic acid into ergostatetraenol (**1**) and other sterols but reduction of the sterol 24-methylene group was not achieved (*6*). Ergostatetraenol (**1**) is a major sterol in several strains of *S. cerevisiae* (*7*), and its efficient incorporation into ergosterol (**2**) by whole-cell cultures (*4, 8*) indicates its importance as a precursor of ergosterol.

RESULTS

[^{3}H]ergostatetraenol (**1**) was obtained from *S. cerevisiae* (strain NCYC 1236) grown in the presence of [2-^{3}H]acetic acid and was incubated with the microsomal fraction of

[1] Part X, *J. Chem. Soc. Perkin I* 88, (1975).



SCHEME 1

TABLE 1

CONVERSION OF ERGOSTATETRAENOL INTO ERGOSTEROL BY THE MICROSOMAL FRACTION

Addition to standard incubation mixture[a]	[^{3}H]sterol recovered in ergosterol fraction (nmol)
1. None	0.84
2. NADH (2 μmoles)	2.17
3. NADH-generating system[b]	2.06
4. NADPH (2 μmoles)	28.32
5. NADPH-generating system[c]	23.28
6. NADPH (2 μmoles) (aerobic)	28.84
7. NADPH-generating system lacking $NADP^+$	1.38
8. $NADP^+$ (2 μmoles)	16.85

[a] Incubation mixtures contained: pH 7.5 Tris–HCl buffer, 60 μmoles; a preparation of microsomal fraction of *S. cerevisiae* containing 11 mg of protein; [^{3}H]-ergostatetraenol (activity, 8.1×10^4 dps/mg), 0.1 μmole added in ethanol (0.05 ml); other additions as stated in a total volume of 1 ml. Incubations were carried out anaerobically (except where stated) at 30°C for 1 h.

[b] NADH-generating system consisted of NAD^+ (2 μmoles) plus alcohol dehydrogenase (100 units) and ethanol (0.05 ml).

[c] NADPH-generating system consisted of $NADP^+$ (2 μmoles) glucose-6-phosphate (5 mg), glucose-6-phosphate dehydrogenase (2 units) plus magnesium chloride (10 μmoles).

S. cerevisiae (commercial strain). In the presence of NADPH or an NADPH-generating system, significant incorporation into ergosterol (**2**), isolated as its trimethylsilyl ether, was observed (Table 1). This reduction only occurred efficiently in the presence of NADPH. When NADH or an NADH-generating system was substituted for NADPH a much lower incorporation was obtained, but this was marginally higher than the very low incorporation which occurred when no nicotinamide nucleotide was added. Some incorporation occurred when $NADP^+$ alone was added. This was presumably due to a slow endogenous NADPH-generating reaction. Ergosterol was confirmed as

TABLE 2

IDENTIFICATION OF ERGOSTEROL AS THE PRODUCT OF ERGOSTATETRAENOL REDUCTION

Addition to incubation mixture[a]	Activity of ergosterol isolated (dpm/mg)	Ergosterol formed (nmoles)
1. NADPH (20 μmoles)	856	66.3
2. None	43	3.4

[a] Incubation mixtures (10 ml) contained: pH 7.5 Tris–HCl buffer, 200 μmoles; resuspended microsomal fraction containing 100 mg protein; [^{3}H]-ergostatetraenol (activity, 8.1×10^4 dps/mg), 1 μmol added in ethanol (0.2 ml); additions as stated above. Incubation was carried out for 2 hr at 30°C; the reaction was stopped, saponified and extracted in the usual way after addition of carrier ergosterol (100 mg). The ergosterol was purified via the 4-phenyl-1,2,4-triazoline-3,5-dione adduct of its acetate (*8*) and, after reduction back to free ergosterol, was crystallized to constant specific activity from chloroform–methanol.

the reaction product by its purification via the 4-phenyl-1,2,4-triazoline-3,5-dione adduct of its acetate (*8*) followed by reduction back to ergosterol. The results, showing the NADPH dependence, are given in Table 2. The NADPH-oxidase activity of the microsomal fraction made it impossible to establish stoichiometry between NADPH oxidation and ergosterol formation.

That the ergostatetraenol reductase activity was located chiefly in the microsomal fraction (105 000 *g* sediment) was shown when the various cell fractions were assayed for activity. Some activity remained however in the 105 000 *g* supernatant fraction (Table 3). In commercially grown baker's yeast mitochondrial development is likely

TABLE 3

DISTRIBUTION OF ERGOSTATETRAENOL REDUCTASE ACTIVITY IN VARIOUS CELL FRACTIONS

Fraction	Volume (ml)	Protein concentration (mg/ml)	Ergostatetraenol reductase activity (nmoles of ergosterol formed/hr/mg of protein)	Percent of initial activity
2000 g supernatant	420	37.1	1.09	100
20 000 g supernatant	415	36.5	0.89	82
105 000 g supernatant	370	26.1	0.16	9
105 000 g sediment	94	38.0	1.93	40

to be repressed (*9*) and it has not yet been established if the enzyme is associated with a membrane fraction or with the promitochondria which are also located in the 105 000 *g* sediment. Thompson *et al.* (*10*) have established that the *S*-adenosylmethionine: Δ^{24}-sterol methyltransferase activity of *S. cerevisiae* is associated with mitochondria and promitochondria.

During fractionation of crude extracts some losses in total activity occurred (Table 3). A recombination of the sediment and supernatant obtained by centrifugation at 105 000 *g* for 2 hr gave no increase in activity compared with the two fractions assayed separately. The loss in activity is therefore due to the instability of the enzyme to the applied conditions rather than to a resolution of cofactors. Storage of a resuspended microsomal preparation at 0–1°C for 45 hr resulted in a loss of 85% of the original activity. Consequently methods for the stabilization of the activity were investigated. Both NADPH and NADH stabilized the activity almost completely at 0–1°C when added to microsomal suspensions (Table 4). Removal of oxygen also proved an efficient

TABLE 4

STABILIZING AGENTS FOR ERGOSTATETRAENOL REDUCTASE[a]

Stabilizing agent	Concentration	Period of storage at 0–1°C (hr)	Percent of initial activity remaining
None	—	24	33
NADPH	1.3 m*M*	24	104
NADH	1.3 m*M*	24	96
Anaerobiosis	—	24	85
Reduced glutathione	10 m*M*	24	42
2-Mercaptoethanol	10 m*M*	24	42
None	—	48	13
Glycerol	10% (v/v)	48	37
Glycerol	25% (v/v)	48	93

[a] Microsomal fraction resuspended in 50 m*M* Tris–HCl buffer, pH 7.5, to a protein concentration of 20 mg/ml was incubated at 0–1°C with stabilizing agent as indicated. A sample (0.6 ml) of the incubation mixture was then withdrawn and assayed for activity as described in the Experimental section. Anaerobiosis was achieved by addition of glucose (2.5 mg) plus glucose oxidase (40 units) to the incubation mixture (1.5 ml) which was then maintained under nitrogen.

means of stabilization and it is therefore unclear whether NADH and NADPH have a direct stabilizing effect or whether they effect the removal of oxygen from solution by the action of their respective oxidase activities. These stabilizing effects must account for the stability of the enzyme under incubation conditions at 30°C. Glutathione and β-mercaptoethanol were not effective stabilizers but glycerol at a concentration of up to 25% (v/v) provided efficient stabilization. Glycerol was therefore added to incubation mixtures to a final concentration of 12.5% (v/v) and at a concentration of 25% (v/v) for short-term storage of resuspended microsomal preparations at 0°C. For longer periods of storage the activity was stable at −20°C in the absence of glycerol. All attempts to solubilize the activity from the microsomal fraction as a prerequisite to further purification either failed or resulted in an almost complete loss of activity.

In the assay system ultimately developed for ergostatetraenol reductase the ergosterol formed was proportional to protein concentration for concentrations up to 10 mg/ml (Fig. 1B) and was linear for incubation times of up to 60 min (Fig. 1A). The activity had a broad pH optimum centering on pH 6.5 (Fig. 1C).

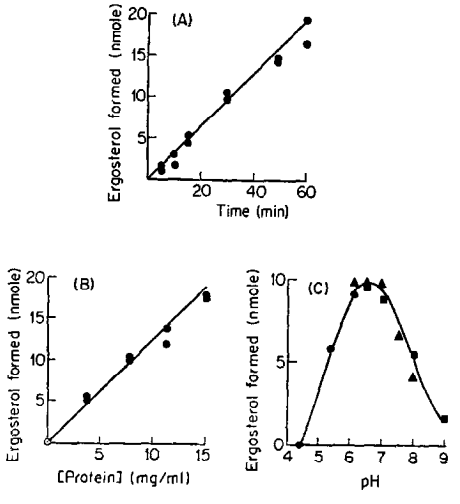


FIG. 1. Relationship between amount of ergosterol formed and (A), time of incubation; (B), concentration of protein, and (C), pH of assay medium.

The activity of the enzyme towards other 24-methylene sterols of yeast was investigated (Table 5). No, or very little, incorporation could be detected when [24-^{3}H]24(25)-dihydro-24-methylenelanosterol (**6**), [2,4-^{3}H]episterol (**4**) or [2,4-^{3}H]fecosterol (**5**) was

TABLE 5

SUBSTRATE SPECIFICITY OF ERGOSTATETRAENOL REDUCTASE

Substrate	Activity (nmoles of 24-methylsterol formed/ hr/mg of protein)
[^{3}H]ergostatetraenol (**1**)	2.53
[28-^{14}C]ergosta-7,22,24(28)-trien-3β-ol (**3**)	0.04
[2,4-^{3}H]episterol (**4**)	<0.03
[2,4-^{3}H]fecosterol (**5**)	<0.03
[24-^{3}H]24,25-dihydro-24-methylene lanosterol (**6**)	0

The various radioactively labeled 24-methylene sterols (100 nmol) were incubated for 1 hr with resuspended microsomal fraction (7.2 mg of protein) in the standard assay mixture containing glucose (5 μmoles) plus glucose oxidase (20 units). After saponification, extraction and addition of the appropriate 24-methyl sterol carrier (1 mg), the sterols, as trimethylsilyl ethers, were worked up as described in the Experimental section. Incorporation into the 24-methyl sterols was estimated by taking the appropriate area of the tlc plate and counting in the usual way.

substituted for ergostatetraenol in the usual anaerobic incubation mixture. Some incorporation into ergosta-7,22-dien-3β-ol occurred when [28-^{14}C]ergosta-7,22,24(28)-trien-3β-ol (**3**) was substituted, but the rate of transformation was only 1.5% of that for ergostatetraenol. It is unlikely that the failure to detect incorporation into the corresponding 24-methyl compounds is due to their further transformation since similar results were obtained when a microsomal preparation of *pol/pol* 2/5 (*11*) was used. In addition, incubations of a microsomal preparation with ergosta-7,22-dien-3β-ol ergosta-7-en-3β-ol and ergosta-7,22,24(28)-trien-3β-ol (**3**) indicated that no, or very little, 5(6)-dehydrogenation or 22,(23)-dehydrogenation occurred. The ergostatetraenol reductase therefore exhibits a marked specificity *in vitro* which has not been indicated by previous *in vivo* studies (*1*, *2*, *11*).

The 24-methyl sterols found in wild-type strains and *pol* mutants may be the products of minor reactions and intermediates on pathways of low flux. Some evidence indicating an inefficient reduction of episterol and fecosterol to 24-methyl sterols in whole cells has been provided by *pol/pol* mutants. Thus fecosterol was the only major sterol isolated from *pol/pol* 2/5 and episterol and fecosterol were found in far greater abundance than

TABLE 6

REDUCTION OF 24-METHYLENESTEROLS BY WHOLE-CELL CULTURES OF *pol/pol* MUTANTS

Experiment number	Mutant used	Enzymes lacking	24-Methylene sterol fed	24-Methyl-sterol into which incorpor-ation was estimated	Percent incor-pora-tion
1	*pol/pol* 2/5	$\Delta^8 \rightarrow \Delta^7$ isomerase	[2,4^{3}H$_2$]fecosterol (**5**)	Ergost-8-en-3β-ol	3.5
		22(23)-dehydrogenase	[^{3}H]ergostatetraenol (**1**)	Ergosterol	48.6
2	*pol/pol* 3/5	5(6)-dehydrogenase	[2,4-^{3}H$_2$]episterol (**4**)	Ergost-7-en-3β-ol	2.7
		22(23)-dehydrogenase	[^{3}H]ergostatetraenol (**1**)	Ergosterol	37.8
3	*pol/pol* 3/5	5(6)-dehydrogenase	[28-^{4}C]ergosta-7,22,24(28)-trien-3β-ol (**3**)	Ergosta-7,22-dien-3β-ol	1.61
		22(23)-dehydrogenase	[^{3}H]ergostatetraenol (**1**)	Ergosterol	26.90

ergost-7-en-3β-ol in *pol/pol* 3/5 although these mutants should possess $\Delta^{24(28)}$-sterol reductase activity. To clarify these conflicting results the ability of whole-cell cultures to reduce the 24-methylene group of various sterols was studied using *pol/pol* mutants. By choice of an appropriate double mutant for each substrate it was possible to obtain comparative data on 24-methylene sterol and ergostatetraenol reduction without the complication of concurrent competitive reactions. The results are given in Table 6. [2,4-^{3}H]fecosterol (**5**) was reduced to ergost-8-en-3β-ol by *pol/pol* 2/5, [2,4-^{3}H]episterol (**4**) was reduced to ergost-7-en-3β-ol and [28-^{14}C]ergosta-7,22,24(28)-trien-3β-ol (**3**) to ergosta-7,22-dien-3β-ol by *pol/pol* 3/5. In each case the amount transformed was very much less than the ergosterol formed from [^{3}H]ergostatetraenol fed in parallel

experiments. The results are in general agreement with those from similar experiments with wild-type strains (*3*, *4*) and confirm that although *pol/pol* mutants have the ability to reduce ergostatetraenol efficiently other 24-methylene sterols are reduced much less readily. In comparative feeding experiments with whole cells the degree of incorporation is not a true reflection of the relative specificities of biosynthetic enzymes and other factors must be rate limiting. These experiments do not eliminate the possibility that *in vivo* more than one enzyme species of differing substrate specificity is involved although the characterization of *pol* mutants has shown that single-gene products catalyse the other terminal steps of the pathway (*2*).

In contrast to previous studies with whole cells which have indicated the possibiliy of multiple pathways of ergosterol biosynthesis in *S. cerevisiae* (*2*, *4*) the strict specificity of ergostatetraenol reductase provides evidence for a major pathway. In addition ergostatetraenol is implicated as the only important immediate precursor of ergosterol. One remaining 24-methylene sterol of yeast ergosta-5,7,24(28)-trien-3β-ol remains to be tested as a substrate of the enzyme.

A recently proposed major pathway of ergosterol biosynthesis based on the sterol (content) of polyene resistant cultures of *S. cerevisiae* omitted ergostatetraenol (*12*). However, the possibility that ergostatetraenol is not sufficiently different in structure from ergosterol to allow polyene resistance was ignored (*2*). In addition a consideration of the specificity shown by ergostatetraenol reductase in the cell-free systems here and our studies of the sterol content of polyene resistant mutants (*2*, *11*) indicate that few assumptions can be made about major pathways of biosynthesis from the sterol content of such mutants.

EXPERIMENTAL

General procedures were as reported previously (*3*). Scintillation counting was performed using 15 ml of toluene containing 2,5-diphenyloxazole (5 g/l) and 1,4-di-[2-(5-phenyloxazolyl)]-benzene (0.2 g/l). When necessary quench corrections were made by the external standard method. Tlc was carried out on silica gel GF 254 or on alumina GF 254, as stated. Silica/silver nitrate and alumina/silver nitrate chromatography refers to plates impregnated with silver nitrate (10%, w/w). Plc was performed on plates 1 mm thick.

Protein estimations were made by a modification of the Lowry method (*13*) with dried bovine serum albumin as standard. All biochemical reagents were used as obtained from commercial sources. Sterols, other than those described below, were available from previous work on ergosterol biosynthesis (*1*, *2*, *4*, *11*, *14*). Ergost-8-en-3β-ol was isolated as its benzoate from *pol/pol* 2/5 (*11*). Ergost-7-en-3β-ol and ergosta-7,22-dien-3β-ol were prepared from ergosterol (*14*). Episterol and fecosterol were isolated from yeast sterol residues as their benzoates by Dr. U. M. Kempe (*1*).

24,25-Dihydro-24-methyllanosterol acetate. A solution of 24,25-dihydro-24-methylenelanosterol acetate (100 mg) in anhydrous benzene (5 ml) containing tris(triphenylphosphine)chlororhodium (50 mg) was hydrogenated at atmospheric pressure for 48 h during which one equivalent of hydrogen was absorbed. More benzene was added and the solution filtered through a column of neutral aluminia (Grade III). Evaporation

of the solvent afforded 24,25-dihydro-24-methyllanosterol acetate as a colourless crystalline solid (86 mg, 86%), mp, 126–128°C (from acetone–methanol), $[\alpha]_D^{20}$ + 54° (*c*, 0.3 in $CHCl_3$), *m/e* 484 (M^+), 469 (100%), and 409. *Anal.* Found: C, 81.79, H, 12.03; $C_{33}H_{56}O_2$ requires C, 81.75, and H, 11.65%.

Saponification with methanolic potassium hydroxide yielded the free sterol, mp, 147–148°C, $[\alpha]_D^{20}$ + 46° (*c*, 0.25 in $CHCl_3$) [mp, 140°C, $[\alpha]_D$ + 47.6°(*17*)].

Ergosta-7,22,24(28)-trien-3β-ol acetate (**3**). This was prepared according to the reported method (*17*), mp, 132–134°C (from acetone–methanol), $[\alpha]_D^{20}$ + 7.0° (*c*, 0.2 in $CHCl_3$) [mp, 133–136°C, $[\alpha]_D$ + 6.4°[18]].

[2,4-^{3}H]episterol and [2,4-^{3}H]fecosterol (with U. M. Kempe). These were prepared by base-catalysed exchange of the corresponding ketone by methods previously described (*15*) and were purified before use by tlc of the trimethylsilyl ethers on alumina/silver nitrate to specific activities: Episterol, 2.8×10^3 dps/mg; fecosterol, 2.3×10^3 dps/mg.

[24-CH_2-$^3H_2$]24(25)-dihydro-24-methylenelanosterol. Wittig reaction of 24,25-dihydro-24-oxolanosterol acetate with [^{3}H]methylenetriphenylphosphorane according to the literature method (*15*) followed by saponification gave the title compound of specific activity 1.4×10^5 dps/mg.

[^{3}H]ergostatetraenol. This was isolated from *S. cerevisiae* (strain NCYC 1236) grown in the presence of [2-^{3}H]acetic acid (25 mCi, specific activity 226 mCi/mmole) on 7 l of yeast complete medium under previously reported conditions (*2*). The [2-^{3}H]acetic acid was added to the medium with the inoculum and growth continued for 24 h. The cells were harvested and saponified and the sterol fraction extracted. Ergostatetraenol was isolated as its benzoate by plc on silica/silver nitrate by the usual methods (*2*). Reduction of the benzoate with lithium aluminium hydride (*8*) and crystallization of the product from methanol to give [^{3}H]ergostatetraenol (63 mg): mp, 108–110°C [112–114°C (*8*)]; specific activity, 8.1×10^4 dps/mg. Batches of the [^{3}H]ergostatetraenol were purified before use by tlc of the trimethylsilyl ether on alumina/silver nitrate developed with 40% benzene–light petroleum (bp, 40–60°C). Elution with benzene, hydrolysis to the free sterol in aqueous methanol, and recrystallization from methanol gave pure material.

[28-^{14}C]Ergosta-7,22,24(28)-trien-3β-ol (with B. R. Worth). This was prepared by the literature method (*2*) giving a product of specific activity 1.9×10^5 dps/mg.

Transformation of 24-methylene sterols by whole-cell cultures. Pol/pol mutants of *S. cerevisiae* (*11*, *19*) were grown anaerobically on yeast complete medium (500 ml) which was inoculated from a slope and shaken at 280 rpm under nitrogen for 60 hr at 30°C. The cells were then sedimented at 2000 *g* for 20 min and the wet-cell paste (2 g) resuspended in fresh culture medium in a 1-l conical flask. Radioactively labelled 24-methylene sterols (1 mg) were taken up in acetone (0.5 ml) and added with efficient stirring to 0.5% (w/v) Tween 80 (5 ml). The acetone was evaporated in a stream of nitrogen before the dispersion was added to the fresh culture medium together with the cell paste. The flask was shaken under aerobic conditions for 24 hr at 30°C before the cells were sedimented. The cell paste was suspended in 15% (w/v) potassium hydroxide in methanol (100 ml), the appropriate 24-methylsterol carrier (100 mg) added, and the mixture saponified by refluxing under nitrogen for 3 hr. After addition of water (200 ml), the sterol fraction was extracted into ether (3 × 100 ml) which was washed with water-

dried ($MgSO_4$) and evaporated. The 24-methylsterol was purified as its trimethylsilyl ether by tlc on alumina/silver nitrate and eluted with benzene. The trimethylsilyl ether was hydrolysed to the free sterol in aqueous methanol extracted into ether and recrystallized to constant specific activity from chloroform–methanol.

Separation and purification of sterols and their trimethylsilyl ethers. Sterol trimethylsilyl ethers, prepared as reported (*19*) were separated by tlc on alumina/silver nitrate developed in benzene–light petroleum (bp, 40–60°C) (ratios given below). The positions of the sterol bands were visualized by uv light. Where necessary the plates were first sprayed with 0.01 % (w/v) rhodamine 6G in acetone. Sterol trimethylsilyl ethers had R_f values at optimum separation: In 5% benzene–light petroleum: ergost-8-en-3β-ol, 0.73; ergost-7-en-3β-ol, 0.63; fecosterol, 0.16; episterol, 0.16; in 15% benzene–light petroleum: ergosta-7,22-dien-3β-ol, 0.81; ergosta-7,22,24(28)-trien-3β-ol, 0.29; in 25% benzene–light petroleum: ergosterol, 0.85; ergostatetraenol, 0.45; in light petroleum: 24(25)-dihydro-24-methylenelanosterol, 0.12.

Preparation and fractionation of cell-free extracts. Commercial *S. cerevisiae* in wet-cake form (500 g) (obtained from British Fermentation Products Ltd.) was suspended in 0.2 *M* Tris–HCl buffer, pH 7.5, cooled to 0–1°C and passed once through a precooled Manton–Gaulin homogenizer at a pressure of 7500 psi. The effluent was quickly cooled back to 0–1°C and centrifuged at 2000 *g* for 20 min. The supernatant, referred to as the crude extract, was centrifuged at 20 000 *g* for 20 min at 2°C and the resultant supernatant fraction further centrifuged at 105 000 *g* for 2 hr at 2°C. The 105 000 *g* sediment, referred to as the microsomal fraction, after separation from the supernatant and floating lipid layer, was resuspended in 50 m*M* Tris buffer, pH 7.5, to a protein concentration of 20 mg/ml. The various fractions were stored, frozen, at −20°C.

Assay of ergostatetraenol reductase activity. The incubation mixtures finally developed contained, in 1 ml: 200 μmoles of Tris–HCl buffer, pH 7.0; 1.5 μmoles NADPH; glycerol to a final concentration of 12.5% (v/v); 100 nmoles of [^{3}H]ergostatetraenol (specific activity, 8.1×10^4 dps/mg) added in ethanol (0.05 ml) and a suitable quantity of cellular fraction. On occasions where stated, 5 μmoles of glucose plus 2 mg of a commercial preparation of glucose oxidase (19.4 units/mg; from *Aspergillus niger*) was added to ensure anaerobic conditions. The reactants were mixed at 0–1°C, the tubes were flushed with nitrogen and stoppered and the reactions started by warming rapidly to 30°C. After the specified time at 30°C (usually 30 min) the reactions were stopped by addition of 10% (w/v) potassium hydroxide in methanol and carrier ergosterol (0.5 mg), and the mixtures were heated at 70°C for 1 hr. The cooled mixtures were extracted with 25% acetone–benzene (3×5 ml) and the combined extracts dried ($MgSO_4$) and evaporated to dryness. The residue was taken up in pyridine (1 ml) and the sterol trimethylsilyl ethers formed by treatment with hexamethyl disilazane (0.1 ml) and trimethylchlorosilane (0.05 ml) at room temperature for 15 min. The solvent was evaporated and the residue taken up in benzene which was then filtered and evaporated. The trimethylsilyl ether of ergosterol was isolated from the residue by tlc on alumina/silver nitrate, developed in 25% benzene–light petroleum (bp, 40–60°C). The layer of trimethylsilyl ether of ergosterol was scraped into scintillation vials and the activity measured. Values obtained were corrected by the values obtained from boiled-enzyme controls.

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