BIOSYNTHESIS OF STEROLS IN YEAST

by

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ABSTRACT

The conversion of lanosterol to ergosterol in the yeast Saccharomyces cerevisiae and of fecosterol to ergosta-7,22-dien-3β-ol in a nystatin resistant strain of the same organism have been investigated. Maintenance of the yeast under anaerobic conditions depleted the sterol content of the organism. A subsequent change to aerobic conditions was accompanied by rapid growth and accelerated sterol production. In the normal yeast strain the composition of the sterol fraction changed with time. Identification of the sterols participating in the gross biogenetic sequence indicated that the structural modifications proceeding from lanosterol involved initial nuclear demethylation at C-14 followed by demethylation at C-4. Alkylation at C-24 can occur before or after complete removal of the C-4 methyl groups. This was ascertained by simultaneous feeding of [25,26-^{14}C] lanosterol and [2,4-^{3}H]-zymosterol to whole yeast cells.

Synthesis of suspected intermediates (^{14}C labelled or ^{3}H labelled) possessing 24-substitution and varying unsaturation, eg. $\Delta^{5,7}$, Δ^{7}, Δ^{8}, Δ^{22}, $\Delta^{24(28)}$, was carried out. Feeding and trapping experiments led to the discovery of six previously unreported intermediates in the biosynthetic sequence. The involvement of these together with previously reported yeast sterols in several alternate pathways in latter stages of the sterol biosynthesis was investigated. The conversion of fecosterol (ergosta-8,24(28)-dien-3β-ol) to ergosterol, which involves introduction of Δ^{5}, Δ^{7} and Δ^{22} unsaturation as well as reduction of the 24-methylene group, was found to occur in

several sequences. Although the Δ^8 to Δ^7 isomerization was found to be reversible, the Δ^5 and Δ^{22} double bonds are not removed once they have been introduced. Neither is the $\Delta^{24(28)}$ methylene group reformed once it is reduced to methyl. Alternative operating sequences were interpreted as evidence for the ability of the enzymes responsible for a particular structural change to modify several closely related substrates. For example, the enzyme(s) responsible for introduction of the Δ^{22} bond must be able to accept sterol substrates containing Δ^8, Δ^7, $\Delta^{5,7}$ or $\Delta^{5,7,24(28)}$ double bonds.

An analogous investigation of the nystatin resistant mutant of Sacch. cerevisiae indicated that sterol biosynthesis in this organism involved the same gross features as in normal strains with the exception that the enzyme system(s) responsible for the introduction of the Δ^5 unsaturation is either blocked or missing. The major sterol produced by this mutant was found to be ergosta-7,22-dien-3β-ol. This sterol is the immediate precursor of ergosterol. This nystatin resistant yeast is the first Sacch. cerevisiae mutant for which an enzymatic deficiency with respect to sterol biosynthesis has been established.

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INTRODUCTION

In vivo synthesis of sterols proceeds via three well-defined phases (Fig. 1):

Phase I: Conversion of acetate (1) into isopentenyl pyrophosphate (2) via mevalonate (3). Head-to-tail condensation of three isopentenyl pyrophosphate residues yielding farnesyl-pyrophosphate (4) and finally tail-to-tail condensation of two farnesyl units to give squalene (5).

Phase II: Cyclisation of squalene via its epoxide generating lanosterol(6) and/or cycloartenol (7).

Phase III: Transformation of lanosterol or cycloartenol into cholesterol and/or phytosterols (9).

The literature related to phases I and II has been reviewed in detail in several excellent publications: the stereochemistry of the enzymatic processes leading to squalene has been investigated in detail and is reviewed by Clayton[1] and by Cornforth and Popjak[2]. Dean[3] has reviewed the enzymology and chemistry involved in squalene cyclization. Talalay[4] has reviewed the enzymology of steroid transformations. Sih and Whitlock[5] have discussed the biochemical mechanisms of steroid metabolism.

Most earlier work has been done on mammalian tissues, mainly with cholesterol biosynthesis in mind. There is little

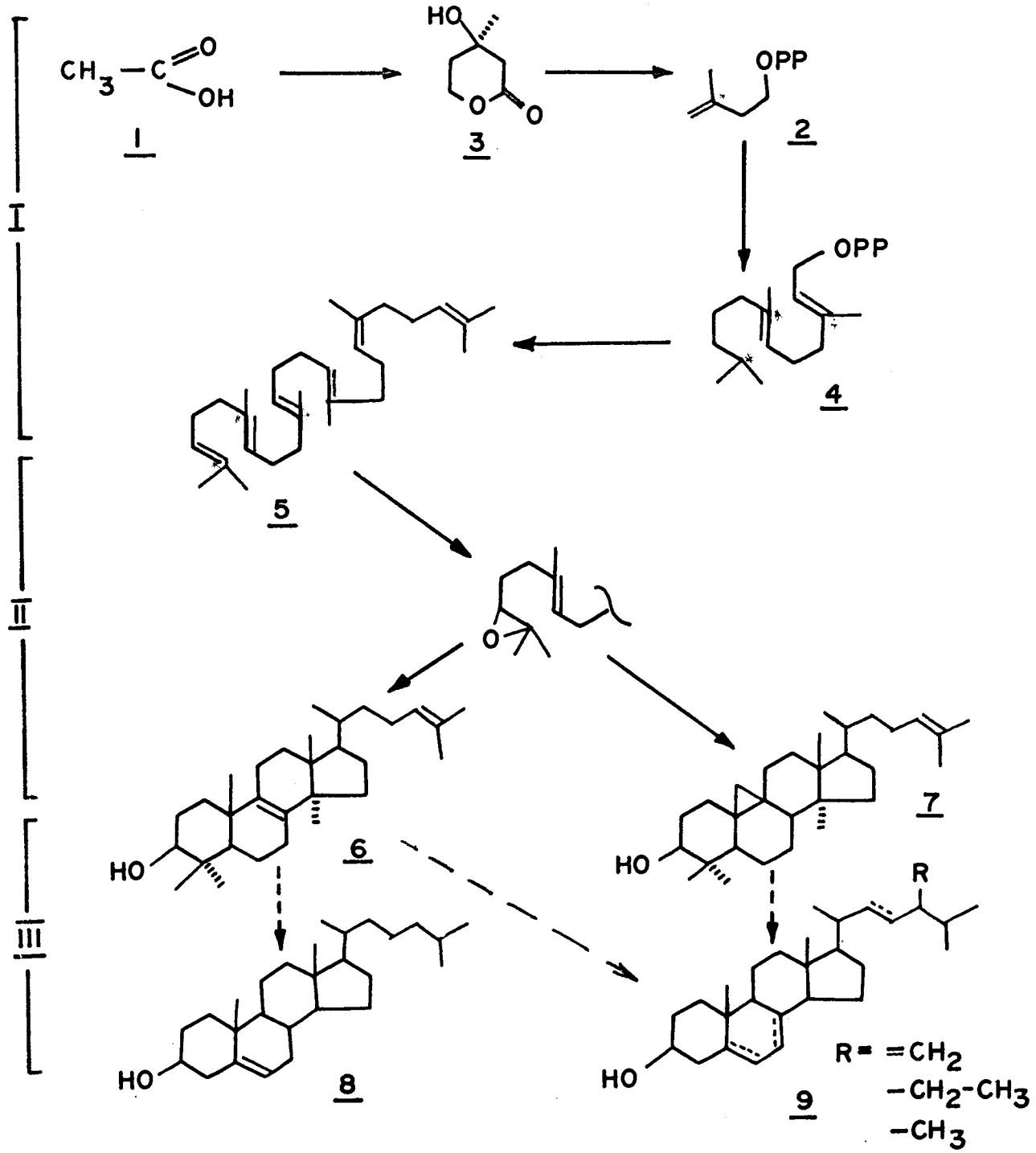


Figure 1: Three Phases of Sterol Biosynthesis

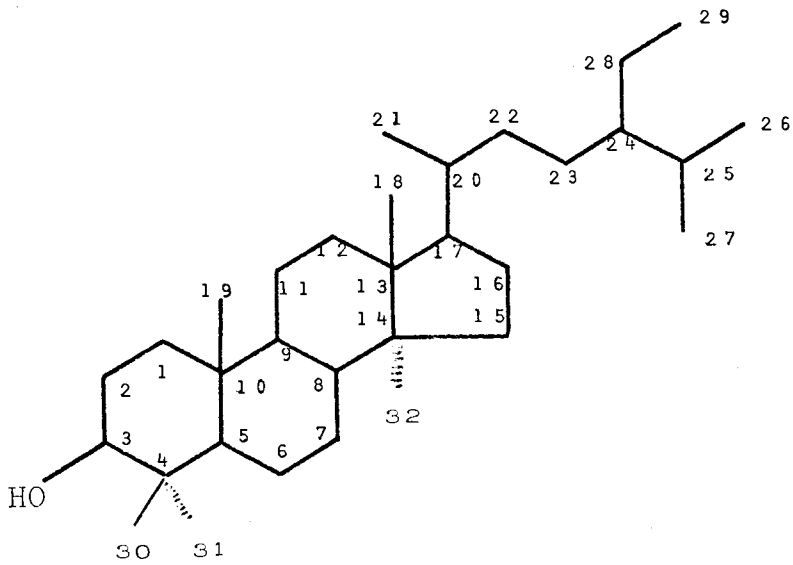


Figure 2: Numbering System of Steroids

doubt that many of the biosynthetic intermediates involved in cholesterol biosynthesis are also precursors of the plant triterpenes and sterols[6,7]. The weight of evidence[1] points to very similar sterol biosynthetic routes in plants and animals to the point of squalene oxide cyclization. The product of this cyclization in mammals is lanosterol while in plants a variety of tetracarbocycles and pentacycles may arise. Of particular interest in connection with sterol biosynthesis is the generation of cycloartenol and lanosterol in this step. Both of these can serve as precursors of phytosterols.

An immense amount of effort has gone into the study of the steps involved in the conversion of lanosterol to cholesterol in mammalian tissue. There are basically five transformations involved in this conversion:

1) Removal of the three methyl groups at C-4 and C-14;
2) Isomerization of the Δ^8 double bond to Δ^7;
3) Introduction of a double bond at Δ^5;
4) Reduction of the double bond at Δ^7; and
5) Reduction of the double bond at Δ^{24}.

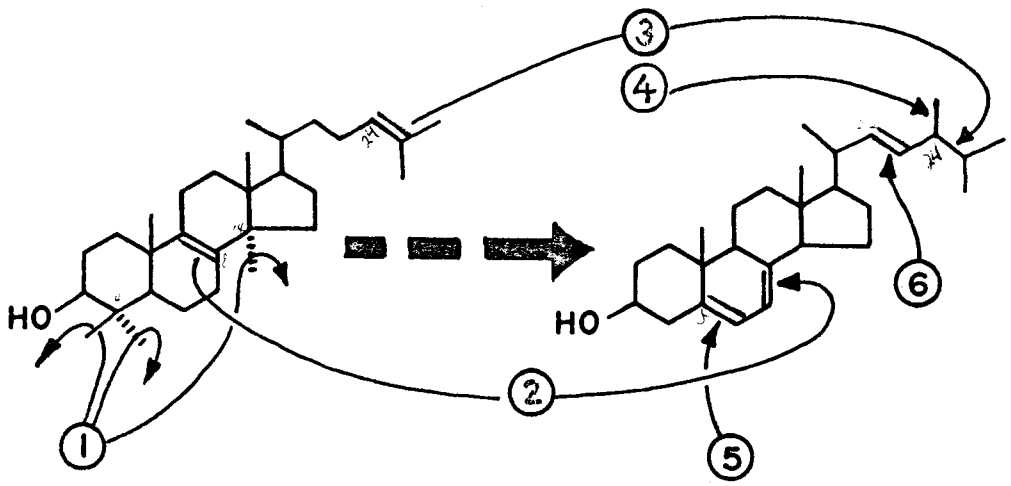
The order in which these transformations occur, largely deduced from tracer experiments, is that in which they are listed above. There is some overlap between transformations and each transformation may be a composite of several enzymatic steps. The details of this sequence are discussed in a recent review by Mulheirn and Ramm[8].

We have undertaken an investigation of the conversion of

lanosterol to ergosterol in the yeast, Saccharomyces cerevisiae. The object of the present study was to attempt to ellucidate the transformation sequence involved in this conversion.

The enzymatic conversion of lanosterol to ergosterol in Saccharomyces cerevisiae requires six general transformations:

1) Removal of the three methyl groups in lanosterol at C-4 and C-14;
2) Isomerization of the Δ^8 double bond to Δ^7;
3) Reduction of the Δ^{24} double bond;
4) Introduction of a methyl group at C-24;
5) Introduction of a Δ^5 double bond;
6) Introduction of a Δ^{22} double bond.



Each of these transformations can be a multistep enzymatic process. If one considers the individual enzymatic reactions to be mutually independent they could conceivably occur in any order. The total number of potential metabolic intermediates involved in the lanosterol to ergosterol conversion would then depend on the total number of possible combinations of enzymatic steps involved.

By analogy with cholesterol biosynthesis[8,9] some of the structural features likely to emerge in biosynthetic intermediates would arise from the following possibilities:

(1) Variation is possible in the order of removal of the methyl groups at C-4 and C-14.

(2) The side chains of intermediates could be methylated at C-24 or not.

(3) Variations in number and position of double bonds could occur eg. Δ^{5}, $\Delta^{5,7}$, Δ^{7}, Δ^{8}, $\Delta^{8,14}$, $\Delta^{7,14}$, $\Delta^{8(14)}$, Δ^{14}, Δ^{22}, Δ^{24}, $\Delta^{24(28)}$, as well as several combinations of these.

(4) Variations of the oxidation state at C-3, which can be either a 3β-alcohol or a ketone, is possible.

(5) Several oxidation states are possible during the removal of C-30, C-31 and C-32, e.g. $-CH_3$, $-CH_2OH$, -CHO, or -COOH.

(6) Additional hydroxyl groups could be present at several positions (e.g. C-5, 6 or 15) during introduction of respective double bonds.

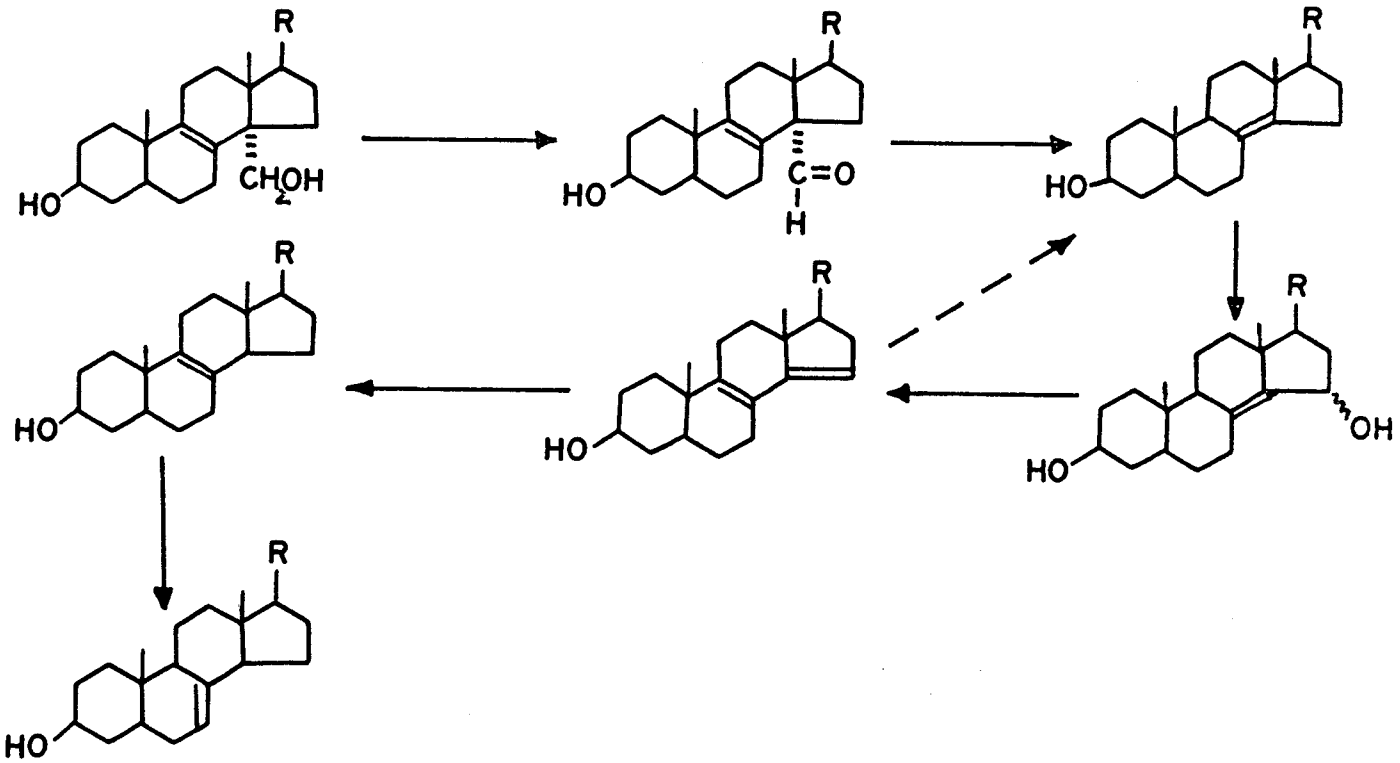
In order to simplify the overall picture and to reduce the total number of potential intermediates to a reasonable number, several reaction sequences were combined into "one-step-transformations". In addition the evidence available in the literature allowed some deductions to be made concerning the occurrence of some transformations prior to or subsequent to others. The rational for condensing some sequences into one-step transformations as well as proposing the occurrence of some transformation prior to others is outlined below:

Steps 1 - 2. The individual steps involved during the oxidative removal of the methyl groups at C-4 and C-14 are not considered as discrete steps. In the case of cholesterol biosynthesis it is considered that the C-14 methyl group is removed initially. The presence in yeast of 4,4-dimethyl zymosterol as well as the apparent absence of 4-desmethyl-14-methyl sterols suggests the 14α-methyl is lost prior to the 4 methyls in yeast also.

Alexander et al.[10] have shown that the C-14 methyl is removed in cholesterol biosynthesis as formic acid. They proposed the sequence $CH_3 \rightarrow CH_2OH \rightarrow CHO \rightarrow$formic acid. The formic acid was envisioned as being derived from the C-14 aldehyde. The loss of this group involved the concomitant introduction of a Δ^{14} double bond. Since no products other than the demethylated sterols can in all probability be expected from any one of the proposed intermediates it seems reasonable to consider such a sequence as one transformation. Schroepfer[9] has pro-

posed a pathway for metabolism of 14α-methyl-Δ^8-sterols in mammalian systems which involves formation of a $\Delta^{8(14)}$ intermediate. This is considered to be converted to an $\Delta^{8,24}$ diene and then to a 14-desmethyl-Δ^8 sterol.

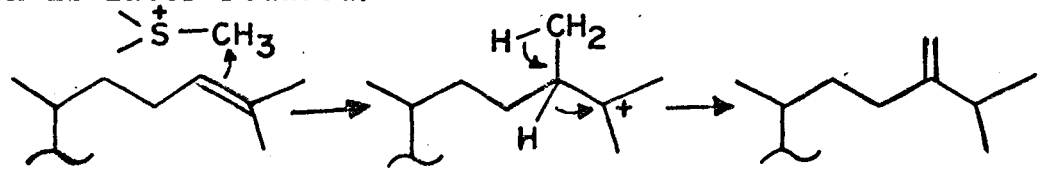
If a parallel pathway operates in yeast, it can be seen that the immediate precursor of a Δ^7 compound would be a Δ^8 compound regardless of what happens during C-14 demethylation.



Based on the above, we consider the isomerization of Δ^8 to Δ^7 as a distinct step and consider that it occurs after loss of the methyl at C-14. The C-4 methyl groups are removed via the sequence $CH_3 \rightarrow CH_2OH \rightarrow CHO \rightarrow COOH \rightarrow CO_2$. This oxidation sequence proceeds initially at the 4α position. The 4β methyl is then isomerized to the 4α position and oxidatively removed. At some time during the oxidative removal steps, the 3β-hydroxyl

is converted to a ketone. After methyl removal the 3β-hydroxyl is regenerated[9,11]. Accordingly we have considered the loss of each methyl at C-4 as a single step.

Steps 3 - 4. The reduction of the Δ^{24} double bond and introduction of the extra carbon at C-24 is considered to be one transformation. Available evidence, reviewed by Lederer[12], suggests the transfer of a methyl group from S-adenosyl methionine to C-24 followed by hydride transfer from C-24 to C-25. Loss of a proton from C-28 gives a 24-methylene intermediate which is later reduced.



Labelling studies have provided the evidence for this sequence. Thus CD_3-methionine yields CD_2-ergosterol in S. cerevisiae[13] while [^{3}H-24]-lanosterol yields [^{3}H-25]-ergosterol in this organism[14].

Gaylor et al.[15] have recently shown that the initial products formed from Δ^{24} sterols by a soluble Δ 24-sterolmethyltransferase system isolated from S. cerevisiae were the corresponding 24-methylene sterols. Hence we consider the initial formation of a 24-methylene derivatives a single step and the reduction of this 24(28) unsaturation is a subsequent and independent step.

Step 5. Although there is some evidence[16,17] to indicate the introduction of the Δ^5 double bond involves prior 5α-hydroxylation and subsequent dehydration, we have considered

this process as a single step. Since no metabolites are present in yeast that possess Δ^5 without Δ^7 unsaturation (Table I), we consider it highly likely that Δ^7 unsaturation is introduced prior to Δ^5 unsaturation.

Step 6. Nothing is known of the single versus multistep nature of the introduction of the Δ^{22} double bond, hence it is considered as a single step.

Once the restrictions outlined above were taken into account we turned to a consideration of the studies of ergosterol biosynthesis in Saccharomyces cerevisiae. Tables I and IIoutline the literature reporting the presence of a number of possible sterol precursors of ergosterol in yeast and their conversion to ergosterol.

The investigations recorded in these tables involved, in most cases, readily available sterols, isolated either from ergosterol mother liquors or obtained from other sources. Only ergosterol was usually recovered. The experiments were carried out with several systems under the different conditions indicated in Tables I and II. The significance of the individual experiments, as far as they are relevant to this work, will be discussed later.

The work cited above was done exclusively with normal strains of Saccharomyces cerevisae. Recently, mutants showing differences in their sterol metabolism have become available. Woods[8] and Ahmed[46,47] isolated various mutants of yeast which are resistant to nystatin, a polyene antibiotic.

Polyene antibiotics have strong fungicidal properties, they are, however, not bacteriostatic. It is now generally accepted that polyene antibiotics act at the level of the cell membranes so that the latter no longer function as selective barriers. In addition it seems that the presence of sterols in natural membranes is a necessary prerequisite for polyene sensitivity (for reviews on the subject see Kinsky[48,49] and Dekker[50]).

Woods[51] found that mutants, resistant to nystatin, differ from nystatin sensitive strains in their sterol content. Parks[37], working with a nystatin resistant mutant, found that this yeast did not contain ergosterol but accumulated a possible precursor of ergosterol which he determined to be ergosta-8,22-dien-3β-ol, 11. This particular compound had not previously been isolated from yeast suggesting that this mutant was lacking the enzymatic system needed for the transformation of this sterol to ergosterol. Since that sterol was efficiently transformed to ergosterol in normal yeast, it seemed that an ergosterol-precusor, usually not detectable, had accumulated.

Bard[52] investigated several phenotypically distinct sets of nystatin-resistant mutants and found that the different mutants varied in sterol content, depending on the degree of resistance. It seemed that for the first time mutants were available which accumulated sterols which were potential intermediates in ergosterol biosynthesis. One mutant designated nys-3 was, therefore, investigated with respect

TABLE I

STEROLS EITHER ISOLATED OR TRANSFORMED IN YEAST

Sterol	Isolation From Yeast	Converted to Ergosterol or to a Precursor of Ergosterol	System and Conditions					Remarks
			O_2	N_2	C	H	E	
4,4,14α-trimethyl-cholesta-8,24 dien -3β-ol (lanosterol)	-H. Wieland et al. 1937[18]	-Schwenk and Alexander 1958[19]	x			x		
	e.g. Ponsinet and Ourisson 1965[20]	-Akhtar et al. 1966[21]			x			
		-Akhtar et al. 1967[14]			x			
		-Akhtar et al. 1968[22]						
		-Akhtar et al. 1969[23]		x	x			
4,4-14α-trimethyl-cholesta-8,24(28) dien -3β-ol (24-methylene-lanosterol)	--	-Barton et al. 1966[24]			x			
		-Akhtar et al. 1966[25]			x			
		-Akhtar et al. 1969[23]	x	x	x			O_2 = aerobic conditions
								N_2 = anaerobic conditions
4-4-dimethyl-cholesta-8,24-dien -3β-ol (14-desmethyl-lanosterol)	Ponsinet and Ourisson 1965[20]	--						C = whole cells
								H = cell homogenate
								E = enzyme

TABLE I (continued)

Sterol	Isolation From Yeast	Converted to Ergosterol or to a Precursor of Ergosterol	System and Conditions					Remarks
			O_2	N_2	C	H	E	
4α,14α-Dimethyl-cholesta-8,24(28)-dien -3β-ol (Obtusifoliol)	--	Barton et al. 1970[26]	x		x			Plants
4α-Methyl-zymosterol	-Barton et al. 1968[27] -Barton et al. 1970[26] -Ponsinet and Ourisson 1965[20]	-Barton et al. 1970[26]	x		x			
4α-Methyl-ergosta-8,24(28)-dien -3β-ol	-Barton et al. -Barton et al. 1970[26]	-Barton et al. 1970[26]	x		x			
4α-Methyl-cholesta-8(14),24-dien -3β-ol	-Barton et al. 1970[26]	--	x		x			Artefact? Barton et al. 1970[26]
Cholesta-8,24-dien -3β-ol (zymosterol)	-Smedley-McLean 1929[28]	-Schwenk et al. 1958[19]	x				x	No incorporation
		-Katsuki et al. 1966[29]	x					No incorporation under anaerob. cond.

TABLE I (continued)

Sterol	Isolation From Yeast	Converted to Ergosterol or to a Precursor of Ergosterol	System and Conditions					Remarks
			O_2	N_2	C	H	E	
Ergosta-8,14-dien -3β-ol		-Akhtar et al. 1969[38]		x				
Ergosta-7,22-dien -3β-ol	-Callow 1931[30] -Wieland et al. 1941[31]	-Akhtar et al. 1968[32]	x	x		x		No conversion
Ergosta-7-en-3β-ol	--	-Akhtar et al. 1969[23]			x			Isolated from Candida utilis Morimoto et al. 1967[34]
Ergosta-8,23-dien -3β-ol (Ascosterol)	-Furst 1966[33]							
Ergosta-8,24(28)-dien -3β-ol (Fecosterol)	-Wieland et al. 1941[31]	--						

TABLE I (continued)

Sterol	Isolation From Yeast	Converted to Ergosterol or to a Precursor of Ergosterol	System and Conditions					Remarks
			O_2	N_2	C	H	E	
Ergosta-7,24(28)-dien -3β-ol (Episterol)	-Wieland et al. 1941[35] -Barton et al. 1948[36]							
Ergosta,8,22-dien -3β-ol	-Parks et al. 1972[37]	-Parks et al. 1972[37]			x			Isolated from a Yeast Mutant
Ergosta-5,7,22,24(28)-tetraen -3β-ol	-Breivik et al. 1954[39] -Petzold et al. 1957[40] -Lampen et al 1962[41] -Longley et al. 1968[42] -Fryberg et al. 1971[43]	-Katsuki et al. 1957[29]	x		x			-Structure of Isolated Sterol Tentative -No. Incorp. Under Anaerobic Cond.
		-Barton et al. 1971[44]	x	x	x			Ergosterol was not Transformed to the Tetraene
Ergosta-7,22-dien -3β,5α-diol	--	-Akhtar et al. 1968[32]	x	x	x	x		No Conversion
4,4-Dimethyl-ergosterol	--	-Barton et al. 1970[26]						No Incorporation into Ergosterol

TABLE II

TRANSFORMATIONS USING ENZYME SYSTEMS ISOLATED FROM YEAST

Substrate	Product	Enzyme	Remarks	Reference
Zymosterol	Fecosterol	Δ^{24}-Sterol methyl transferase purified 600 fold		Moore and Gaylor 1969[53]
	No product	Same enzyme as above		Moore and Gaylor 1970[15]
4,4-Dimethylzymosterol	2% Methylation product		Transformation determined by the amount of ^{14}C incorporated from ^{14}C-methionine products were not characterized.	
4α-Methylzymosterol	5% Methylation product			
Zymosterol	100% Methylation product			
Ergosta-7,22-dien-3β-5α-diol	Ergosterol	Partially purified homogenate		Topham and Gaylor 1967[16]
Ergosta-7,22-dien-3β,5α-diol	Ergosterol	5α-Hydroxysterol dehydrogenase		Topham and Gaylor 1972[17]

TABLE II (continued)

Substrate	Product	Enzyme	Remarks	Reference
5α,8α-epi-Dioxy ergosta-6,22 dien -3β-ol	Ergosterol	5α-Hydroxy-sterol dehydrogenase		
Lanosterol	Demethylated C_{27}-sterol	Sterol demethylase	Δ^7-Sterols are not demethylated	Moore and Gaylor 1967[45]

to sterol content as well as with regard to operative sterol biosynthetic pathways.

Taking into account the restrictions discussed above and the sterol metabolites previously formed in Saccharomyces cerevisiae, the network shown in Figure 3* can be constructed. It represents the most likely potential pathways for the transformation of lanosterol to ergosterol.

(*Figure 3: See fold-out at the end of the theses.)

THE PROBLEM

As is obvious from Fig. 3, a multitude of potential pathways exist whereby lanosterol can be converted to ergosterol in yeast. The problem of prime importance is to distinguish between the operation of a unique pathway and the operation of competing alternative pathways. Assuming that several pathways are in fact operative, the determination of their relative dominance under varying conditions is important. Information available from the literature allowed the construction of the scheme as shown in Fig. 4. This scheme is identical in construction to the one shown in Fig. 3 with the exception that only those compounds are shown which had been isolated from Saccharomyces cerevisiae prior to this work. The scheme described in Fig. 3 subsequently served as a working model.

The general procedure used to establish an overall sequence of transformations was to determine the precursor-product relationship of the individual members of the sequence (Figs. 3 and

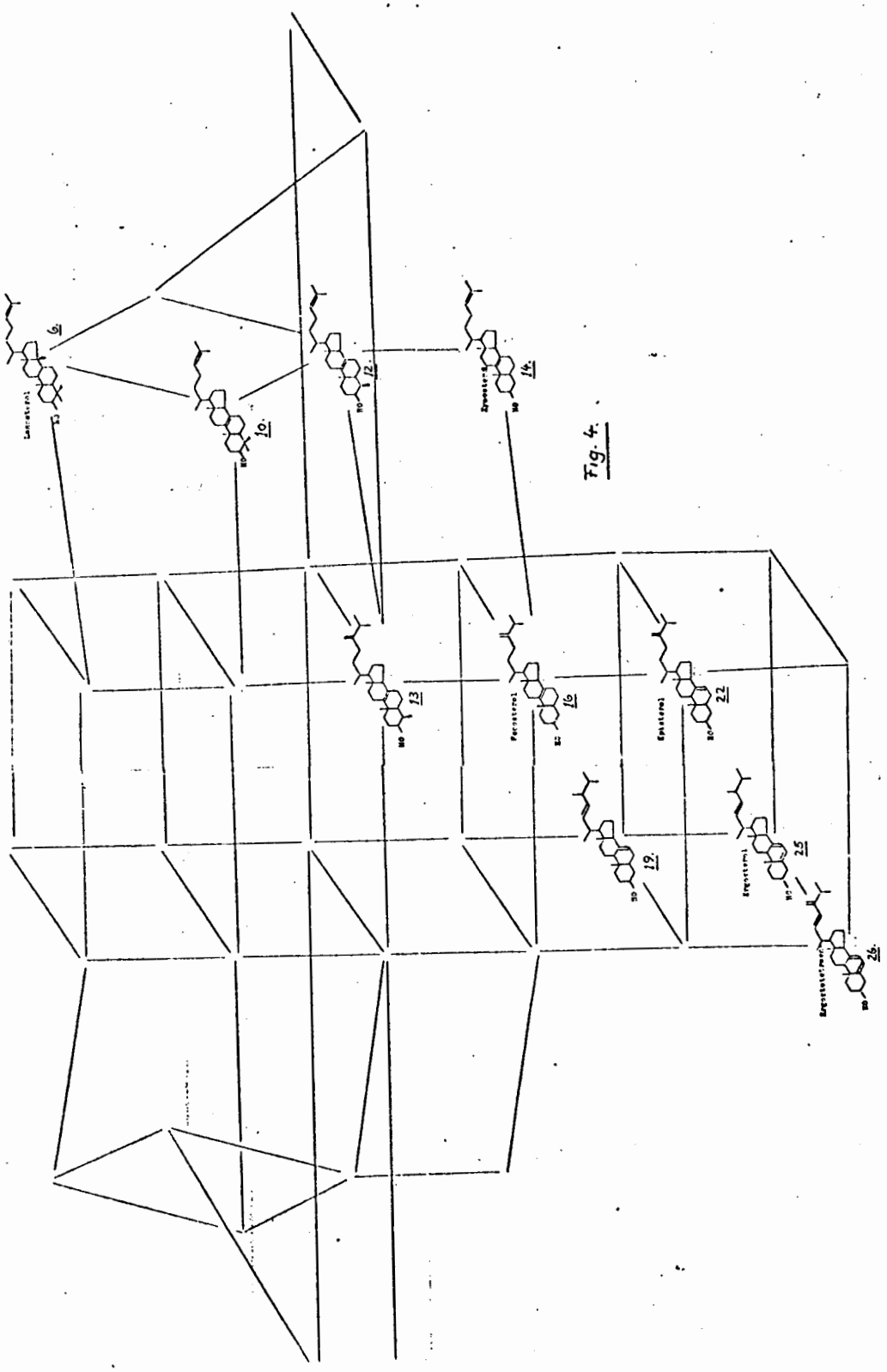


Fig. 4.

4). The proof of involvement of individual intermediates in the biogenesis of ergosterol was considered to be established when all of the following criteria were met:

(1) The presence of the intermediate in yeast must be established and its identity must be confirmed.

(2) The intermediate must be converted to ergosterol by living yeast.

(3) The intermediate must be isolated in labelled form from incubation experiments by trapping.

Although these criteria satisfactorily identify metabolic intermediates, they do not establish the exact position of a particular sterol in a proposed metabolic pathway. This information is only obtained by investigation of four additional aspects:

(1) The compound must be observed to be formed from its proposed immediate precursor.

(2) It must be converted to the sterol or sterols suspected to be its direct transformation product(s).

(3) The possibility that individual steps are reversible must be considered.

(4) The possibility of a compound being a branchpoint of two or more pathways must be considered.

The general methods available for obtaining the required intermediates, as well as information concerning the sequences in which they are involved, are given below:

(1) A thorough search of the yeast Saccharomyces cerevisiae

should yield the major sterol intermediates if their steady state concentrations are high enough to allow detection.

(2) If the system responds to environmental changes one might be able to find optimal conditions which increase the steady state concentrations of intermediates normally present in too low a concentration to allow isolation.

(3) Suspected intermediates can be either isolated from sources where they are more abundant or they can be synthesized chemically. Tracer experiments can then be carried out using these compounds for trapping labelled intermediates of low concentration.

(4) Mutants possessing distinct enzymatic blocks which give rise to accumulation of a particular intermediate can be utilized.

(5) The enzymes, catalysing particular transformations, can be isolated and tested with suspected substrates.

(6) Metabolic inhibitors, blocking specific steps in the proposed sequence are available, and can in some cases be used to cause accumulation of intermediates. These compounds can then be isolated, identified and their positions in the pathway assessed.

The solution of the problem on hand was approached in the following way. The first phase involved the chemical synthesis of the greater part of the desmethyl sterols shown in the lower

part of the scheme in Fig. 3. The second phase consisted of a thorough search of the total sterol fraction of yeast for potential intermediates. In a third phase the synthetic and the naturally occurring sterols were investigated with respect to their involvement in the biosynthesis of ergosterol. Biosynthetic pathways were investigated in normal as well as in a mutant strain of S. cerevisiae using radioactive tracers.

CHEMICAL SYNTHESIS OF INTERMEDIATES

Comparison of Fig. 3 and Fig. 4 reveals that at the outset of this study relatively few of the intermediates which could conceivably be involved in the conversion of lanosterol to ergosterol had been detected in yeast. In order to elaborate the possible biointerconversions in the circumscribed portion of Fig. 3 we decided to synthesize the suspected intermediates. This approach had the following advantages over a detailed search for these compounds in yeast cultures:

(1) Crude analyses of yeast sterol mixtures revealed that most biosynthetic intermediates were present in rather low concentration. Since appreciable quantities (< 100 mg) were required of each intermediate to be tested in several trapping experiments, synthesis had the obvious advantage of supplying the quantities necessary.

(2) An initial synthesis would allow an unambiguous structure assignment of new, and previously known inter-

mediates which were subsequently isolated and were shown to have identical physical properties.

(3) Acquisition of a compound by synthesis would permit characterization of its chromatographic properties. This would subsequently facilitate its detection and isolation in the event it turned out to be a natural yeast metabolite.

(4) Relatively direct synthetic procedures had to be devised in any event for the preparation of radioactive compounds later needed in tracer experiments.

As can be seen from Fig. 3 the required intermediate possess Δ^8, Δ^7 and $\Delta^{5,7}$ ring systems with varying structures and degrees of unsaturation in the side chain. Table 3 lists the sterol intermediates synthesized or isolated in labelled and unlabelled form during this work.

The majority of the necessary sterols possessing a Δ^8 double bond were synthesized starting from readily available ergosta-7,22-diene acetate (27) as follows (Fig. 5):* Treatment of 27 with mercuric acetate in dioxane[54] gave 7,9(11), 22-ergostatrien acetate, 28. The crude triene was epoxidized with m-chloro-perbenzoic acid in $CHCl_3$ to give 3β-acetoxy-9,11α-epoxy-ergosta-7,22-diene (29). Rearrangement of the epoxide with ferric chloride[56] gave 3β-acetoxy-11-keto-ergosta-8,22-diene (30). Reduction with $LiAlH_4$ in refluxing ether

* The experimental procedures were identical to those reported in the referred literature and will therefore not be described further. All compounds had the expected n. m. r. spectra and the reported melting points.

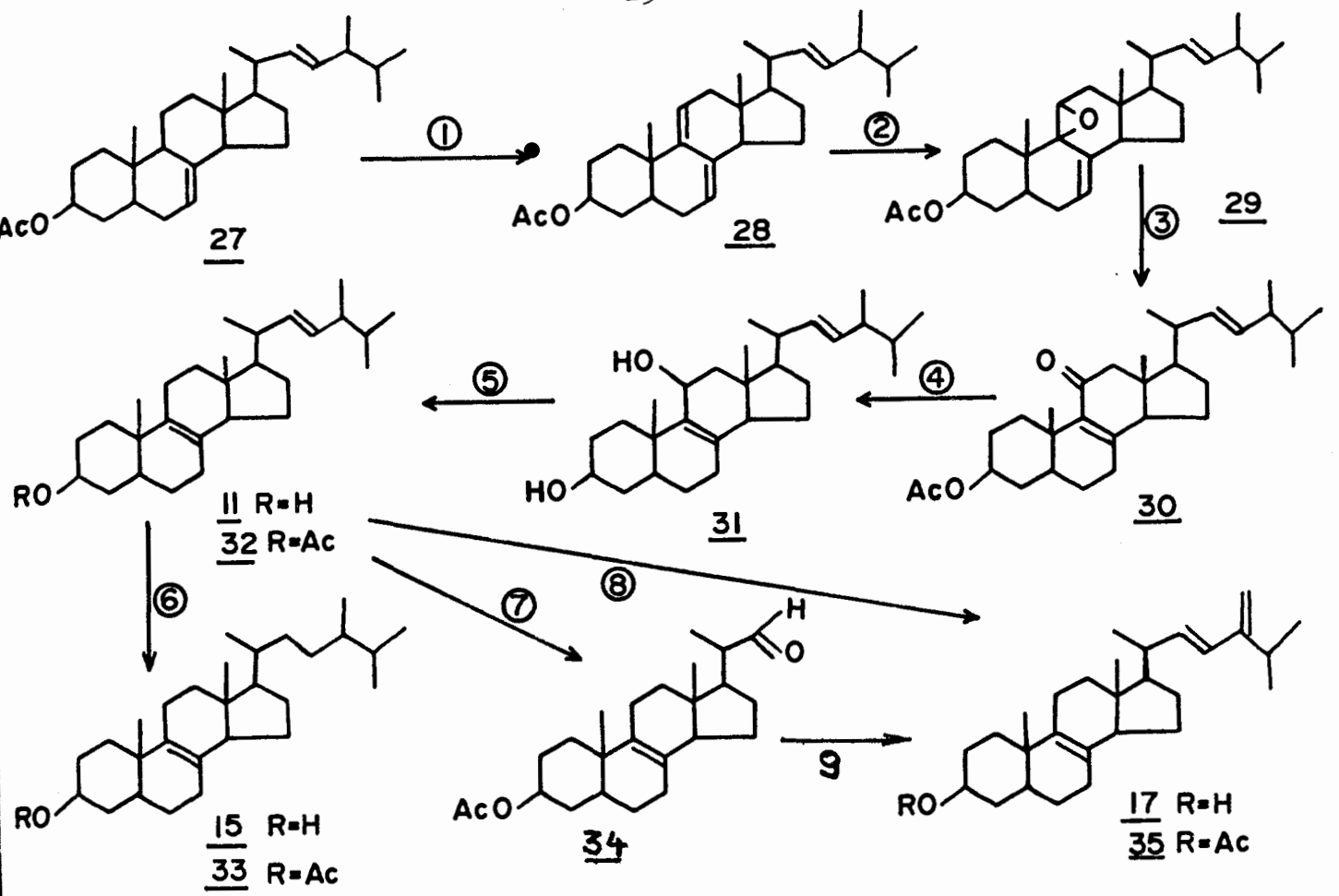


Figure 5: Synthesis of Δ^8-sterol Intermediates.

(1) Mercuric acetate. Saucy et al.[54]
(2) m-chloro perbenzoic acid. Heusser et al.[55]
(3) Ferric chloride. Heusser et al. [57]
(4) $LiAlH_4$. Hallsworth et al.[57]
(5) Li/ethylamine[57]
(6) Pd/C[57]
(7), (8), (9) see experimental.

gave ergosta-8,22-diene-3β, 11β-diol (31). The diol was finally treated in ethylamine with an excess of lithium[57] to give the diene (11). Hydrogenation of the corresponding acetate[57](32) gave 3β-acetoxy-ergost-8-ene (33). Ozonolysis of 32 gave 34 which was converted to 35 by a Wittig reaction[43]. An attempted conversion[58] of 32 to 35 by bromination-dehydrobromination was unsuccessful.

Fecosterol (16) was isolated from yeast sterol mother liquors as described in a succeeding section. Labelled fecosterol was synthesized according to the scheme outlined in Fig. 6.

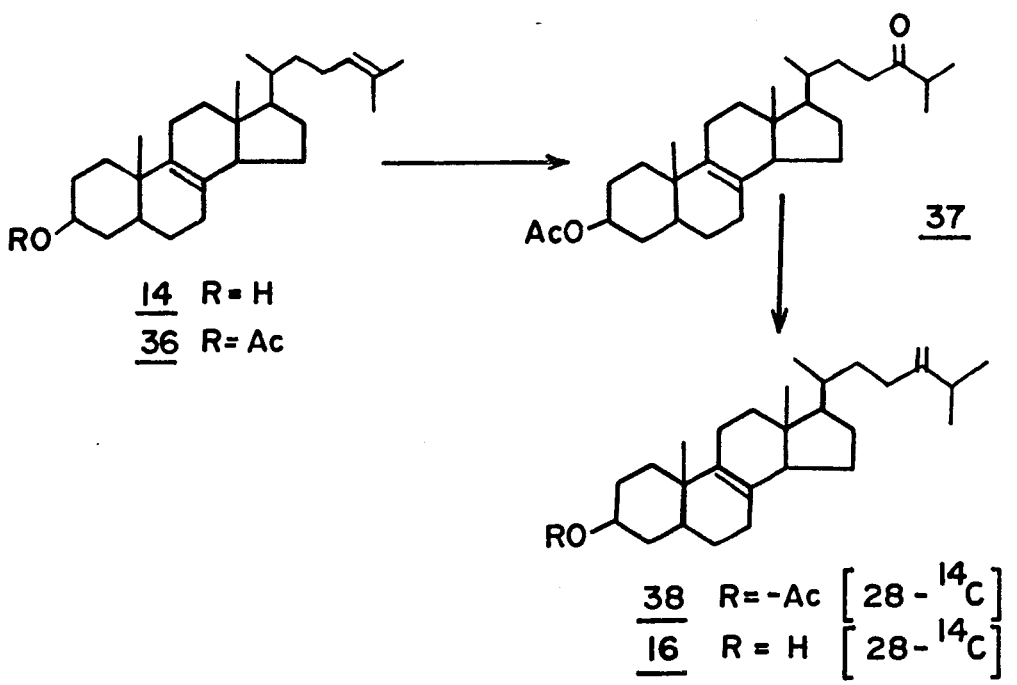


Figure 6: Synthesis of Fecosterol

Preliminary experiments[43] had been executed prior to this work which resulted in the development of an efficient procedure for synthesis of Δ^7 and $\Delta^{5,7}$ sterols with various side chain structures. The sequence used for synthesis of most of the Δ^7 intermediates is illustrated in Fig. 7. The synthesis leading to 56 is relatively direct, the only noteable feature being the observation that C-20 retained its configuration in the conversion of 55 to the aldehyde (56).

Two methods for the reconstruction of the desired new sidechains were available:

(a) the entire $\Delta^{22,24(28)}$ side chain (21) could be introduced in one step by a Wittig reaction using 2-methylene-3-methyl-butan-1-triphenylphosphorane

or

(b) a Δ^{22}-24-keto side chain (38) could be constructed in an initial step by condensation of the aldehyde with 3-methylbutan -2-on -1-triphenylphosphorane. In a second reaction, the 24-methylene group could then be introduced (39 → 40 or 38 → 41) by a conventional Wittig reaction using methyltriphenylphosphorane.

Since [^{14}C]-methyl iodide is readily available and the introduction of radio label into the desired compound is desirable as late as possible in a reaction sequence, method (b) was generally preferred.

The Δ^{22} double bond of the Δ^{22}-24-keto-side chain can readily be reduced (10% Pd/$BaSO_4$) without attack of nuclear

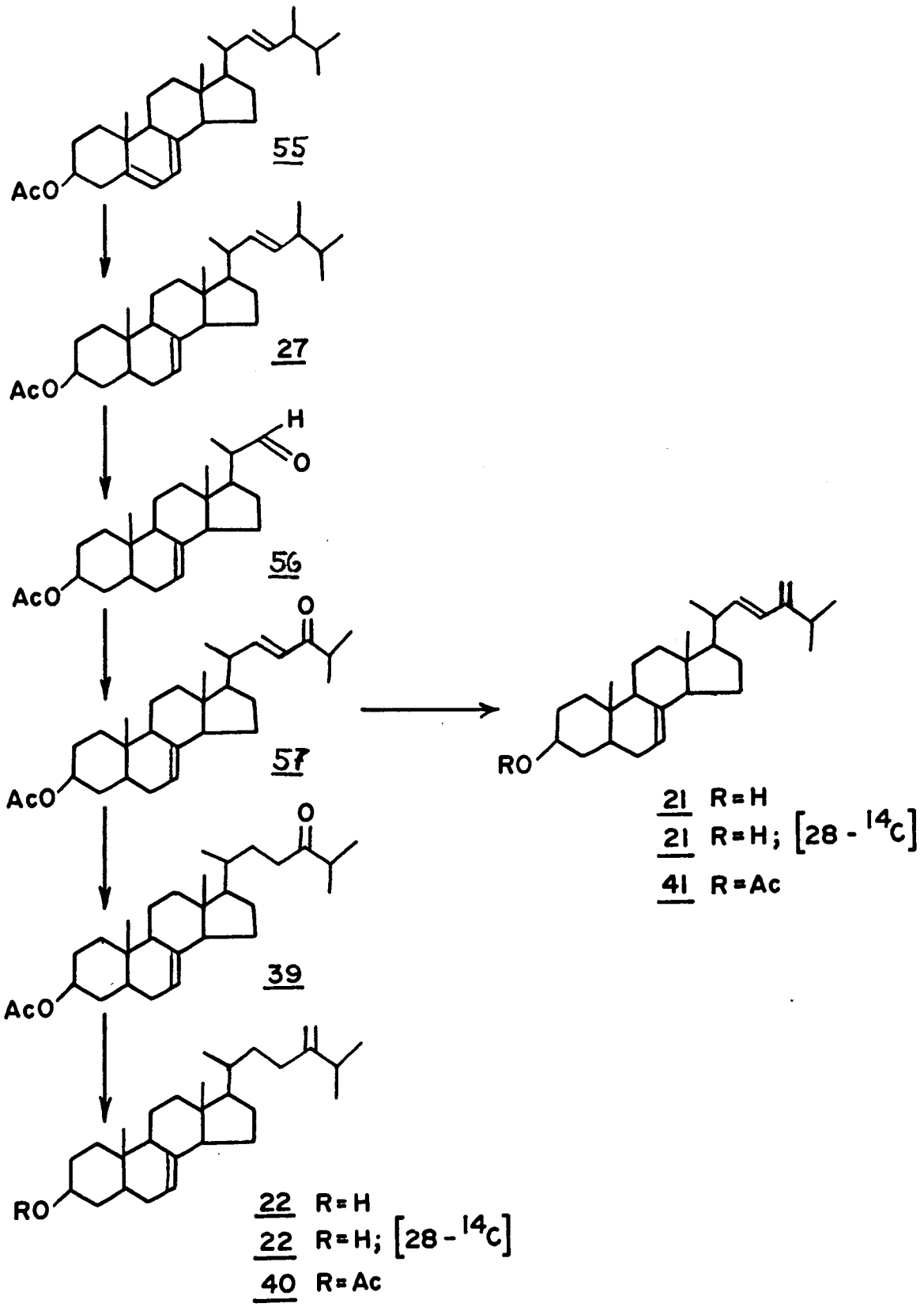


Figure 7: Synthesis of Δ^7-sterol Intermediates.

double bonds (57 → 39) at either the 5 or 7 position.

Reduction of ergosterol acetate (55) with Raney Ni gave initially 27 which on further reduction gave 42. Hydrolysis of 42 gave 20 which upon oxidation with Jones reagent gave 43. Base catalyzed exchange of 43 in ^3H_2O followed by borohydride reduction gave tritiated 20 (Fig. 8).

The $\Delta^{5,7}$ sterol intermediates were prepared by several routes. The $\Delta^{5,7,22,24(28)}$tetraen -3β-ol (26) was prepared in labelled and unlabelled form as described earlier[43]. Labelled ergosterol was obtained from yeast cultures grown on [U-C^{14}]-acetate. The $\Delta^{5,7}$-dien -3β-ol (24) was synthesized from labelled and unlabelled ergosterol via reduction of the ergosterol acetate-maleic anhydride adduct as described in the literature[63]. The $\Delta^{5,7,24(28)}$-trien -3β-ol (23) was synthesized in labelled and unlabelled form according to the scheme outlined in Fig. 9 (45 → 23).

In addition to the sterols described above, labelled lanosterol and zymosterol were synthesized. Lanosterol was synthesized with ^{14}C in C-26 and C-27 according to the procedure of Akhtar et al.[14]. Zymosterol was labelled with ^3H at C-2 and C-4 by base catalyzed exchange of zymosterone in ^3H_2O followed by reduction of the carbonyl function to the alcohol.

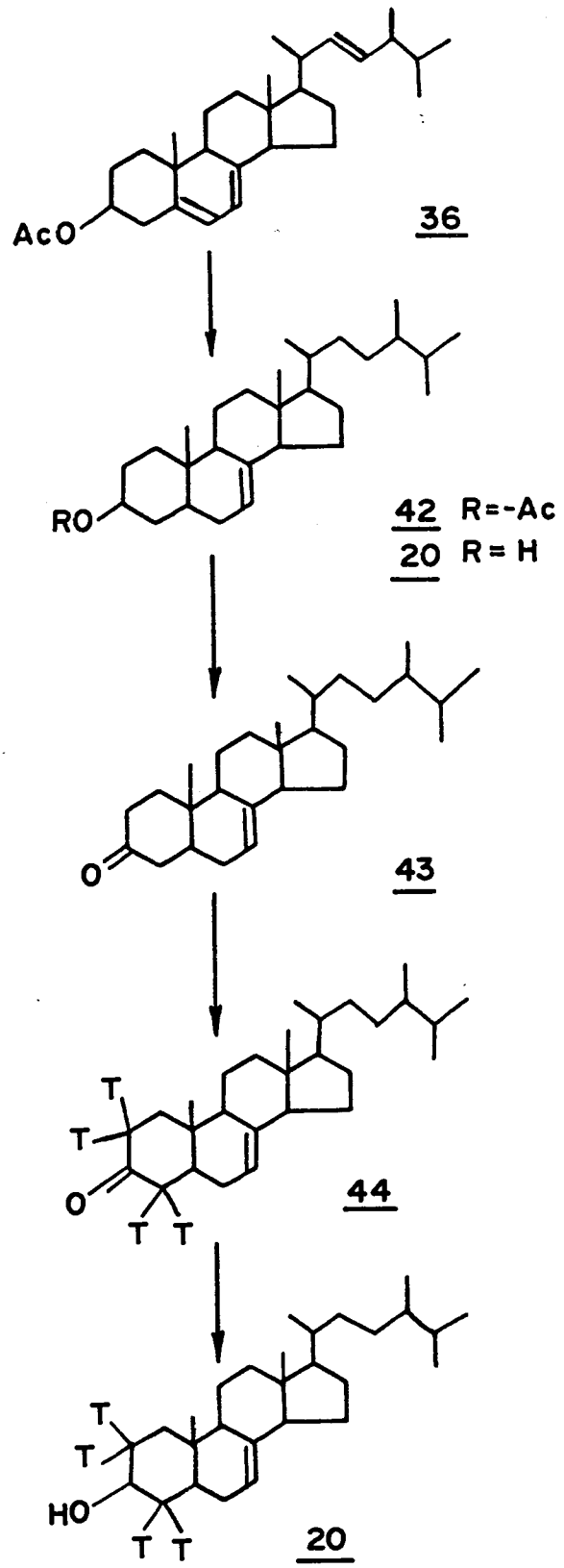


Figure 8. Synthesis of Ergost-7-en-3β-ol.

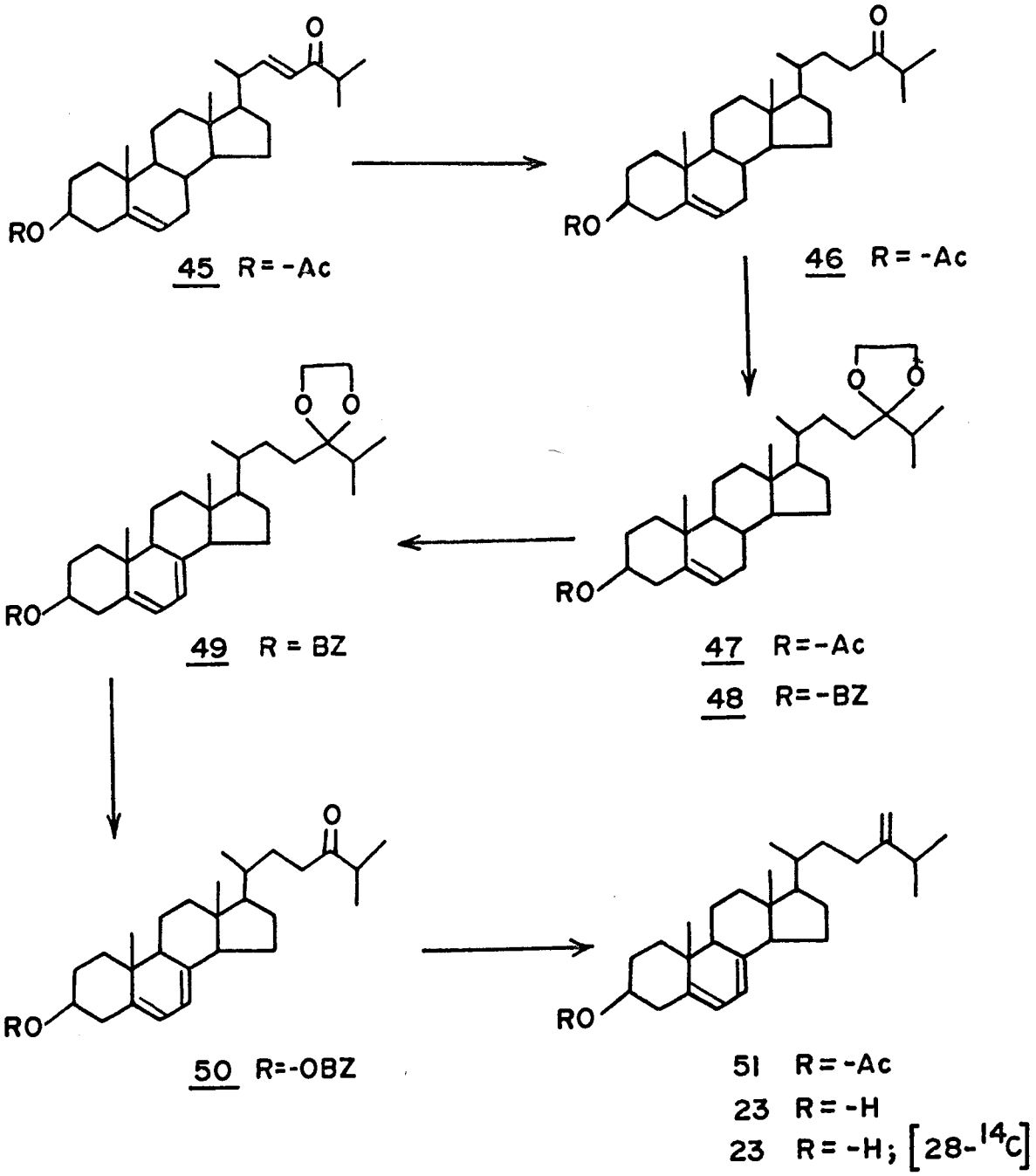


Figure 9: Synthesis of Ergost-5,7,24(28)-trien-3β-ol.

TABLE III

STEROLS SYNTHESIZED AND/OR ISOLATED DURING THIS WORK

Sterol	Normal	Mutant	Synthesized Unlabelled	Synthesized With Label at:	Remarks
Lanosterol (6)	P	P/N		26,27-^{14}C	
Zymosterol (14)	P	P/N		2,4-^{3}H	
Fecosterol (16)	P	P/N		28-^{14}C	
Ergost-8-en-3β-ol (15)	N	N	F[7]		
Ergost-7-en-3β-ol (20)	P/N	P/N	R	2,4-^{3}H	Previously isolated from Candida[34]
Episterol (22)	P	P/N	R	28-^{14}C	
Ergosta-8,22,24(28)-trien -3β-ol (17)	N	N	R		
Ergosta-7,22,24(28)trien -3β-ol (21)	N	N	R	28-^{14}C	
Ergosta-8,22-dien -3β-ol (11)	P/N	P			
Ergosta-7,22-dien -3β-ol (19)	P	P/N		2,4-^{3}H	commercial product
Ergosta-5,7,24(28)-trien -3β-ol (23)	P/N	A	R	28-^{24}C	previously isolated from Phycomyces blakesleeanus[59]

TABLE III (continued)

Sterol	Normal	Mutant	Synthesized Unlabelled	Synthesized With Label at:	Remarks
Ergosta-5,7-diene-3β-ol (24)	(A)	A	R	U-^{14}C	tentatively identified in Agarillis bisporus[60] Holtz et al. 1972
Ergosta-5,7-22,24(28)-tetraen-3β-ol (26)	P	A	R	28-^{14}C U-^{14}C	
Ergosterol (25)	P	A		U-^{14}C	
Ergosta-7,22-diene-3β-5α-diol (52)			F[2]		
Ergosta-8(14),22-dien-3β-ol (53)	-	-			Synthesized according to Laubach et al.[62]
Ergost-8(14)-en-3β-ol	-	-			do
Ergosta-8,14,22-trien-3β-ol (55)					Synthesized according to Fieser et al.[61]

A: absent, P: previously isolated from yeast, N: new in yeast, P/N: previously isolated but new in this system, R: synthesis under experimental.

F [1]) This compound was synthesized according to methods available from the literature (see text)

[2]) Synthesized according to Topham and Gaylor 1967, and references cited therein.

ISOLATION OF STEROLS FROM NORMAL YEAST

With most of the potential desmethyl-sterol intermediates at our disposal, attention was directed to the investigation of the 4,14-desmethyl sterol fraction of yeast. Three sources of sterol mixtures were available: (a) mother liquors of ergosterol crystallization*, (b) the unsaponifiable material from base hydrolyzed commercial yeast, and (c) the sterol fraction of laboratory grown yeast cultures. It became apparent at the very early stage of the investigation that the extracts from the commercial yeast, when directly extracted as supplied, was an inadequate source of ergosterol precursors. The commercial yeast (ale brewers yeast obtained from a local brewery) was usually harvested during its stationary growth phase. It became evident that yeast extracted at such a growth stage contained mainly ergosterol (25) and ergosta-5,7,22,24(28)-tetraen-3β-ol (26), these two compounds having reached rather high steady state concentrations. In order to establish the presence or absence of the previously synthesized and postulated sterol intermediates in mixtures derived from natural sources, mother liquors from which the bulk of ergosterol had been removed by crystallization were investigated.

The solution* was evaporated and the residue partly purified by acetylation and base hydrolyses to give a semi-crystalline mixture of the free sterol alcohols. This mixture was separated chromatographically on an alumina column into

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* Kindly supplied by Mycofarm-Delft, Division of Royal Netherlands Fermentation Industries Ltd., Delft, Holland.

five major fractions as illustrated in Fig. 10.
Fraction 1 contained squalene and a clear oil consisting of a number of low-boiling compounds which had g. l. c. retention times close to that of squalene. Squalene was identified by comparison of its n. m. r. with that of authentic material. The remaining oily mixture was not further investigated.
Fraction 2 contained essentially a single compound which was identified by n. m. r., m. s. and g. l. c. and comparison with authentic material as lanosterol (6).
Fraction 3 contained a mixture of at least four compounds as evidenced from g. l. c. The two major components were determined to be 4,4dimethyl-zymosterol (10) and 4α-methyl zymosterol (12) on the basis of relative g. l. c. retention times[63] and m. s.[64]. To the third compound was tentatively assigned the 4α-methyl-ergosta-8,24(28)diene-3β-ol (13) structure based on g. l. c. retention time and m. s. The structures of 10 and 12 were later confirmed by comparison with authentic compounds[65].
Fraction 4 was a mixture of mono-, di- and triunsaturated 4,14-desmethylsterols.
Fraction 5 contained ergosterol (25), tetraene (26), and a third unidentified sterol.

Fractions 4 and 5 were acetylated individually. Preliminary examination of the mixtures by m. s. showed strong possible parent ions at 442, 440, 438, 436 and 426 mass units. The UV spectrum of fraction 4 showed absorption at approximately 330 - 340 nm but no absorption at higher wavelength. Fraction

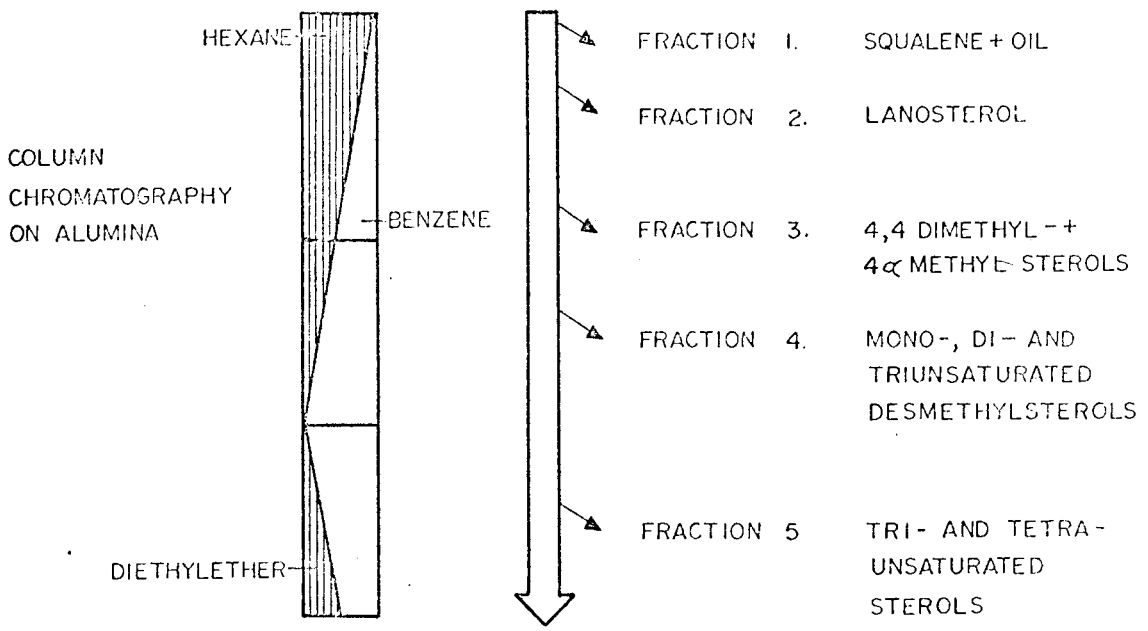


Figure 10: Column Chromatography of Yeast Sterols

5 showed the typical ergosterol (25) and tetraene (26) absorptions[39]. The two fractions were further separated by 15% $AgNO_3$-silica gel column chromatography and/or preparative T. L. C. The sterols in fraction 4 were eluted from the column with increasing amounts of diethylether in hexane. Three subfractions were collected (4-1, 4-2, and 4-3 as illustrated in Fig. 11). Fraction 4-1 contained mainly 3β-acetoxy-ergost-7-ene (40). The structure was assigned on the basis of the following evidence. The compound was expected to be in this fraction as found by previous separations using synthetic mixtures. The melting point, g. l. c. retention time, n. m. r. and mass spectra of this compound were identical to the reference sample prepared above (Fig. 8). The corresponding Δ^8 isomer, 3β-acetoxy-ergost-8-ene (33), which was expected, if present, to appear in the same fraction could initially not be isolated although there was a very small peak with the correct relative retention time present in g. l. c. trace of this fraction. Its presence therefore initially remained in doubt (see later trapping experiments). Fraction 4-2 was a mixture of three compounds (g. l. c.) and was therefore further purified by preparative T. L. C. Two bands were recovered. The band with the higher R_f had the same R_f value as 5,6-dihydroergosterol acetate (27). The recovered material gave no melting point depression when mixed with authentic 5,6-dihydroergosterol acetate (Fig. 7). The n. m. r. and m. s. of the isolated and of the synthetic

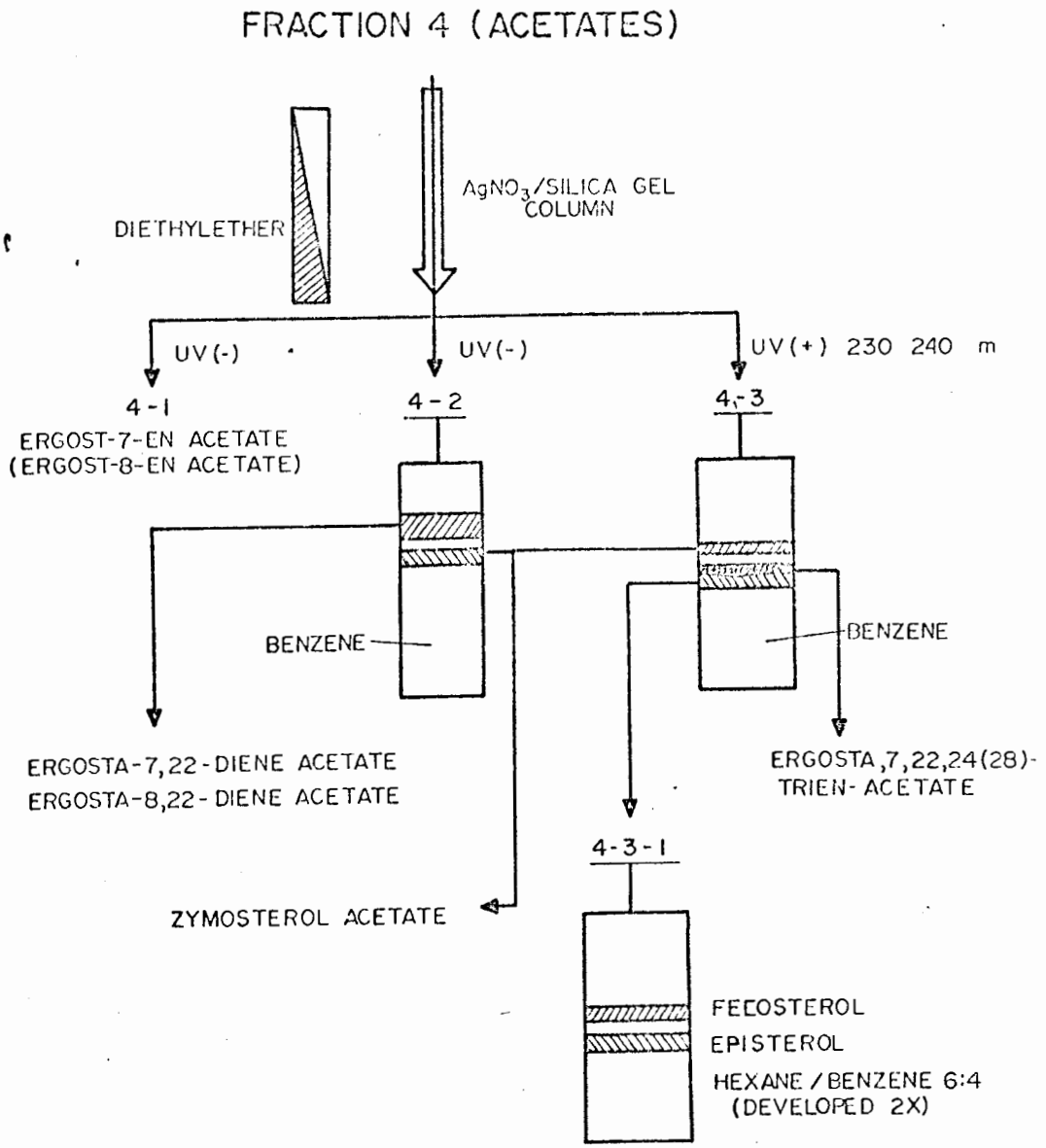


Figure 11: T. L. C. Fractionation of Sterol Acetates

material were identical.

The material from the slower moving band had a M^+ at m/e 426. A strong peak at m/e 313 indicated an unsaturated C_8 side chain. The free sterol showed a corresponding peak at m/e 271. The n. m. r. spectrum of the acetate showed angular methyl singlets at δ 0.62 and δ 0.98. Two singlets at δ 1.62 and δ 1.70 were assigned to methyl groups on an unsaturated carbon. The only absorption in the olefinic region was a broad peak at δ 5.0 - 5.2. Based on this evidence and the melting point of the free sterol and the acetate, this compound was identified as zymosterol acetate (36).

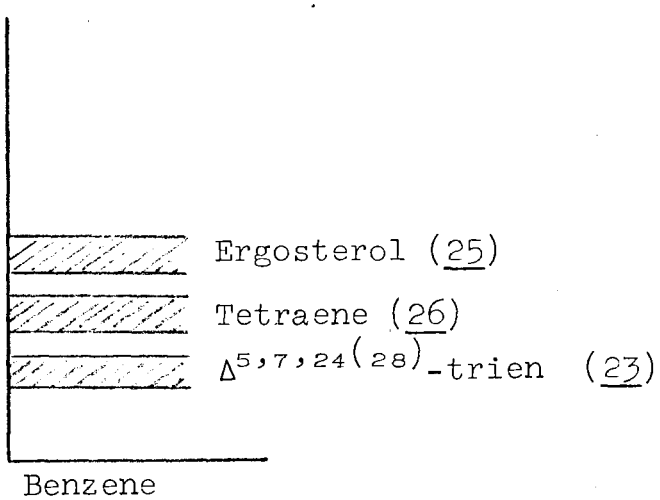
A third compound which co-chromatographed with 5,6-dihydroergosterol acetate could not be isolated in pure form due to lack of material. The relative retention time on g. l. c. corresponded to that of 3β-acetoxy-ergosta-8,22-diene (32). Comparison of the m. s. of the natural sterol obtained by g. l. c. - m. s., with the mass spectrum of the synthetic $\Delta^{8,22}$-diene (Fig. 5) showed that these spectra were identical. Fraction 4-3 was a mixture of four compounds and was separated by preparative T. L. C. using benzene as solvent; the mixture separated into two bands. The faster moving band had the same R_f value as zymosterol acetate (36) and when isolated was identical with authentic zymosterol acetate (Fig. 6). The slower running band, when viewed under UV light, showed a dark band of higher R_f than a partially separated yellow band of lower R_f (see experimental for preparative T. L. C.). The two bands were recovered separately and rechromatographed two times,

each time separating the dark from the yellow band. The dark band co-chromatographed with 3β-acetoxy-ergosta-7,22,24 (28)-triene (41) on T. L. C. After purification by T. L. C. (2x) and crystallization from methanol, the sterol acetate gave no melting point depression when mixed with synthetic triene (Fig. 7). The UV and n. m. r. spectra of 41 isolated above were identical with the spectra of the synthetic sterol acetate (Fig. 7). The mass spectra showed M^+ m/e 438 and significant peaks at 423, 395, 378, 363, 313 of intensities identical to those of the synthetic sterol acetate. The g. l. c. retention times on two different columns (QF-1, XF-60) were the same as for the synthetic compound. Based on this evidence, the presence of ergosta-7,22,24(28)triene-3β-ol (21) was established as a naturally occurring sterol.

The third band from fraction 4-3 contained two compounds which could be separated cleanly by T. L. C using benzene-hexane (4:6) as solvent and running the plates twice. The two sterol acetates were identified as fecosterol acetate (38) and episterolacetate (40) based on the following evidence: The slower running band co-chromatographed with synthetic episterol acetate (Fig. 7) on T. L. C. and g. l. c. and the mixed melting points of isolated and synthetic materials showed no depression. In addition the n. m. r. spectra of materials derived from the yeast and via synthesis were identical.

The material from the faster running band gave an i. r. spectrum which showed bands at 1640 and 890 cm^{-1}. These bands

are characteristic of 1,1-disubstituted ethylenes. The mass spectrum had M^+ m/e at 440 (100%) and significant peaks at (m/e) 425, 380, 365, 313, 255 and 213. The n. m. r. spectrum of this compound was identical to that of fecosterol acetate (38) prepared earlier (Fig. 7). Although the melting point of isolated fecosterol (136.5 - 137.5°) did not agree with the value reported by Wieland et al.[35] it was identical to the melting point of the fecosterol synthesized from zymosterol. Fraction 5 contained three sterol acetates.



The three compounds were found to co-chromatograph on T. L. C. with ergosterol (25), tetraene (26) and ergosta-5,7,22(24)-trien -3β-ol (23). The first two compounds were isolated and their identity confirmed by comparison with authentic samples.

Ergosta-5,7,24(28)-trien -3β-ol (23) had not been reported to occur in yeast. The identity of the isolated compound was proven by comparison of the T. L. C. and relative retention times of the isolated sterol with those of a synthetic sample.

Furthermore the correspondence of the n. m. r. and i. r. spectra of the isolated and synthetic samples indicated structure 51 for the isolated acetate. Thence the corresponding alcohol, 23, occurs in yeast. This sterol has been previously reported in Phycomyces blakesteeanus[59]. The presence of ergosta-5,7-dien -3β-ol (24) expected in this fraction could not be demonstrated.

No attempt was made to determine the relative amounts of the individual sterols isolated from mother liquors from which ergosterol had been largely removed. Meaningful information concerning the relative amounts of the sterols in a crude non-saponifiable preparation can only be obtained by investigation of the total sterol fraction from an actively growing system.

ISOLATION OF STEROLS FROM THE MUTANT STRAIN

Only a limited amount of unsaponifiable material from the nystatin resistant strain was available because, as will be discussed later, this yeast can not be cultured in large batches. Separations were therefore carried out entirely by preparative T. L. C. The overall procedure is shown in Table IV. The same procedure was later used for time course studies and in the biosynthetic experiments. The individual sterols were identified using the same criteria as above.

A preliminary investigation of the crude sterol extract by g. l. c. revealed the presence of one major sterol (~80%)

TABLE IV

TLC-SEPARATION OF STEROL ACETATES ($AgNO_3$/SILICA GEL)

A : benzene			B : solvent as indicated			
BAND	Rf	G.l.c. analyses rel. ret. times[1])	Solvent System[3])	BAND	G.l.c. analyses	RTT
1	Origin	[2])				
2[4])	0.2-0.35	2 peaks	B/EA 9:1	2-1	ergosta-5,7,24(28)-dien-3β-ol	2.2
				2-2	ergosta-5,7,22,24(28)-tetraen-3β-ol	2.1
3	0.35-0.4	1 peak	B	3-1	ergosterol	1.88
4	0.4-0.55	2 peaks	B/H 2:3 2x	4-1	fecosterol	1.99
					episterol	2.15
5	0.6-0.55	1 peak	B/H 4:1	5-1	ergosta-7,22,24(28)-trien-3β-ol	2.05
6	0.65-0.6	1 peak	B/H 1:1	6-1	zymosterol	1.64
7	0.85-0.65	4 peaks	B/H 1:5 2x	[5])	ergosta-7,22-dien-3β-ol	1.83
				7-1	ergosta-8,22-dien-3β-ol	1.68
					ergost-7-en-3β-ol	2.17
				7-2	ergost-8-en-3β-ol	1.97
8	0.85-1.0	Mixture of squalene, lanosterol and 4,14-methylated sterols			lanosterol	2.4
					4α-methylzymosterol	1.82
					4,4-dimethylzymosterol	2.28

[1]) Relative retention times are relative to β-cholestanol on 1.5% QF-1.

[2]) Normal yeast: complex mixture of polar material
Ergosterol less strain: very little material left at origin

[3]) B: benzene, H: hexane, EA: ethyl acetate

[4]) Band 3 and 4 contained no detectable amount of material with pure nys-3 strain.

[5]) Band 7-1 and 7-2. The respective compounds can be further separated by continuous T. L. C. (24 - 48 hours) using 10% benzene as solvent.

which, after purification, was identified as ergosta-7,22-diene-3β-ol (19). The second most abundant sterol was indeed the Δ8,22 diene, 11, as reported earlier by Parks et al.[37]. The mixture showed UV absorption in the region 220 - 240 mμ but no significant amount of ergosterol could be detected. Mass spectral analysis of the crude mixture showed possible molecular ions at 426 (>3%), 412 (>3%), 400 (30%), 398 (100%, 396 (14%), 384 (28%). The crude sterol fraction was acetylated and analyzed using a combination of T. L. C. - g. l. c. - m. s. In a typical separation eight bands were recovered (see under A in Table 4). The individual bands were rechromatographed as under A since some material from neighbouring bands was usually isolated with the recovered band. The corresponding bands were pooled and rechromatographed as under B. Only band 5 showed strong UV absorption in the region 220 - 240 mμ. When cultures were worked up which had partly reverted (see "Culturing Methods" for an explanation) band 3 showed the typical ergosterol UV absorption.

In case of the mutant bands 1, 2 and 3 contained no significant amount of material. Band 4 contained fecosterol acetate and episterol acetate in a ratio of 1:25 . These two acetates were readily separated by T. L. C. and identified by comparison of their spectra with spectra of authentic compounds. Band 5 contained mainly 7,22,24(28)-trien , 41, identified by comparison with authentic material (Fig. 7). G. l. c. revealed however a minor component (less than 5%

of the total material in band 5) with the same retention time as synthetic triene, 35.

The minor compound could not be isolated by T. L. C. in pure form. Collection from g. l. c. of enough material for mass spectral and ultra violet analysis showed it to be identical with the synthetic material. Attempts to demonstrate the same compound in the normal strain failed.

Band 6, after crystallization, gave pure zymosterol acetate (36). Band 7 contained four components with the relative g. l. c. retention times corresponding to 7-ergosterol-42, $\Delta^{8,22}$ ergostadiene - 32, and $\Delta^{7,22}$-ergostadiene - 27. This mixture was further separated by T. L. C. into two bands containing monounsaturated and diunsaturated sterols.

The double bond isomers in each band could finally be separated by continuous T. L. C. (Preparative T. L. C. plates were covered with a second plate and clamped together. The top was extended with filter paper to the outside of the developing chamber.) The plates were usually run for twenty-four hours. The compounds were recovered and identified by comparison with authentic samples. The two major $\Delta 7$ isomers could also be obtained pure by crystallization.

The n. m. r. data reported[37] for the isolated ergosta-8, 22-diene-3β-ol, 11, did not agree with values obtained from synthetic material. A sample of the synthetic 11 was compared with the sterol isolated elsewhere and found to be identical.*

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* This comparison was carried out by Dr. T. Bond, University of California, San Diego.

Analyses of the spectra (220 MHz) of more plentiful synthetic material showed that some of the initially reported chemical shifts were misinterpreted.

In order to exclude the possibility that the tetra-substituted double bond of 11 could be located at the $\Delta^{8(14)}$ position, the isolated ergosta-8,22-diene, 11 and ergost-8-ene, 15, were compared with the corresponding 8(14) isomers (See Table 3). Melting point, g. l. c. and n. m. r. data differed substantially in both cases. Band 8 contained principly squalene and 4- or 4,14-methylated compounds (e.g. 10, 12, 13). This fraction was not further investigated.

The isolation and identification of the new sterols 15, 17, 20, 21, and 23 completed the desmethyl sterol part of Fig. 4. The only exceptions are compounds 18 and 24. No evidence for the presence of 18 has been obtained. Preliminary examinations of the sterol fraction obtained from a culture of the mutant which had partly reverted and therefore produced ergosterol seemed to suggest the presence of ergosta-5,7-diene-3β-ol (g. l. c.).

BIOSYNTHESIS

1. Time course studies: Results and Discussion

The projected biosynthetic studies necessitated the acquisition of most of the ergosterol precursors shown in the lower part of Fig. 3. Parallel with chemical syntheses of these compounds we investigated culture conditions conducive to achieving higher than usual levels of sterol intermediates than occurred in yeast under normal conditions.

It was known from the experiments of Klein et al.[67] that sterol formation could be induced in Saccharomyces cerevisiae by vigorous aeration of cells that had previously been grown under strictly anaerobic conditions. Under these conditions, S. cerevisiae becomes auxotrophic for sterols, and squalene accumulates (Klein[68]). New and vigorous sterol synthesis can be induced by commencement of aerobic conditions[67]. This has been found to be due to the necessity of molecular oxygen for the formation of 2,3-oxidosqualene, the immediate precursor of lanosterol[69,70]. In addition several of the steps involved in the transformation of lanosterol to ergosterol are also oxygen dependent, e.g. removal of the extra methyl groups, introduction of double bonds and methylation at C-24. These steps are consequently also inhibited under anaerobic conditions.

Although the lack of oxygen provides adequate reason for the lack of production of sterols in yeast, little is known about the process giving rise to the actual depletion of sterols under anaerobic conditions. From the general behaviour described

above two methods emerge by which intermediates in the sterol biosynthesis scheme could be induced to accumulate in higher proportions than would normally be the case. Firstly, if ergosterol is an obligatory intermediate in the anaerobic consumption process resulting in depletion of yeast sterols, one would expect some ergosterol precursors to occupy more prominent positions in the yeast sterol mixtures remaining after anaerobic maintenance. This would be true of those sterol intermediates that could not be transformed to ergosterol anaerobically.

Secondly, after depletion of the sterol content by anaerobic maintenance, and commencement of aerobic conditions, initial sterol production should be accompanied by accumulation of initial intermediates. Depending on the rates of interconversion, some intermediates could occupy more prominent positions in the sterol mixture at early stages rather than at later stages.

Although the dependence of total sterol production upon culture conditions has been well investigated, little information is available regarding variations in the composition of the sterol fraction as a function of growth conditions. Bronn[71] and Kodicek *et al.*[72] reported an intermediate accumulation of lanosterol (6) during fermentation. Langley *et al.*[73] reported ergosta-5,7,22,24(28)tetraene-3β-ol (26) and zymosterol (14) as the major sterols and minor amounts of ergosterol in cells grown batchwise at 25°. Hunter and Rose[74] found a slight variation in the tetraene/ergosterol ratios depending on the temperature at which the yeast was grown. Petzold[75] found a change of the tetraene/ergosterol ratio of 7:3 in batch cultures to a ratio

of 3:7 in continuously grown cultures. Preliminary experiments designed primarily to determine optimal culturing conditions confirmed, in general, the above observations.

Yeast harvested during its stationary growth phase contained ergosterol (25) and tetraene (26) as major sterols as well as small amounts of zymosterol (14) and lanosterol (6). The former two accounted for more than 80% of the total sterol fraction. When the same yeast was cultured under nitrogen for 80 hours, the major portion of the sterols disappeared. The composition of the small amount left had change appreciably, although there were traces of ergosterol left, as judged from U. V. spectra. The major sterol was now ergosta-7,22-diene-3β-ol (19) followed in decreasing amounts by ergosta-7,22,24(28)-triene-3β-ol (21), lanosterol (6), zymosterol (14) and ergosta-8,22-diene-3β-ol (11). Upon aeration, the total sterol content increased rapidly at the beginning and levelled off after approximately 20-30 hours. The lanosterol content reached its maximum at approximately 2 hours. The ergosterol/tetraene ratios varied with time from approximately 2:3 at 10 hours to 4:3 at 24 hours.

These preliminary experiments seemed to indicate that significant changes in sterol composition did occur upon anaerobic-aerobic treatment. Time course studies were therefore carried out on anerobically pretreated yeast cultures which were resuspended in fresh medium and aerated. (See experimental section for details). Aliquots of cell suspensions were removed at set time intervals for dry weight determinations and sterol analyses. The sterol fraction of each aliquot obtained from base hydrolysis

was acetylated and total sterol content determined by g. l. c. (The total area under the curve was evaluated planimetrically.) The acetates were separated by T. L. C. into eight bands which were then analysed by g. l. c. (peak height x width at peak half height, relative to a known amount of added standard). Difficulties in precisely controlling all culture conditions e.g. anaerobic pretreatment, total cell population, aeration and particularly the ratio of viable cells to dead cells caused appreciable differences from one experiment to the other. After several experiments it became clear that the major differences between individual experiments concerned the time at which the maximum concentration of the individual sterols appeared. In general, it was observed that the slower the maxima of individual sterols were achieved the lower were their observed concentrations. When the total sterol content increased very slowly, no actual accumulation of intermediates could be observed. The results obtained from two extreme experiments (A and B) are shown in Fig. 12. Fig. 13 illustrates the results obtained from one particular experiment in which the concentrations of several components of the sterol fraction were followed with time.

Squalene increased briefly at the beginning and then decreased sharply as the total sterol content increased. When sterol production levelled off, squalene synthesis approximately paralleled sterol synthesis. Lanosterol and 4,14-methylated sterols increase during early stages and reach a maximum at 2 to 3 hours. Lanosterol reaches its steady state concentra-

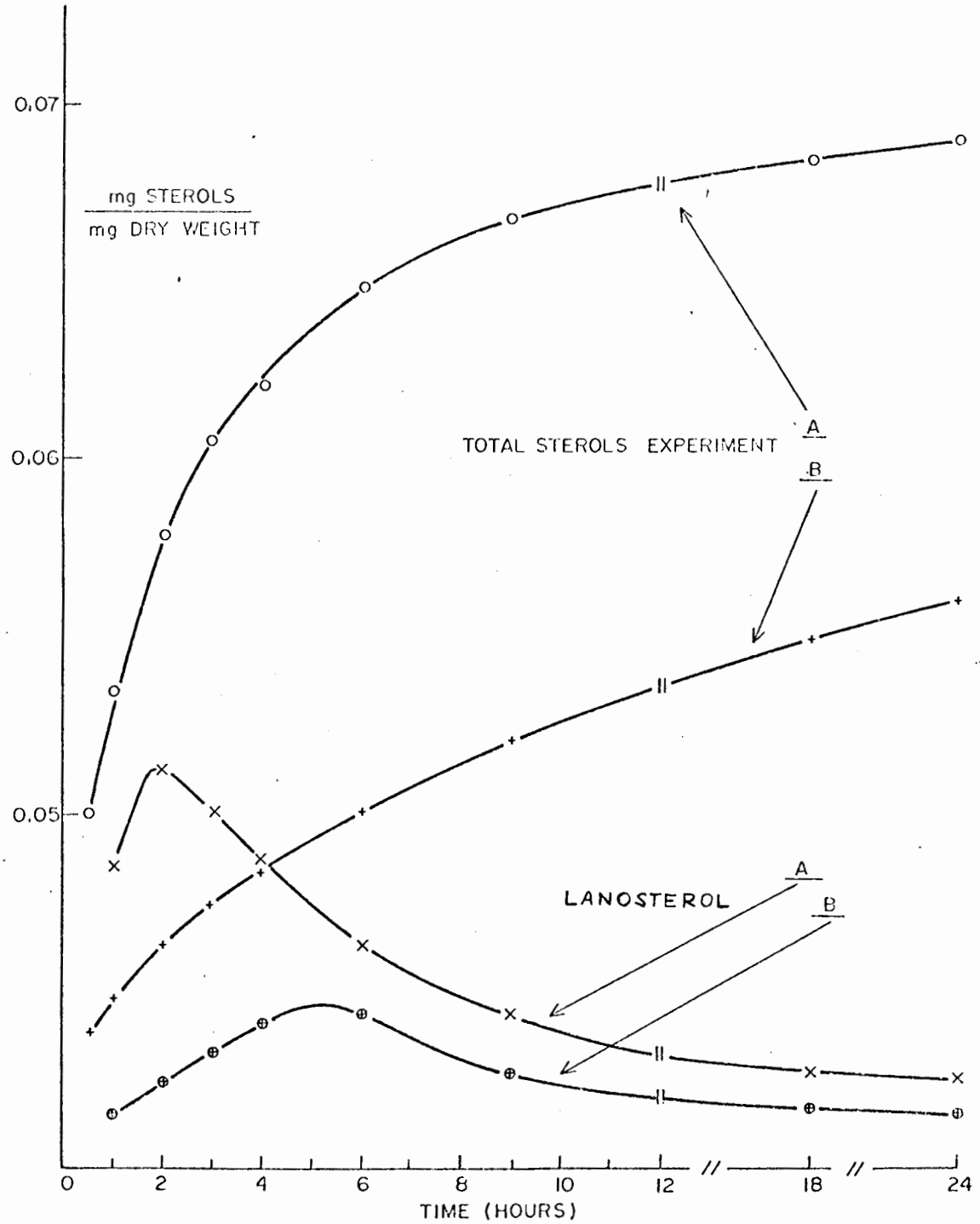


Figure 12: Variation of Sterol Content with Growth Conditions

tation (10 - 15% of the total sterol fraction) at approximately 10 hours. The concentrations of 4-mono- and 4,4-dimethyl sterols vary in a similar manner, however, after initial attainment of rather high proportional concentrations they drop to rather low concentrations. The 4,14-desmethyl sterols, with the exception of ergosta-5,7,24(28)-trien -3β-ol (23), increase slowly towards their steady state concentrations. Episterol, ergosta-7,22,24(28)-trien -3β-ol, and fecosterol (plotted in Fig. 13 as their sum for clarity) increase steadily with relative concentrations which deviate only slightly from the ratio of 4:2:1 during the whole period investigated. Noteworthy is the low concentration of ergost-7-en-3β-ol compared with the other desmethyl sterols. The concentration of ergosta-5,7,24 (28)-trien-3β-ol behaves similarly to the other 4,14-desmethyl sterols initially but later falls to lower values and is practically absent after 10 hrs.

An analogous experiment was performed with the nys-3 mutant (Fig. 14). It was expected that precursors of the major sterol (ergosta-7,22-dien-3β-ol) of this organism would accumulate during the early growth phase. Experiments however revealed that this yeast strain did not respond in the same manner as normal yeast to the anaerobic-aerobic treatment. Squalene accumulated more slowly in the mutant than in the normal strain under anaerobic conditions. Although under aerobic conditions both normal and nys-3 strains produced comparable amounts of sterols, the sterol content decreased much more slowly in the mutant than in the normal strain under anaerobic maintenance.

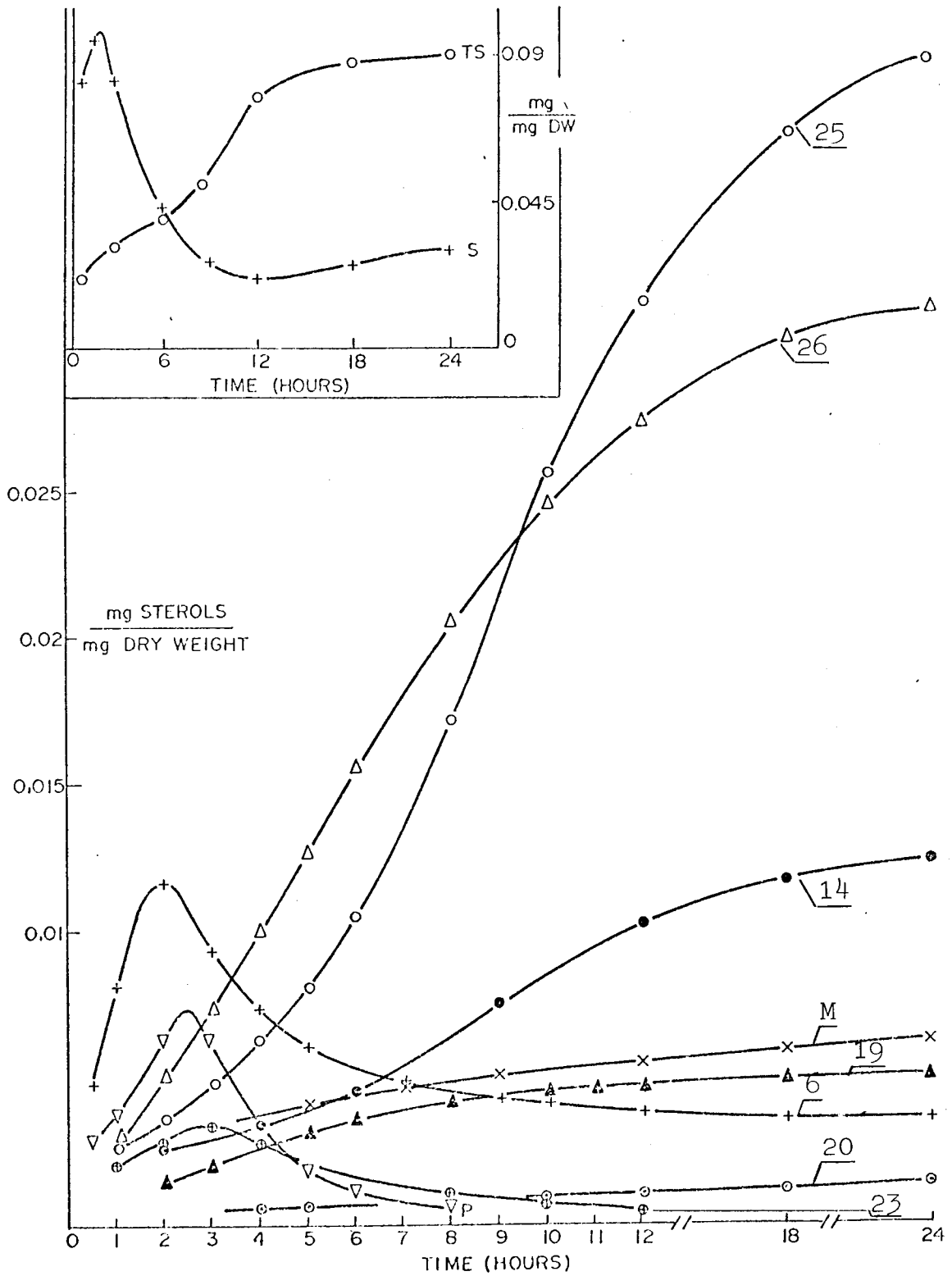


Figure 13: Variation in the Sterol Composition in Normal Yeast with Time

To Figure 13:

25: Ergosterol, 26: Ergosta-5,7,22,24(28)-tetraen-3β-ol,

14: Zymosterol, M: Episterol 22 + Fecosterol 16 + Ergosta-7,22,24(28)-trien-3β-ol 21, 6: Lanosterol

19: Ergosta-7,22-dien-3β-ol

20: Ergosta-5,7,24(28)-trien-3βol

P: 4,and 4,4-methyl-sterols 10,12 and 13.

The insert shows total sterols (TS) and squalene (S) of the same experiment on a reduced scale.

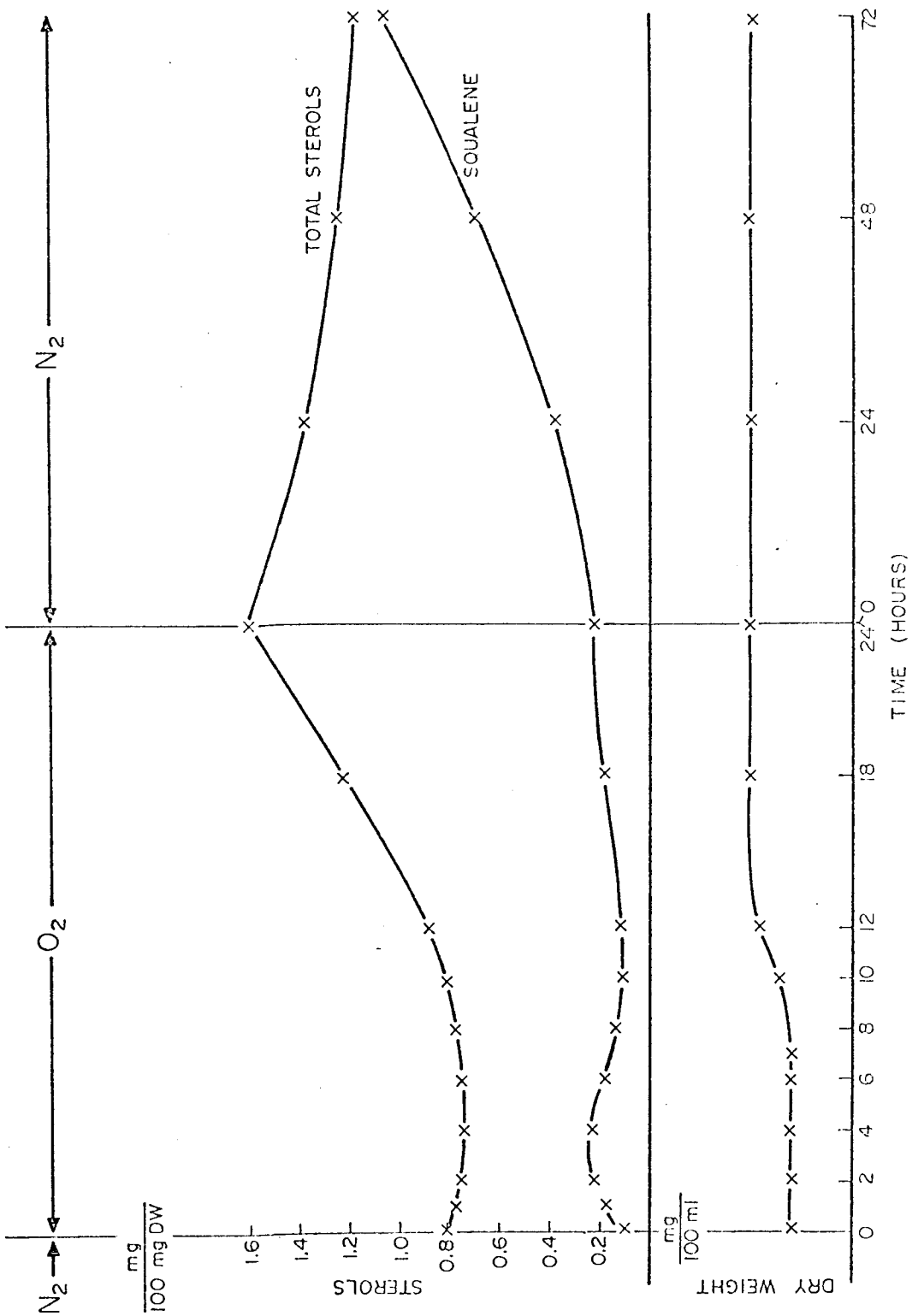


Figure 14: Variation of Sterol Content in Nys-3 with Time

After 24 hrs (anaerobic), the sterol content of the normal strain had decreased to less than 5% of its original value while in the mutant approximately 80% of the sterols remained. Unlike the normal strain, the composition of the remaining sterol fraction in the mutant, after anaerobic treatment, remained essentially unaltered. Furthermore the composition of the small amount of sterols left after anerobic treatment of normal yeast was essentially the same as the sterol distribution in the nys-3 strain. If only $\Delta^{5,7}$ diene systems were subject to rapid anaerobic degradation in the normal strain, the anerobic sterol content of this organism could well become closely matched to that of the mutant which contains only Δ^{7} (and Δ^{8}) sterols. This situation would be expected to arise if no conversion of Δ^{7} (and Δ^{8}) to $\Delta^{5,7}$ sterols were possible under anerobic conditions. Since Akhtar *et al.*[32] have shown that introduction of the Δ^{5} double bond requires oxygen, no $\Delta^{7} \rightarrow \Delta^{5,7}$ conversion is indeed possible anerobically. The fact that sterols of the mutant are metabolized at all in anaerobic conditions indicates, however, that Δ^{7} sterols are also subject to the anerobic degradation process. The rate of degradation of Δ^{7} sterols would appear, from the above evidence, to be significantly slower than that of the $\Delta^{5,7}$ analogs.

An equally valid rationale for the correspondence of the sterol composition in the nys-3 and normal strain, after anaerobic treatment of the normal strain, is that the nys-3 mutant cells are present at all times as a small percentage of the total cell population in a normal yeast culture.

Evidence has been reported that the development of polyene antibiotic resistance is based on a selection of naturally occurring mutants rather then on adaptation[76]. One would therefore expect a small population of mutant cells to be present in normal yeast. Since nys-3 is only depleted of sterols very slowly under nitrogen as shown above, the sterol fraction isolated from the anerobically maintained normal strain could represent the sterol fraction of the mutant. Both of the above described factors, e.g. rapid degradation of $\Delta^{5,7}$-dienes as well as presence of small amounts of mutants in normal cultures, do in all probability, contribute to the overall effect.

2. Tracer Experiments: Results and Discussion

The tracer experiments were designed to yield information concerned with the biointerconversions (shown in Fig. 3) involving the 4,14-desmethyl sterol region . As is illustrated, five Δ^8-sterols (11, 15 - 18) could be formally derived from 4α-methyl precursors. Although all of these, with the exception of 18, have been shown to occur in yeast, only two of their 4α-methylated precursors have thus far been isolated, e.g. 12[26] and 13[26]. Formally, compound 12 yields zymosterol (14), and sterol 13 gives fecosterol (16) upon 4-demethylation. Using Figure 3 as a model, fecosterol could itself be transformed in four ways: either to the Δ^8 sterol 15, to the $\Delta^{8,22,24(28)}$-triene 17, to episterol (22) and to zymosterol (14). Some evidence for the reversibility of the C-24 alkylation has recently been reported[77].

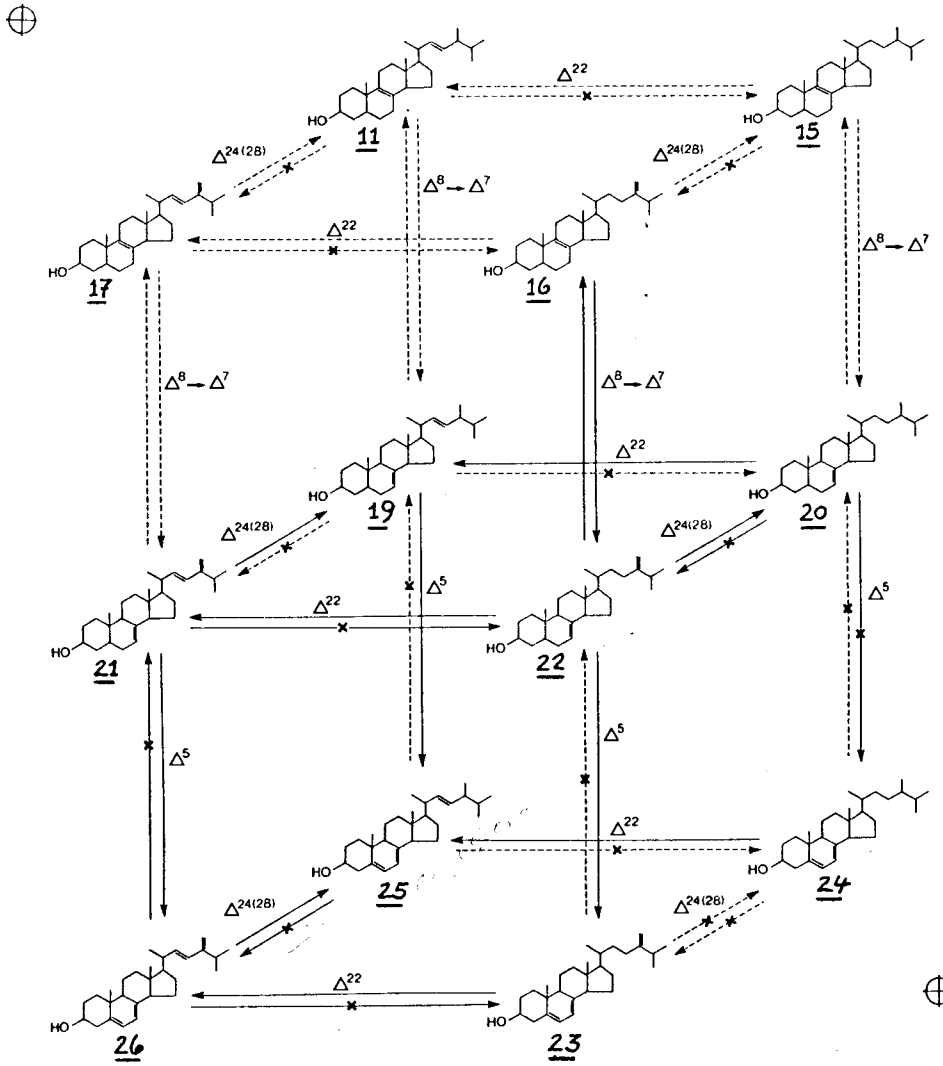
Due to the great number of possible interconversions, neither fecosterol nor zymosterol seemed to be logical choices to initiate investigations of the interconversions possible in this region of Fig. 3. Isomerization of the Δ^8 double bond in fecosterol to Δ^7 gives episterol (16 → 22). Since episterol occupied a more central position in the interconversion model depicted by Fig. 3, we initiated incubation-trapping experiments directed at the determination of the sequence(s) operating in the conversion of episterol to ergosterol. The results of these experiments are summarized in Table V and illustrated in Fig. 15.

TABLE V

ACTIVITY IN ISOLATED METABOLITES*

LABELLED STEROL ADDED TO WHOLE CELLS	22	20	23	21	19	24	26	25
22	-	0.32 0.21	0.9 0.4	6.1	-	-	5.4 17.6	5.1 2.9 4.6
20	0	-	-	-	11.6 10.45	< 0.01	0 < 0.001	15.2 16.4
23	-	-	-	0	-	-	7.1	3.5
21	< 0.001	-	-	-	0.23 0.4	-	9.7 10.0	3.7 4.1
24	-	0	0	-	-	-	-	19.2
26	-	-	0	0	-	-	-	17.8

*The given values are % incorporation based on the total activity recovered in the nonsaponifiable fraction. In cases where more than one value is obtained they are from different, independent experiments. No given value means that this particular compound was not investigated for activity in that particular experiment. For details see experimental part.


11
15
17
16
19
20
21
22
25
24
26
23
HO
Δ22
Δ24(28)
Δ8→Δ7
Δ5

Feeding labelled 22 resulted in radioactivity in 20, 21, 23, 26 and 25 (Experiments #1, 2 and 3). Incorporations were low in 23 and 20 (less than 1%). In 21, 25 and 26, tracer incorporation ranged from 2.9 to 17.6%. The specific activities were comparable for 21, 23, 26 and 25 ($\sim 10^3$ cpm/mg). Although the incorporation of 22 into 20 was rather low, 20 itself was efficiently converted to the diene 19 and to ergosterol (10.4 to 16.4%, specific activity $\sim 10^4$ cpm/mg) as determined by two separate feeding experiments (Experiments 4 and 5). No activity was found in either 22, 26 and 24 upon feeding radioactive 20. Despite the fact that no significant activity was associated with the $\Delta^{5,7}$-diene 24 upon feeding 20, it was converted rather efficiently to ergosterol when fed in labelled form to yeast (19.2%; Experiment 6). Ergosterol was the only labelled sterol recovered from this experiment. Feeding of labelled 21 (Experiment #7) produced, as expected, labelled 19, 25 and 26. No activity was found in 22. Likewise labelled ergosterol (25) was obtained when labelled 23 (Experiment #8) and labelled 26 (Experiment #9) were fed.

None of the transformations investigated were found to be reversible, eg. 23 ↛ 22, 21 ↛ 22, 26 ↛ 23, 26 ↛ 21, 20 ↛ 22 and 25 ↛ 26. Since activity was found in 19 and 25 but not in

21 or 26 when 20 was fed (Experiment #4 and 5), the transformation of 21 to 19 can consequently not be reversible. It appears therefore that once the C-22 and C-5 double bonds are introduced they are not subsequently removed (i.e. $\Delta^{22} \rightarrow$ 22H, 23H: 26 ↛ 23, 21 ↛ 22 and Δ^{5} ↛ 5H, 6H: 26 ↛ 21, 25 ↛ 19). Nor does it seem likely that a reintroduction of a methylene at C-24(28) occurs once it is reduced to methyl (24-Me → $\Delta^{24(28)}$: 20 ↛ 22, 25 ↛ 26).

Episterol (22) is transformed in at least three ways: reduction of $\Delta^{24(28)}$, introduction of Δ^{22}, and of Δ^{5}. Each one of these changes gives rise to a different intermediate which then undergoes one of the two remaining transformations that are required for their conversion to ergosterol. The relative importance of the individual sequences discovered is as yet rather difficult to assess. The principle difficulty involved has been the interpretation of the available data in terms of a high or low rate of turnover of each intermediate. The procurement of these parameters will determine the efficiency cited above provided the amount of activity associated with each intermediate, its specific activity and effective pool size.

Time course studies (Fig. 13) indicate three types of behaviour with respect to pool size. The concentrations of lanosterol, 4 and 4,4-methylated intermediates and the $\Delta^{5,7,24(28)}$ triene, 23, increase rapidly to attain an early maximum concentration, then subsequently decrease rapidly to

very low values. The concentrations of most Δ^7 and Δ^8 intermediates increase steadily until apparent steady state concentrations are reached. The concentrations of ergosterol and tetraene, 26, increase slowly at first, then accumulate at an increasing rate during the middle of the time course (24 hr). Finally these two sterols reach high concentrations in the sterol fraction as would be expected of final products of metabolism.

The relatively low concentration of 4,14- and 4-methylated precursors present after the initial increase and subsequent rapid decrease is, in our view, most likely due to rapid turnover. These compounds are precursors of the desmethyl sterols and must therefore be produced at least as fast as the increase of the subsequent products.

The case of triene 23 is not as clear cut as the case of 4-methylated sterol precursors. After seven to eight hours, the usual time interval of the tracer incubation experiments, its concentration is rather low. The total radioactivity associated with this triene upon feeding episterol (22) was also low (0.4%, Experiment #2). Its specific activity is however comparable with that of the other intermediates formed from 22. This information does not allow one to differentiate between rapid and slow turnover. Such a combination of results could be due to fast influx and fast outflow leaving little activity in the pool at any time. Specific activity under these conditions would remain high since the pool is not diluted with material derived from other sources. Slow

influx, coupled with slow outflow would create the same situation. It is however noteworthy that a decrease in production of tetraene 26 follows the decrease of formation of 23. A possible interpretation of the above is that 26 is formed efficiently from 23 as well as from 21. With time, the net production of 23 decreases with a concomitant decrease in rate of formation of 26. This leads to the suggestion that initially 22 → 23 → 26 > 22 → 21 → 26 but later 22 → 21 → 26 > 22 → 23 → 26. It is very difficult from the incubation experiments to determine the efficiency of the 22 → 20 → 19 and 22 → 21 → 19 sequences. Each route has one apparently efficient step as judged from the high incorporation percentages and low pool sizes of intermediates becoming labelled during the course of the experiments (i.e. 22 → 21 and 20 → 19). Finally, the facility of the 21 → 19 → 25 vs 21 → 26 → 25 sequence cannot be assigned on the basis of presently available data. The determination of the efficiencies of these newly uncovered sequences awaits further investigations (see Proposed Research).

In subsequent experiments (Experiment #10) the intermediary role of episterol (22) was established by feeding labelled fecosterol (16). Activity was found in episterol as well as in tetraene (26) and ergosterol as was expected.

One of the possible precursors of fecosterol is zymosterol. Very little was known about its (zymosterol) involvement in the biosynthesis of ergosterol in whole cells of yeast. Schwenk et al.[19] failed to obtain incorporation of zymosterol into ergosterol by cell free homogenates under aerobic condi-

tions. The same system did incorporate squalene and lanosterol. Masters[78] investigating growth supporting efficiencies of a number of sterols in yeast kept under anaerobic conditions, found that zymosterol when incubated together with [$^{14}CH_3$-methyl]-methionine was transformed into a new sterol (possibly fecosterol, g. l. c.) containing radioactivity but no activity was found in ergosterol. Katsuki et al.[29] found that zymosterol was converted to ergosterol under aerobic but not under anaerobic conditions.

Moore and Gaylor[53,15] isolated and purified a Δ^{24} sterol trans-methylase which showed stoichiometry between the disappearance of zymosterol and the appearance of fecosterol. They were able to demonstrate that zymosterol was superior to 4,4-dimethyl zymosterol and 4-methyl zymosterol as a substrate for this system. If the relative rates observed in this system are an indication of the ability of whole cells to alkylate these substrates then the introduction of the C-24 methyl group most likely occurs after nuclear demethylation, e.g. at the zymosterol stage. The isolation of 4α-methyl-ergosta-8,24(28)-diene-3β-ol[24,26] (13) from whole yeast cells suggests that C-28 may be added before complete demethylation of the ring structure. Feeding experiments have shown[26] that this compound is efficiently converted to ergosterol. If both compounds, zymosterol and 4α-methyl-ergosta-8,24(28)-diene-3β-ol, are indeed ergosterol precursors in whole cell yeast preparations, then two alternative pathways must be operative (as can easily be seen in Fig.15) since zymosterol (14) and 4α-methyl

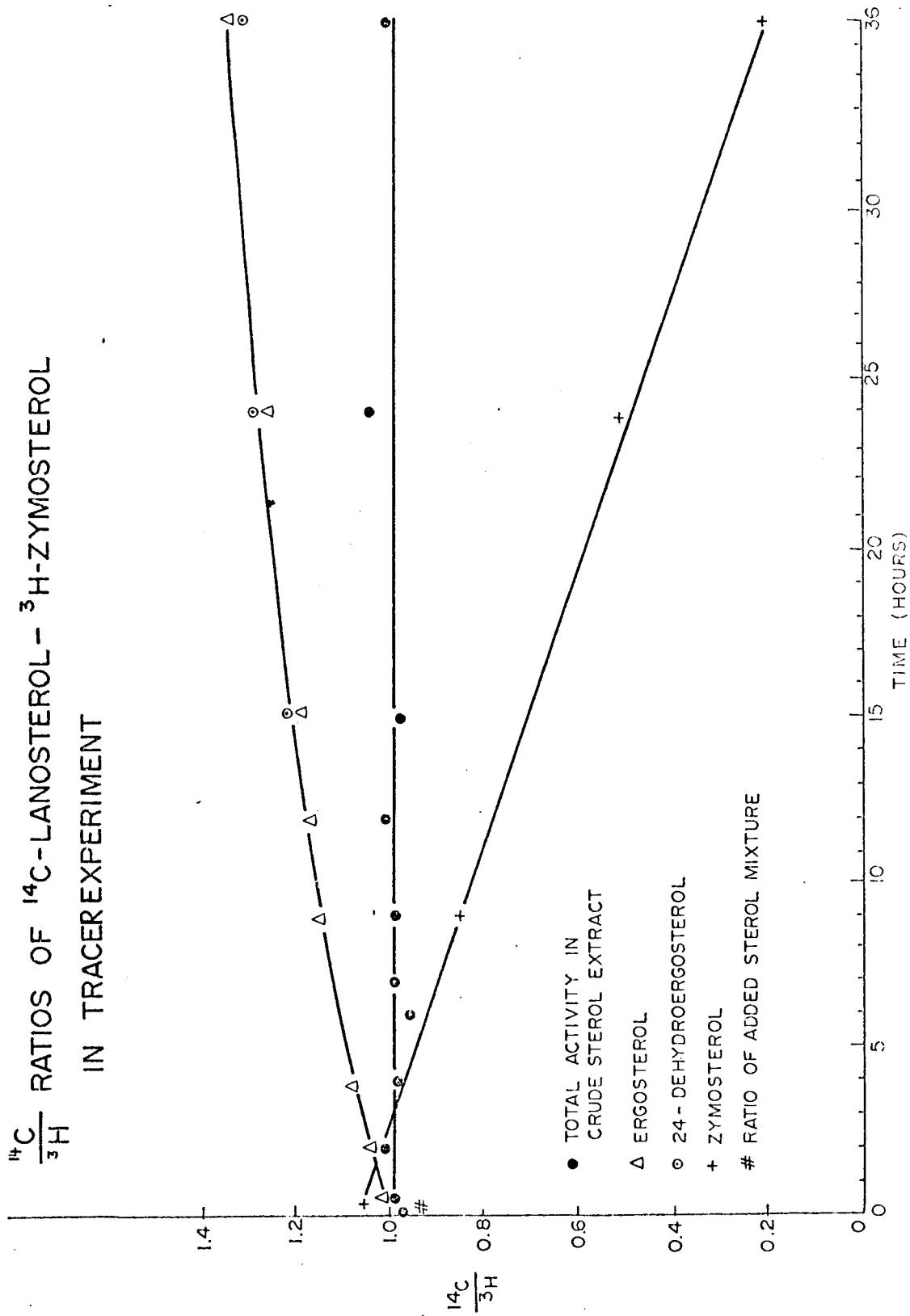


Figure 16: Variation in $^{14}C/^{3}H$ in Zymosterol and Ergosterol Upon Feeding 26,27-^{14}C Lanosterol and 2,4-^{3}H-Zymosterol

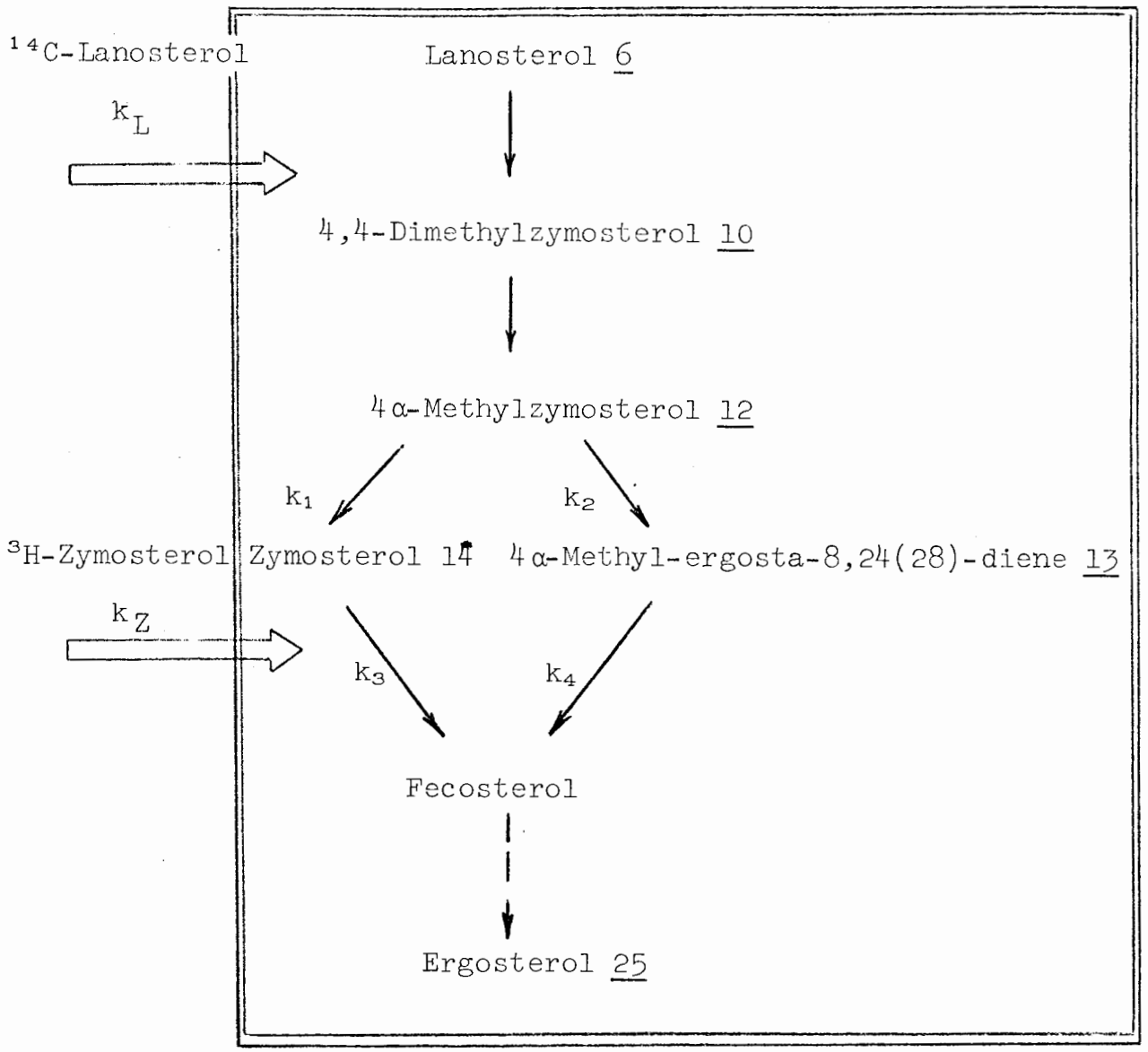


Figure 17: Proposed Model for Demethylation Alkylation Sequence.

ergosta-8,24(28)-diene-3β-ol (13) are not inconvertible. A preliminary experiment showed that tritium labelled zymosterol was efficiently incorporated into ergosterol after only 6 hours incubation with whole yeast cells. In order to further study the importance of zymosterol as an ergosterol precursor, a double label tracer experiment was carried out.

A mixture of 26,27-^{14}C-lanosterol and 2,4-^{3}H-zymosterol was fed to a growing Saccharomyces cerevisiae culture which had, prior to feeding, been depleted of endogeneous sterols by anaerobic growth. Aliquots were removed at set time intervals washed with buffer‡, and analyzed for $^{14}C/\,^{3}H$ ratio in the total sterol fraction, in zymosterol, tetraene-26 and in ergosterol. The results are shown in Fig. 16.

In the total sterol fraction the $^{14}C/\,^{3}H$ ratios were found to remain constant over the period of the experiment (36 hours). Since the weight as well as the activity of the two added precursors was close to unity, this observation is interpreted as indicating that the rates of diffusion (k_L and k_Z Fig. 17) of lanosterol and zymosterol into the cells are of the same order of magnitude. Although lanosterol and zymosterol both appear to be efficient precursors in whole yeast cells,

‡ The buffer wash was required to remove extracellular lanosterol and zymosterol. This was especially important in the case of zymosterol since any labelled extracellular zymosterol present during the hydrolytic work up of the cells of the culture would be mixed with intracellular zymosterol. This would give rise to abnormally low $C^{14}/\,^{3}H$ ratios in zymosterol in the early stages of incubation. The buffer wash used efficiently removed extracellular sterols. These were found to be in water soluble form (Parks et al.[79], Anding et al.[80]).

the rates at which they are incorporated into final product vary considerably with time. The steadily decreasing $^{14}C/^{3}H$ ratio observed in zymosterol is interpreted in terms of efficient conversion of zymosterol to ergosterol during early stages but less efficient conversion as the system reaches its normal equilibrium stage. The steadily increasing $^{14}C/^{3}H$ ratio in ergosterol indicates that ^{14}C-lanosterol is incorporated into ergosterol via a pathway not involving zymosterol (e.g. 12 → 13 → 16 → 25). In zymosterol, in addition to the relative amount of tritium increasing with time, the absolute amount of zymosterol increases. If Fig. 3 represents the actual state of affairs, the only route by which ^{14}C can pass into zymosterol in this experiment is via the 4α methyl precursor 12. In order that there may be an increase in the tritium content in zymosterol relative to ^{14}C, the rate of the lanosterol → zymosterol transformation must be smaller than the rate of diffusion of ^{3}H-zymosterol from the exogenous pool into the cells. If one considers the biosynthetic route from lanosterol to fecosterol to branch at the 4α-methyl-zymosterol (12) stage, the differing $^{14}C/^{3}H$ ratios in zymosterol compared to ergosterol represent competition between k_1 vs k_2 and k_3 vs k_4. Relevant to the interpretation of the present experiment in terms of Fig. 16 are the reports by Gaylor et al.[45] that zymosterol competitively inhibits the nuclear demethylation of 4α methyl sterols (i.e. slows processes such as k_1 and k_4). Hunter and Rose[74] further suggest that the synthesis of zymosterol (i.e. lanosterol → zymosterol) is

moderated by the accumulation of ergosterol. Since both zymosterol and ergosterol accumulate under the present conditions it is to be expected that whatever their initial values, k_1 and k_4 decrease during the course of this experiment.

The effect of zymosterol and ergosterol concentration on the rate of methyl transfer (k_2 and k_3) has been observed. Katsuki and Bloch[4] describe evidence suggesting that upon increasing zymosterol concentrations, the incorporation of [^{14}C-methyl]-methionine into ergosterol is decreased.

Working with a soluble Δ24-sterol methyltransferase isolated from yeast, Moore and Gaylor[15] corroborated this observation. They found that at high concentrations of zymosterol, the methyltransferase system was inhibited by addition of ergosterol whereas at lower concentrations of zymosterol, addition of ergosterol had a stimulatory effect. These observations would suggest that from their initial values, k_2 and k_3 would decrease as zymosterol and ergosterol increase in concentration.

The present experiment clearly shows that both pathways, 12 → 13 → 16, and 12 → 14 → 16 operate at all times. Their relative importance however is different. The low concentration of 13 throughout the latter course of the experiment means $k_4 \geq k_2$. At early stages k_3 is much greater than k_2 because zymosterol is the better substrate for transmethylase and in addition ergosterol stimulates transmethylation. As zymosterol increases, it starts to inhibit demethylation of

12 (k_1) and so slows or moderates its own production, i.e. product inhibition. Ergosterol begins to inhibit methylation of zymosterol (k_3) which in turn could make 24-methylation of 12 (k_2) more competitive with its 4α-demethylation (k_1).

Since 13 does not accumulate, its demethylation (k_4) does not appear to be inhibited by the accumulation of zymosterol. It would seem that whatever their initial relative contributions, the route via 14 is expected to be inhibited to a larger extent than that via 13 with the accumulation of zymosterol and ergosterol. The changes in $^{14}C/^{3}H$ ratios of zymosterol and ergosterol revealed in Figure 16 are easily interpreted in terms of the above.

3. Isomerization of the Δ^8-Double Bond

Tracer experiments were subsequently carried out with the nys-3 mutant. Isolation experiments had already established the absence of ergosterol and the presence of $\Delta^{7,22}$diene 19 as the major sterol. Incubation with labelled episterol (22) was expected to yield labelled $\Delta^{7,22}$-diene 19, $\Delta^{7,22,24(28)}$-triene 21 and Δ^7-monoene 20 but no activity was expected in ergosterol. This expectation was confirmed (Experiment #11). In addition, the $\Delta^{8,22}$-diene-11 was checked for activity. This compound as well as fecosterol, were found to contain label. Radioactivity in these Δ^8 intermediates can be explained only if $\Delta^8 \rightarrow \Delta^7$ isomerization is reversible.

Gaylor et al.[66] have investigated several aspects of enzyme system isolated from rat liver which isomerizes Δ^8 isomers. They found that although the rate of isomerization of 4α-methyl sterols was comparable to that of 4-desmethyl sterols, the presence of a 14α-methyl group prevented isomerization. In addition they found the isomerization to be irreversible. Working with liver microsomes, it was shown that the 9α-hydrogen, introduced when Δ^8 was isomerized to Δ^7, originated from the medium and not via an intramolecular hydrogen migration from C-7[81]. Furthermore, when cholest-7-en-3β-ol was reisolated after incubation in the presence of tritiated water, activity was found to be incorporated at the C-9 position[82]. This requires the reversibility of the $\Delta^8 \rightarrow \Delta^7$ transformation in microsomes. The lack of reversibility in the case of the

isolated enzyme but not with the whole cell system suggests that two different enzymes catalyse the forward and reverse reaction. No analogous information is available for yeast systems. Isomerization of the Δ^8 double bond involves stereospecific loss of the 7β hydrogen atom in mammalian tissues but in yeast the 7α proton is eliminated[83,84] indicating that there is some divergence between the two systems.

In a further experiment (Experiment #12), labelled fecosterol was incubated with nys-3. Activity was found to be associated with 15, 17 and 22 as well as with the $\Delta^{7,22}$-diene-19. These results indicate that modification of the side chain at the Δ^8 level can occur in any order before isomerization of Δ^8 to Δ^7 takes place. If one assumes that not only the episterol → fecosterol transformation but other $\Delta^8 \rightarrow \Delta^7$ isomerizations are reversible, a logical rationale for the constant ratios of the Δ^8, Δ^7 isomeric pairs observed in the mutant yeast strain emerges.

Having established the reversibility of the $\Delta^8 \rightarrow \Delta^7$ transformation in the mutant it became significant to determine its reversibility in the normal strain. Isolation procedures suggested the presence of the Δ^8-sterol-15 as well as of the $\Delta^{8,22}$-diene-11. Feeding of ^{14}C-episterol-22 to normal yeast produced labelled $\Delta^{8,22}$-diene-11 (Experiment 13). The incorporation of activity into sterol 11 was however low compared with that obtained in nys-3. The suggestion is put forward that the rather high concentration of $\Delta^{7,22}$-diene in the mutant

exerts an influence on the isomerization equilibrium, e.g. $\Delta^7 \rightarrow \Delta^8$ isomerization is more prevalent in the mutant than in the normal yeast where the concentration of Δ^7-sterols is low.

4. Introduction of Δ^5-Double Bond

Isolation procedures as well as tracer experiments carried out in this study established the fact that the enzymatic block in the mutant nys-3 is due to the absence and/or the inhibition of the enzymatic system introducing the Δ^5 double bond (e.g. no transformations occur between the Δ^7 and the $\Delta^{5,7}$ level). Two alternative mechanisms have been proposed for the introduction of the Δ^5 double bond. One involves a hydroxylation-dehydration mechanism, while the second involves the direct elimination of two hydrogens from carbons 5 and 6. Topham and Gaylor[16] showed that ergosta-7,22-dien-3β,5α-diol (56) is converted to ergosterol under anaerobic conditions by a cell-free extract of S. cerevisiae. These same workers isolated an enzyme from S. cerevisiae which catalyses the conversion of the hydroxylated intermediate to ergosterol. Akhtar et al.[32] however failed to incorporate [3-^{3}H] ergosta-7,22-dien-3β,5α-diol into ergosterol in yeast whole cells under anaerobic conditions. Under aerobic conditions, the same compound was efficiently transformed to ergosterol. These workers then proceeded to incorporate 5,6-dihydro [5α,6α-$^3H_2$]-ergosterol into ergosterol. The biosynthesis of ergosterol from the latter resulted in complete loss of tritium from both the 5α- and the 6α-position. They therefore con-

cluded that the introduction of the 5,6 double bond in ergosterol proceeded via a cis-removal of 5α- and 6α-protons rather than via a hydroxylation - dehydration mechanism. Topham and Gaylor[17] recently reported additional evidence for the dehydration mechanism. They were able to show that 3α-^{3}H-ergosta-7,22-dien-3β-ol is converted to ergosterol with loss of the 3α-^{3}H. The amount of substrate disappearance, calculated from tritium loss, was equivalent to the amount of ergosterol formed. A mechanism was proposed which is consistent with the loss of the 3α-hydrogen as well as with the elimination of the cis hydrogens at C-5 and C-6 (Figure 18).

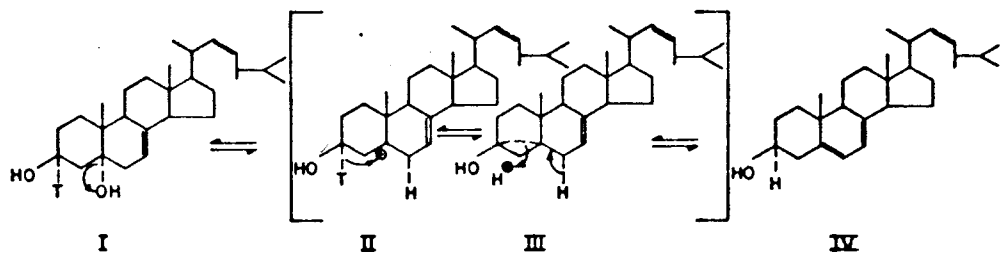


Figure 18: Possible Reaction Mechanism for the Conversion of Ergosta-7,22-dien-3β-5α-diol to Ergosterol (Topham and Gaylor[17]).

Since neither of the two reported investigations unequivocally demonstrated the obligatory intermediacy of a 5α-hydroxylated compound, we attempted to establish the involvement of such a sterol in ergosterol biosynthesis by a trapping experiment.

Labelled episterol was fed to growing, sterol depleted, yeast as usual. Unlabelled ergosta-7,22-dien-3β-5α-diol was added to the harvested cells and the mixture hydrolyzed in base. The re-isolated diol was radioactive. This observation demonstrates that a 5α-hydroxy intermediate is indeed involved in the introduction of a Δ^5 double bond.

CONCLUSIONS

This investigation has conclusively shown the presence of multiple pathways in the biosynthesis of ergosterol in the yeast, S. cerevisiae. The elucidation of the individual biosynthetic sequences described in this investigation have been achieved by application of three principal lines of investigation: 1) chemical synthesis of potential biogenetic intermediates, 2) an extensive search in yeast sterol fractions for such compounds and 3) the involvement of these compounds as biosynthetic precursors of ergosterol by requisite feeding and trapping experiments.

The initial construction of a biogenetic model describing a variety of potential pathways between lanosterol and ergosterol was found to be of prime importance throughout this work. It became immediately obvious from this model that several new sterols, so far not isolated from yeast, had to be postulated if particular sequences were to be invoked. A number of such sterols, as suggested by the model network, were synthesized chemically. The subsequent investigation of the yeast sterol fraction did indeed reveal the presence of most of these hitherto non-implicated sterols. As can be seen in Fig. 3, the number of possible transformations decreases proceeding from the top to bottom. The structures of the isolated yeast sterols do however suggest an increase of the operative transformations towards the end of ergosterol biosynthesis.

Only one 4,4,14-trimethyl intermediate (lanosterol), one

4,4-dimethyl sterol, and two 4-mono-methyl sterols have so far been detected. But, as has been shown in this investigation, practically all possible desmethyl sterols occur in this organism. The noticeable exceptions are desmethyl sterols not methylated at C-24, eg. ring B modified derivatives of zymosterol (14) or cholesta-8,22,24-trien-3β-ol (18). These have not been observed nor are they likely to occur in this yeast.

In general, it seems that enzymes catalyzing 24-methylation and nuclear demethylation are far more specific than enzyme(s) involved in introduction and reduction of double bonds. The enzymatic studies by Gaylor and co-workers[11], have shown that Δ^{24} sterol-methyl transferases as well as nuclear demethylases have rather strict structural specificities. If these transformations catalyzed by purified enzymes are representative of transformations in whole cells, this could be the reason for the observance of the operation of relatively few of the transformations in the upper part of the model illustrated in Figs. 3 and 4. In the desmethyl sterol region, transformations seem to occur in any possible order giving rise to the different pathways shown in Fig. 15.

Since each one of the routes observed represents a different possible ordering of the sequence in which each functionallity, is introduced, it is interesting to view the results from a different perspective. Assuming each sterol transformation is mediated by a single enzymatic system, then the relative rates of the individual transformations in various sequences are in

essence the substrate specificities of each enzyme system. The particular enzymatic systems with which one is concerned in the present cases are those introducing the Δ^7, Δ^5 and Δ^{22} unsaturation as well as that reducing the $\Delta^{24(28)}$ double bond. These systems, regardless of their nature, unitary or otherwise, can and do obviously operate on several closely related substrates.

The relative importance of the observed pathways seems, however, to depend considerably on the conditions one imposes on the system. Two major situations seem to exist. One in which the normal steady state sterol distribution prevailing in systems in their stationary growth phase is obtained, and a second by which the sterol depleted system reaches its normal sterol content during an accelerated growth period. This second situation suggest a rather intricate regulatory mechanism. Whether or not the rather fluid system observed under forced growth represents the true picture is difficult to assess. The general pattern which seems to emerge at this state is the following: The Δ^5 double is one of the last transformations to occur, although some crossover with the reduction of the $\Delta^{24(28)}$ double bond occurs also. In a first approximation, pathways involving the $\Delta^{24(28)}$ double bond seem to be more important than those involving fully saturated side-chains. The introduction of the Δ^{22} double bond can occur at the Δ^8, Δ^7 as well as at the $\Delta^{5,7}$ level. The only transformation which was found to be reversible to a significant extent was the $\Delta^8 \rightarrow \Delta^7$ isomerization. This step could be part of a regulatory mechanism.

Since it was found that a) Δ^7-4-methyl sterols are not demethylated in yeast[45], b) no Δ^7-methylated sterols have been found in yeast[20] (they do occur in plants and animals), and c) 4,4-dimethyl ergosterol was not incorporated into ergosterol by whole yeast cells[26], it can be concluded that nuclear demethylation takes place at the Δ^8 level.

Methylation at C-24 can not occur at the lanosterol level since 24-methylene lanosterol has been excluded as an intermediate by trapping experiments[26]. The most likely level of methylation is at the zymosterol stage. It has been reported to be the most highly suitable substrate for the methylating enzyme. In addition, it has been shown in this work that a sequence involving zymosterol forms a significant, if not the major pathway involved in the transformation of lanosterol to Δ^8 sterols. The information available so far seems to indicate that the major route(s) to ergosterol involves the sequence shown in Fig. 19.

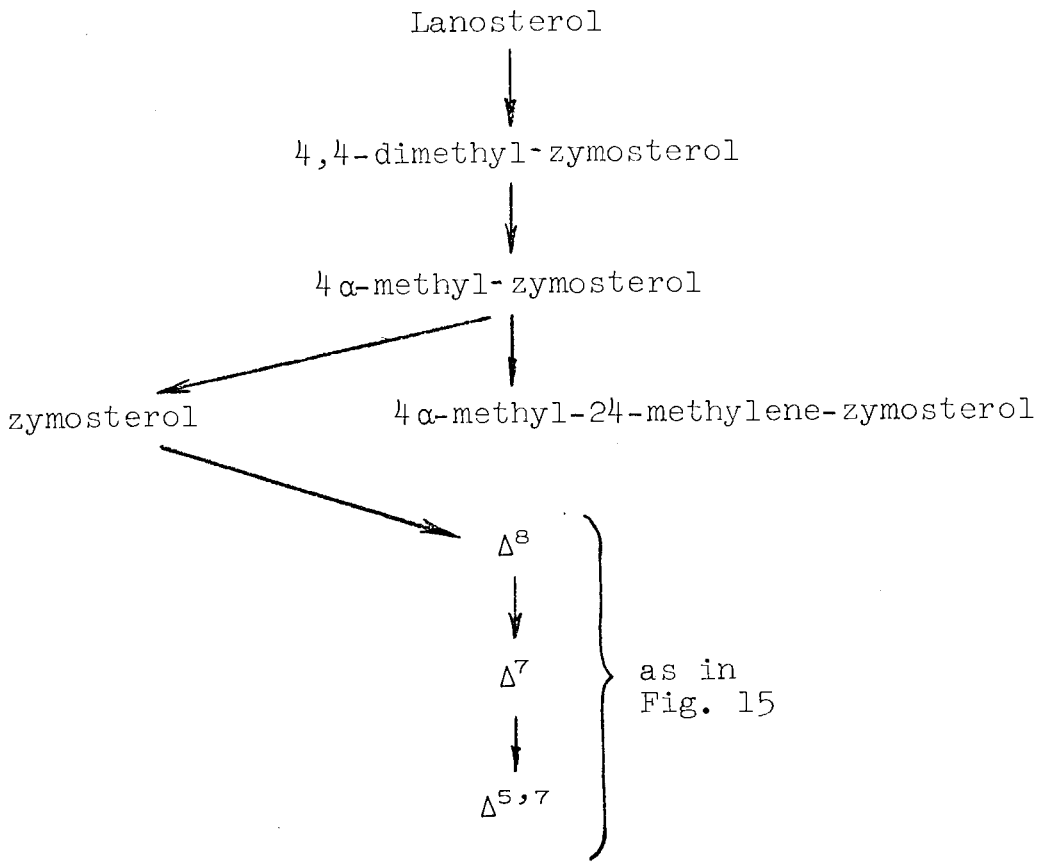
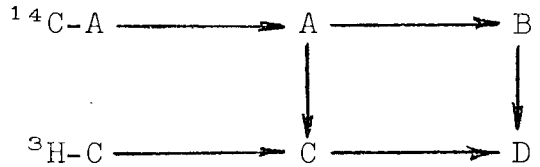


Figure 19: Proposed Operative Biosynthetic Pathway from Lanosterol to Ergosterol

PROPOSED RESEARCH

Several aspects of ergosterol biosynthesis in yeast (or other microorganisms) which evolved from this work await further investigation. The most obvious being the investigation of the relative importance of the pathways on a quantitative basis. One experiment carried out during this study, e.g. the lanosterol-zymosterol double label experiment, seems to indicate the feasibility of a similar approach to the determination of the importance of alternate biosynthetic routes. By feeding simultaneously two different labelled precursors (^{14}C and ^{3}H) at the most efficient network positions one of the precursors can act as an internal standard. As illustrated below, feeding ^{14}C-A and ^{3}H-C leads to the establishment of a $^{14}C/^{3}H$ ratio in C which on comparison with the $^{14}C/^{3}H$ ration in D provides sufficient information to estimate the relative importance of the A → C → D route vs the A → B → D route.



In order to quantitatively calculate the contributions of each pathway a workable mathematical model has however to be elaborated.

A second point intimately connected with the sterol biosynthesis in yeast as well as other sterol producing microorganisms is the interrelation of sterol content and polyene antibiotic resistance. The composition of the sterol content

changes with increasing degrees of resistance as well as with the type of antibiotic used[52]. The nys-3 mutant investigated here is the first S. cerevisiae mutant for which sterol biosynthesis has been investigated in detail. The lack of ergosterol and other ring B conjugated sterols as well as the accumulation of $\Delta^{7,22}$-diene and the presence of all other normally occurring sterols in the nystatin resistant strain may indicate that nystatin does not interact with sterols other than those possessing ring B conjugation such as ergosterol. Investigation of the sterol content and sterol biosynthesis of mutants possessing different degrees of resistance as well as of strains resistant to different antibiotics should provide additional information on the relationship between antibiotic resistance and sterol content. Such studies might aid in the elucidation of the mode of action of polyene antibiotics at the molecular level.

Study of the so-called methionine-requiring strains is an additional area of interest. If suitable growth can be obtained without addition of methionine one would expect C-24-unmethylated sterols to accumulate, e.g. zymosterol or even ring B modified derivatives of zymosterol. Such compounds have not been isolated in yeast although they are common in cholesterol biosynthesis.

EXPERIMENTAL

Instruments and materials. M. pts. were obtained on a Fisher-Johns m. p. apparatus and are uncorrected. Spectra were obtained on the following instruments: Perkin-Elmer 457 (IR), Unicam SP 800 (UV), and Varian A 56/60 or XL-100 NMR spectrometer. NMR results are reported as δ using TMS as internal standard (δ=0). Ionising voltage for MS, Perkin-Elmer Hitachi RMU-7, was 80eV if not specifically mentioned otherwise. Stigmasterol was generously supplied by the Upjohn Company, Kalamazoo, Michigan. Yeast was donated by Carling Breweries Limited in Vancouver.

Separation of Yeast Sterols by Column Chromatography

Free sterols were separated on alumina (activity II). The sterol mixture (approx. 10 g) dissolved in a minumum amount of benzene was added to the column (200 - 250 g of alumina) made up in hexane. Elution was started with pure hexane. Benzene was added automatically until mixture had changed to pure benzene, then ethyl ether was added until a 1:1 solution of benzene-ethyl ether had been obtained. In a typical run ca 15 l of solvent were used. Fractions of 10 ml were collected automatically. The individual fractions were analyzed by T. L. C., g. l. c. and/or UV and the corresponding fractions were set aside and fractionated in a second run.

Sterol acetates were separated on silica gel coated with 15% $AgNO_3$. Elution was carried out in an analogous way using hexane/ether as solvent.

Thin-layer Chromatography (T. L. C.):

Plates for preparative chromatography were made up by spreading a mixture of: 18 g $AgNO_3$

40 g silica gel G (Merck)

0.1 g rhodamine 6 G

65 - 70 ml H_2O

20 ml ethanol

over 4 plates (20 x 20 cm). Unless otherwise stated, benzene was used as solvent for the separation of sterol acetates. The sterol containing regions were revealed as dark red bands on pale-red background under daylight. Under UV-light, sterol acetates with isolated double bonds appeared as bright yellow bands while those with conjugated double bonds gave dark bands. The individual bands were recovered from the plates and the material extracted four times with diethyl ether by resuspension and centrifugation. The ether was evaporated, the residue taken up in 10 ml hexane-ether (4:1, v/v) and percolated through a column (10 x 1 cm) of celite:silica gel (1:1, w/w) to remove the extracted rhodamine. The column was washed with additional 50 ml of the same solvent mixture. The solvent was evaporated giving the crude sterol acetates.

Test experiments with several radioactive compounds showed a recovery of at least 85% using the above method. For analytical plates the rhodamine 6 G was omitted and the spots made visible by spraying with either $SbCl_3$ in glacial acetic acid or a mixture of 0.5% vanillin in H_2SO_4-ethanol 4:1 v/v and heating for 5 - 10 min at 120° C.

Authentic samples were spotted on one side of all plates as references.

Gas Chromatography (g. l. c.)

A Varian Model 2100 chromatograph equipped with an all glass system and a H_2 flame ionization detector was used. Standard chart speed was 5 inches per hr. The glass columns (3.4 m x 25 mm) were packed with either 3% QF-1 or 3% XF-60 on Chromosorb G-AW-DMCS (80 - 100 mesh). Flow rate of helium was 60 ml hr^{-1}. Retention times are relative to dihydrocholesterol. Quantitative determinations were carried out according to the method of Rozanski.[96]

Base Hydrolysis and Extraction of the Nonsaponifiable Fraction:

The yeast cells were harvested by centrifugation and the wet weight determined. The cells were resuspended in ca 50 ml dist. H_2O and transferred into a hydrolysis mixture made up as follows: 15 g KOH

20 ml H_2O

Ethanol to give a total of 100 ml.

This solution (100 ml) was mixed with wet cells (2 g). The mixture was stirred at reflux under an atmosphere of nitrogen for 3 hrs. An equal amount of cold dist. water was added and the mixture extracted with four 100 ml portions of heptane. The heptane extract was washed with water until neutral and dried over anhydr. Na_2SO_4. Evaporation of the solvent gave the crude nonsaponifiable material.

General Procedure for the Incorporation Studies

The radioactive sterol of known activity (1 - 5 mg) was emulsified in Tween 80 (~50 mg) and dissolved in 1 ml of acetone. This mixture was diluted with dist. water (~3 ml) and added to the incubation vessel. The acetone was removed with a stream of nitrogen. Sterilized growth medium (1.5 l) M-2 was added and the solution stirred vigorously for 10 min. Anaerobically pretreated yeast cells were added and the culture grown as described above. At the end of the incubation time, the cells were collected by centrifugation (20 min at 2500 rpm), resuspended in phosphate buffer, centrifuged and hydrolyzed and extracted as above.

The crude nonsaponifiable material was dried under vacuum, weighed and dissolved in heptane. The solution was made up to 50 ml and a 0.5 ml aliquot removed to determine the total incorporation. The remainder of the solution was evaporated and acetylated in pyridine acetic anhydride (2:1 v/v) overnight at room temperature. The acetates were worked up the usual way and the crude acetates dissolved in 50 ml of heptane. Total activity of the acetates was determined. The heptane was evaporated and the acetates separated on T. L. C. into the single fractions as described above.

The single fractions were dissolved in 25 ml of heptane and a 0.5 ml aliquot removed for counting giving the total activity in the band. A known amount was injected into g. l. c. and the total content of the component in question determined as above. Unlabelled material was usually added and the sterol then

purified and crystallized to constant activity. The reported % incorporation into the individual precursors is based on the actual amount of the particular sterol present as determined by g. l. c.

Yeast Growth Conditions:

1. Normal Strain

Yeast Saccharomyces cerevisiae, of an unknown strain (ale brewers yeast) was obtained fresh from a local brewery each time before use. The yeast was pretreated under anerobic conditions, then resuspended for aerobic growth. For the anaerobic growth phase the liquid medium (M-1) had the following composition (Turner et al., 1965[97]):

1) DIFCO Malt Extract	0.5%	
2) B. B. L. Yeast Extract	1.5%	
3) NH_4Cl	0.1%	
4) KH_2PO_4	0.68%	0.1 molar in phosphate pH 6.4
5) K_2HPO_4	0.87%	
6) Glucose	2.5%	

Components 1-5 and glucose were dissolved in 500 ml of water each in a 1-liter flask and sterilized separately for 15 min at 120°. Immediately after removal from the autoclave the solutions were combined and cooled to 30°. The flask was fitted with Bunsen valve and gas inlet tube and flushed with nitrogen for 10 min then 50 ml yeast cream (ca 8 g dry weight) in phosphate buffer were added. The flask was filled complete-

ly with sterilized buffer, flushed with nitrogen for additional 10 min and kept at 30° in static culture for 80 hrs. The culture was flushed approx. every 12 hrs with nitrogen for 5 min. Then the yeast was harvested by centrifugation, washed twice with phosphate buffer, resuspended in buffer, refrigerated for 10 - 15 hrs and then used for the aerobic growth phase. The yeast cells were added to 1.5 l sterilized growth medium (M-2) containing per liter: Glucose 40.0 g

K_2HPO_4 8.7 g

KH_2PO_4 6.8 g

The yeast was grown in a 4 l flask on a Virtis fermenter under the following conditions:

Temp.: 30°

Air: 4 - 5 l min^{-1}

Stirring: 400 RPM

Time: as called for by the individual experiment.

Dow Corning Antifoam A Spray was used to prevent foaming.

2. Mutant Strain

A Saccharomyces cerevisiae yeast mutant designated nys-3[37] obtained from Prof. L. W. Parks was used. The strain was originally isolated by Woods.[51] The cells were maintained on YEPD-Agar slants and recultured every 3 - 4 weeks. Aerobic cultures were grown on a fermenter in one liter batches in medium M-1.

Aeration was 4 - 5 l/min, stirring was set at 400 rpm and the temperature was kept at 30° C. The cultures were inoculated with a small culture grown overnight on a shaker

in the same medium. The cells were harvested after ca 20 hrs by centrifugation and base hydrolyzed. When anaerobic-aerobic growth was desired, cells obtained as above were washed with phosphate buffer, resuspended in fresh medium of the same composition and kept in a still culture under nitrogen for ca 70 hrs. The cells were then harvested, washed with buffer and resuspended in a medium containing glucose (4%) and phosphate buffer (0.1 molar). Fermenter conditions were the same as above.

When large quantities of cells were produced in a series of larger batches, each time using cells from the previous batch for inoculation, the sterol content changed. Increasing amounts of ergosterol were found. This could be prevented by inoculating each batch from stock cultures and by keeping the batches small. Crude sterol mixtures containing detectable amounts of ergosterol (UV) were therefore not considered representative of this mutant and were set aside. Nystatin resistance on solid medium was determined as described by Bard[52]. When resistance was determined in liquid medium, nystatin was added as a Tween 80 emulsion in water to a medium of the same composition as above (M-1).

Preparation of 3β-Acetoxy-ergosta-8,22-diene, 32.

This sterol was prepared from 31[57]. It had the following physical properties: m. p. 168 - 169° (lit.[57] 166 - 169°), n. m. r. ($CDCl_3$): δ 0.58 (CH_3-C-18, s), 0.94 (CH_3-C-19, s), 0.82, 0.92, 1.02 (CH_3- at C-20, C-24, C-25, d, J = 7), 2.02 (CH_3-COO- , s), 5.2 (H-C-22, H-C-23, broad multiplet).

Preparation of 3β-Acetoxy-ergost-8-ene, 33.

This sterol was prepared by hydrogenation of the diene 32 in ethyl acetate in the presence of Adam's catalyst[57], m. p. 158 - 159° (lit.[57] 156 - 158°). N. m. r. ($CDCl_3$): δ 0.60 (CH_3-C-18, s), 0.95 (CH_3-C-19, s), 0.80, 0.87, 0.91 (CH_3- at C-20, C-24, C-25, d, J = 7) and 2.02 (CH_3-COO-, s).

Preparation of 3β-Acetoxy-ergosta-8,22,24(28)-triene, 35.

(a) Attempted direct preparation.

A solution of 3β-acetoxy-ergosta-8,22-diene (150 mg) in dry ether (50 ml) at 0° was treated with an equimolar amount of Br_2 in glacial acetic acid (0.5 ml). Bromine was taken up immediately. The reaction mixture was poured into water and the ether extract washed until neutral. The extract was dried over Na_2SO_4 and evaporated. The pale yellow oil obtained was taken up in toluene to which 1,5-diaza-bicyclo [4, 3, 0] non-5-ene was added and the solution refluxed for 18 hours[58]. After work up a mixture of several compounds was obtained as evident from g. l. c. The mixture showed a strong UV absorption in the region typical of a 7,9(11) diene and only slight absorption near 230 nm. The major product (ca 70%

of the mixture) had a rel. ret. time on g. l. c. identical with 3β-acetoxy-ergosta-7,9(11),22-triene. One of the minor products had the same ret. time as the desired product obtained by the method described below. The mixture was not further investigated.

(b) Preparation via the 22-aldehyde.

1) When 3β-acetoxy-ergosta-8,22-diene (32) was ozonized in a solution of CH_2Cl_2 containing 2% pyridine at -70° and subsequently worked up using zinc and acetic acid as described earlier[43], a product was obtained which did not show the expected n. m. r. spectrum. The chemical shifts for the angular methyl groups corresponded to the calculated values corresponding to the isomeric aldehyde, 3β-acetoxy-23,24-di-nor 5α-chol-8(14)-en-22-al. The product was not further investigated.

2) The 3β-acetoxy-ergosta-8,22-diene was ozonized according to the modified method of Pappas et al.[85] The diene was ozonized at -70° in a solution of CH_2Cl_2 containing 0.5% pyridine. Ozone was passed through the solution until a pale blue color persisted (~5 min). The system was flushed with N_2 and an excess of $(CH_3)_2S$ in methanol was added. The solution was stirred and allowed to rise slowly to r. t. The solution was evaporated, water was added and the aldehyde extracted with hexane and dried over Na_2SO_4. The product, 34, was approx. 93% pure by g. l. c. and was used without further purification. N. m. r. ($CDCl_3$): δ 0.61 (CH_3-C-18, s), 0.95

(CH_3-C-19, s) and 9.57 (aldehyde H, d, J = 3 Hz).

Ergosta-8,22-24(28)-trienyl-3β-acetate (35) was prepared from this aldehyde according to the method described earlier[43]. for preparation of ergosta-5,7,22-24(28)-tetren -3β-ol. An excess of 2-methylene-3-methyl- butan -1-triphenylphosphorane in THF was reacted with the above aldehyde (250 mg). The usual work up, acetylation and purification on T. L. C. yielded a product which melted at 131 - 133°. υ_{max} (KBr) 1722 and 890 cm^{-1}; λ_{max}^{EtOH} 230 mμ (ϵ=28500) and 240 mμ shoulder (ϵ = 16800); n. m. r. ($CDCl_3$): δ 0.57 (CH_3-C-18, s), 0.93 (CH_3-C-18), 1.05 ((CH_3)$_2$-C-25, CH_3-C-20 d, J = 7 Hz), 2.01 (CH_3-COO, s) 4.8 (CH_2 = C-24, d, J = 2 Hz), 5.51 (H-C-22, d of d, J = 16 Hz, J = 8 Hz) and 5.90 (H-C-23, d, J = 16 Hz).

Preparation of [28-^{14}C]-Ergosta-8,24(28)-dien -3β-ol, 16.

To 500 mg zymosterol acetate (36, 1.17 m mole) in 15 ml of THF and 3 ml of water, at 0°, NBS (250 mg, 1.4 m mole) was added with stirring. The mixture was stirred for 2 hrs, poured into water and extracted with ether. The extract was dried over Na_2SO_4 and evaporated. The product was crystallized several times from methanol to give 370 mg of 24-oxo-5α-cholest-8-en-3β-yl-acetate (37), pure by g. l. c. N. m. r. ($CDCl_3$): δ 0.62 (CH_3-C-18, s), 1.0 (CH_3-C-19, s) and 1.06 (CH_3-C-20, d, J = 7 Hz). The ketone (100 mg) was reacted with the ylide prepared from [^{14}C]-methyl triphenyl-phosphonium iodide and BuLi in THF analogous to methods described earlier. After the usual work up, acetylation, purification by T. L. C.

and crystallization from methanol, fecosterol acetate (38), pure by g. l. c., was isolated. The physical data were identical with those of fecosterol acetate isolated from yeast. Base hydrolysis gave fecosterol (16) which had an activity of 6.9×10^5 cpm/mg.

Preparation of 3β-Acetoxy-5α-ergosta-7,22-dien, 27.

Ergosterol acetate (36), (15 g, 0.034 mole) in 400 ml of thiophene free benzene, was hydrogenated in a 500 ml glass container at 40 psi with ca 10 g Raney Ni. After 2 hrs, 1.1 m of H_2 were taken up. The catalyst was filtered through a short column of Celite 535 and the benzene evaporated. The residue was recrystallized from ethyl acetate-methanol to yield 92% 27, m. p. 184.5 - 187° (lit. m. p.[86] 182.5 - 186.8°). υ_{max} (KBr) 1731, 1250 (-OAc), 1028 and 965 cm^{-1}; n. m. r. ($CDCl_3$): δ 0.55 (CH_3-C-18, s) 0.80 (CH_3-C-19, s), 0.82 ((CH_3)$_2$-C-26,27, d, J = 6.0 Hz), 0.91 (CH_3-C-28, d, J = 6.0) 1.01 (CH_3-C-20, d, J = 6.0), 2.01 (CH_3-CO-, s), 4.4 - 5.05 (H-C-3, m), 5.-5 - 5.3 (H-C-7, H-C-22, H-C-23, m).

Preparation of 3β-Acetoxy-5α-cholest-7-en-22-al, 37

The aldehyde 37 was prepared by ozonolysis according to Sakai et al.[87] from the acetate 27. It had m. p. 144 - 145°. υ_{max} (KBr) 2710, 1730 (-CHO), 1732 and 1250 (-OAc) cm^{-1}; n. m. r. ($CDCl_3$): δ 0.58 (CH_3-C-18, s), 0.82 (CH_3-C-19, s), 1.13 (CH_3-C-21, d, J = 6.5 Hz), 2.01 (CH_3-COO-, s), 2.1 - 2.5 (H-C-20, m), 4.9 - 5.0 (H-C-3, m) 5.1 - 5.33 (H-C-7, m) and 9.58 (H-CO-, d J = 3Hz). The n. m. r. spectrum of 37 is identical with that

reported by Sucrow et al.[88]

Preparation of 3β-Acetoxy-5α-cholesta-7,22-dien-24-one,38.

The aldehyde 37 was reacted in DMSO with 3-methylbutane-2-one-1-triphenylphosphorane for 60 hrs at 80°. The reaction mixture was treated with 10% aqueous H_2SO_4 and the product extracted with ether. The ether extract was washed several times with H_2O and dried over Na_2SO_4 (anhydr). The product was purified on a silica-gel column using 10% ethyl ether in hexane as eluting solvent to give 4.2 g (67.2), m. p. 143 - 144°. Melting point and spectra were identical with the values reported by Sucrow et al.[88]

υ_{max} (KBr) 1732 (-OAc), 1698, 1674, 1628 and 993 (-CH=CH-CO-) cm^{-1}; $\lambda_{max}^{methanol}$ 222 mμ (ε = 19550); n. m. r. ($CDCl_3$): δ 0.575 (CH_3-C-18, s), 0.815 (CH_3-C-19, s), 1.1 ((CH_3)$_2$-C-25, CH_3-C-21, d, J = 7.1 Hz), 2.01 (CH_3-COO-, s), 2.81 (H-C-25, sept, J = 7.1 Hz), 4.4 - 4.9 (H-C-3, m), 5.05 - 5.28 (H-C-7, m), 6.06 (-C=CH-C=O, d, J = 16 Hz) and 6.74 (H-C=C-, d of d, J = 16, J = 8.5 Hz).

Preparation of 3β-Acetoxy-5α-cholest-7-en-24-one,39.

A solution of 1.2 g (2.73 mmole) of ketone 38 in 50 ml ethyl acetate was hydrogenated at r. t. and 50 psi pressure over 10% palladium/$BaSO_4$[89]. The suspension was filtered through a column of Celite 535 and recrystallized from methanol to give m. p. 97 - 98°.[88] υ_{max} (KBr) 1731, 1250 (-OAc) and 1716 (C=O) cm^{-1}; n. m. r. ($CDCl_3$): δ 0.525 (CH_3-C-18, s),

0.81 (CH_3-C-19, s), 1.08 ((CH_3)$_2$-C-25, CH_3-C-20, d, J = 7 Hz), 2.01 (CH_3COO-, s) 2.4 (-CH_2-25, t, J = 5.5 Hz), 2.61 (H-C-25, sept., J = 7 Hz), 4.45 - 4.95 (H-C-3, m), and 5.15 (H-C-7, m).

Preparation of 3β-Acetoxy-5α-ergosta-7,24(28)-dien, 40.

To a suspension of methyltriphenylphosphonium iodide (2.4 g, 10 mmole) in 50 ml dry tetrahydrofuran, a solution of BuLi in heptane (2.2 mmole) was added. The mixture was left to react under dry nitrogen for 1 hr at r. t. The ketone 39 (0.9 g, 2.05 mmole in THF) was added and the reaction mixture stirred for 1 hr at r. t. and refluxed for 2.5 hrs. Excess reagent was decomposed with wet THF. The mixture was then poured into 500 ml MeOH/H_2O (1:5) and the product extracted with ether. The ether extract was washed several times with H_2O and saturated NaCl soln., dried over anhydr. Na_2SO_4 and evaporated. The crude product was reacetylated in pyridine-acetic anhydride (2:1) at r. t. for ca 12 hrs. The acetate was worked up in the usual way by rapid filtering through a short silica gel column using hexane-ether (9:1) as solvent. Crystallization from ethanol gave 0.55 g (60.5%) of product 40, m. p. 136 - 137°. Two more recrystallizations brought the m. p. to 140 - 140.5° (lit.[90] m. p. 140). No m. p. depression was observed when the synthetic product was mixed with episterol acetate isolated from yeast.

υ_{max} (KBr) 887 cm^{-1}; n. m. r. ($CDCl_3$): δ 0.54 (CH_3-C-18, s), 0.81 (CH_3-C-19, s), 1.03 ((CH_3)$_2$-C-26,27, CH_3-C-21, d, J = 6.5 Hz), 2.01 (CH_3-CO-, s), 4.70 (CH_2=, d, J = 2.5 Hz) and

5.15 (H-C-7, broad s). Spectra of the synthetic product and of the natural compound were identical. Hydrolysis of episterol acetate 40 gave the free alcohol 22, with m. p. 130 - 130.5° (lit.[90] 131).

Analysis: Calcd. for $C_{28}H_{46}O$: C 84.35, H 11.63
Found: C 84.29, H 11.50.

Preparation of [28-^{14}C]-Ergosta-5α-7,24(28)-diene-3β-ol, 22.

This sterol was prepared as above from ketone 39 using [^{14}C]-methyl-triphenylphosphonium iodide. The compound had identical spectra with that prepared above and had an activity of 6.2×10^5 cpm/mg.

Analysis: Calcd. for $C_{28}H_{46}O$: C 84.35, H 11.63
Found: C 84.27, H 11.64.

Preparation of 3β-Acetoxy-5α-ergosta-7,22,24(28)-triene, 41.

Ketone 38 (1.0 g; 2.28 mmole) was reacted in THF with a 5-fold excess of the ylide, generated from methyltriphenylphosphonium iodide and BuLi in THF, in the same manner as for compound 40. The same work up and reacetylation gave 652 mg (65%) 41, m. p. 134.5 - 136.5. υ_{max} (KBr) 1640, 975, 965 and 890 cm^{-1}; λ_{max}^{EtOH} 232 mμ (ϵ = 33100); n. m. r. ($CDCl^3$): δ 0.54 (CH_3-C-18, s), 0.80 (CH_3-C-19, s) 1.06 ((CH_3)$_2$-C-25, CH_3-C-20, d, J = 7 Hz), 2.01 (CH_3-COO-, s), 2.48 (H-C-25, sept., J = 7 Hz), 4.8 (CH_2=C-24, d, J = 2Hz), 5.14 (H-C-7, broad s), 5.52 (H-C-22, d of d J = 16 Hz, J = 8 Hz) and 5.92 (H-C-23, d, J = 16 Hz). Hydrolysis of the acetate 41 gave the free

alcohol 21 with m. p. 121 - 124°.

Analysis: Calcd. for $C_{28}H_{44}O$: C 84.79, H 11.18

Found: C 84.55, H 11.01.

I. r. and n. m. r. spectra of the synthetic and natural sterols were identical.

Preparation of [28-^{14}C]-Ergosta-5α-7,22,24(28)-trien-3β-ol, 21.

This compound was prepared as above from ketone 38 using [^{14}C]-methyl-triphenylphosphonium iodide. The triene had the same properties as 21 above and showed an activity of 4.52×10^5 cpm/mg.

Analysis: Calcd. for $C_{28}H_{46}O$: C 84.35, H 11.63

Found: C 84.51, H 11.48.

Preparation of Ergost-7-en-3β-ol, 20.

Ergosterol acetate 36 (2.0 g) dissolved in thiopene-free benzene (70 ml) was hydrogenated at r. t. and 45 psi using freshly prepared Raney Ni until 2.2 mole equiv. were absorbed. The mixture was filtered over Celite 535, the solvent evaporated and the compound recrystallized from ethanol. The compound was separated from unreacted starting material (~6% by g. l. c.) on $AgNO_3$ coated silica gel. M. p. 162 - 164 (Lit. 162 - 164°[91]; 153[34]; 158 - 160[92]). N. m. r. ($CDCl_3$): δ 0.54 (CH_3-C-18, s), 0.78 (CH_3-C-28, d, J = 6.5 Hz), 0.81 (CH_3-C-19, s), 0.85 (CH_3-C-26,27, d, J = 6.5 Hz), 0.93 (CH_3-C-21, d, J = 6.5 Hz), 2.0 (CH_3-COO-, s) 4.4 - 4.95 (H-C-3, m) and 5.18 (H-C-7, m).

The acetate was hydrolyzed with 2% K_2CO_3 - 10% H_2O in methanol to give the alcohol, m. p. 145 - 146° (lit. 141 - 145[34]; 148[92]).

N. m. r. ($CDCl_3$): δ 0.525 (CH_3-C-18, s), 0.79 (CH_3-C-19, s), 0.79 (CH_3-C-28, d, J = 6.5 Hz), 0.825 (CH_3-C-26, 27, d, J = 6.5), 0.92 (CH_3-C-21, d, J = 6.5 Hz) 1.675 (HO-, s, D_2O exch.), 3.2 - 3.9 (H-C-3, m) and 5.16 (H-C-7, m).

Preparation of Ergost-7-en-3-one, 43.

A solution of ergost-7-en-3β-ol (20) (750 mg) in 100 ml of acetone was cooled to 10° C and treated for 15 min with 1.2 ml of Jones reagent . Excess reagent was decomposed with isobutanol and the mixture poured over ice. The ketone was extracted with ether and the extract washed with water, dried over anhydr. Na_2SO_4 and evaporated. Crystallization from ethanol gave 610 mg ketone 43, m. p. 164.5 - 165.5°.

υ_{max}^{KBr} 1708 c=0 cm^{-1}, n. m. r. ($CDCl_3$): δ 0.56 (CH_3-C-18, s), 0.78 (d, J = 6.5), 1.0 (CH_3-19, s) and 5,1 - 5, 3 (H-C-7, m).

Preparation of [2,3-^{3}H]-Ergost-7-en-3-one, 44.

The ketone 43 (100 mg) was dissolved in 8 ml of THF and 4 ml of benzene. Alcoholic KOH (2 ml of 5% KOH in methanol) and 0.15 ml of tritiated water (3.75 mc) were added. The mixture was kept at r. t. for 44 hrs, then added to an excess of water and extracted with ether. The ether extract was washed several times with water, dried over anhydr. Na_2SO_4,

evaporated and the residue recrystallized from methanol, 82 mg m. p. 162.5 - 164.5°. No depression of the m. p. was observed when mixed with the original ketone.

Preparation of [2-^{3}H]-Ergost-7-en-3β-ol, 20.

The tritiated ketone (80 mg) was dissolved in 25 ml of methanol and 20 mg $NaBH_4$ added. The mixture was stirred at r. t. for 1 hr, added to an excess of water and extracted with ether. The usual work up and recrystallization from ethanol gave 41 mg of alcohol 20. The compound had an activity of 3.2×10^5 cpm/mg.

Preparation of [28-^{14}C]-Ergosta-5,7,22,24(28)-tetraen 3β-ol, 26.

The tetraene (26) was prepared via Wittig reaction from 3β-benzoxy-5,7,22-triene-24-one and [^{14}C]-methyl-triphenyl phoshonium iodide as described earlier[43]. The compound had an activity of 7.2×10^5 cpm/mg.

Preparation of [^{14}C]-Ergosterol:

Labelled ergosterol was obtained biosynthetically by incubating yeast aerobically in the presence of [U-^{14}C] acetate. The harvested cells were hydrolysed and ergosterol recovered as the acetate. Hydrolysis, purification by T. L. C. and six crystallizations gave the pure compound (by g. l. c.) with a constant activity of 3.0×10^4 cpm/mg.

Preparation of Ergosta-5,7-dien -3β-ol (24).

This compound was prepared from ergosterol acetate via

the maleic anhydride adduct and hydrogenation over Adam's catalyst as described earlier[94], m. p. of acetate 155 - 156°. The labelled compound was prepared in an analogous manner using [U-^{14}C] ergosterol obtained biosynthetically as described above. The activity was 2.9 x 10^4 cpm/mg.

Preparation of 3β-Acetoxy-cholest-5-en-24-one, 46.

A solution of 1.2 g (2.7 mmole) 3β-acetoxy-5,22-cholestadien-24-one 45 [43] in 50 ml ethyl acetate was hydrogenated at r. t. and 50 psi pressure for 35 min, using 10% palladium/$BaSO_4$ [89] as catalyst. The mixture was filtered through a celite 535 column and the solvent evaporated. Recrystallization from methanol gave 1.14 g 46 (95%) m. p. 128.5 - 129.5° (lit. m. p. 128°).

υ_{max} (KBr): 1728 (C=O acetate), 1710 (C-24, ketone), 1040 and 800 (Δ5) cm^{-1}; n. m. r. ($CDCl_3$): δ 0.675 (CH_3-C-18, s), 1.01 (CH_3-C-19, s), 1.075 (CH_3-C-21, $(CH_3)_2$-C-25, d, J = 6.8 Hz), 2.00 (CH_3-COO-, s), 2.4 (H-C-25, hep., J = 6.8 Hz), 4.30 - 4.85 (H-C-3, m) and 5.25 - 5.47 (H-C-6, m).

Preparation of 3β-Acetoxy-5-cholesten-24-ethylene ketal, 47.

A solution of 1.0 g (2.2 mmole) ketone, 46, 3 ml diethylene glycol and 150 mg p-toluenesulfonic acid in 800 ml of dry benzene was refluxed for 60 min. Benzene was then slowly distilled off over a period of ca 10 hrs until 500 ml distillate was collected. The benzene solution containing the product was washed with conc. $NaHCO_3$ soln. then with H_2O until neutral, dried over anhyd. $CaCl_2$ and evaporated. The crystalline resi-

due (0.97 g, 88.5%) was recrystallized from acetone, m. p. 134.5 - 135°.

υ_{max} (KBr) 1728 (acetate C=O), 1040 and 798 (Δ5) cm^{-1}; n. m. r. ($CDCl_3$): δ 0.69 (CH_3-C-18, s), 0.925 (CH_3-C-21, d, J = 6.5 Hz), 1.02 (CH_3-C-19, s), 2.02 (CH_3-COO-, s), 3.95 (-O-CH_2-CH_2-O-, s), 4.3 - 4.9 (H-C-3, m) and 5.3 - 5.55 (H-C-6, m)..

Preparation of 3β-Benzoxy-5-cholestene-24-ethylene ketal, 48.

The acetate 47 was hydrolyzed by refluxing in 2% K_2CO_3 in 10% aqueous methanol for 1 hr and the free alcohol obtained was benzoylated with benzoyl chloride in pyridine. After the usual work up, purification over a short alumina column and recrystallization from acetone, the compound had m. p. 146 - 148°.

υ_{max} (KBr) 1710 (benzoate C=O), 1250, 110 (C-O-), 1040 and 798 (Δ5) cm^{-1}; n. m. r. ($CDCl_3$): δ 0.695 (CH_3-C-18, s), 0.92 ((CH_3)$_2$-C-25, CH_3-C-20, d, J = 6.5 Hz), 3.96 (-O-CH_2-O- s), 4.6 - 5.2 (H-C-3, m), 5.4 - 5.6 (H-C-5, m) and 7.3 - 8.25 (aromatic H, m).

Preparation of 3β-benzoxy-5,7-cholestadiene-24-ethylene ketal, 49.

A solution of 0.9 g (1.8 mmole) of 3β-benzoxy-5-cholesten-24-ethylene ketal (48) in 50 ml CCl_4 was heated to reflux and 0.34 g NBS (2.2 mmole) added. The mixture was refluxed for 10 min. then cooled in ice and the precipitated succinimide removed by filtration. The filtrate was evaporated and the oil

obtained taken up in xylene. This solution, containing the bromosterol, was added dropwise to a vigorously boiling solution of 0.7 g trimethylphosphite in 20 ml xylene. After refluxing for 90 min the xylene was distilled off at 75° under vacuum and the crystalline residue recrystallized from acetone to give 0.55 g (61%) of ketal 49 with m. p. 140 - 142°.

υ_{max} (KBr) 1710 (C=O benzoate), 1250, 1110 (-C-O-), 1600, 1580, 1065, 1025, 832 and 800 (Δ5, 7) cm^{-1}; n. m. r. ($CDCl_3$): δ 0.64 (CH_3-C-18, s), 0.93 ((CH_3)$_2$-C-25, d, J = 6.8 Hz), 1.05 (CH_3-C-19, s), 1.10 (CH_3-C-20, d, J = 7.0 Hz, 3.96 (-O-CH_2-CH_2-O, s), 4.75 - 5.3 (H-C-3, m), 5.3 - 5.75 (H-C-5, H-C-6, m) and 7.3 - 8.25 (aromatic H, m).

Preparation of 3β-Benzoxy-cholesta-5,7dien -24-one, 50.

To a solution of 0.9 g (1.65 mmole) of ketal 49 in THF, 5% aqueous H_2SO_4 was added until the solution became turbid. The solution was clarified with additional THF and stirred at r. t. for 90 min., neutralized with Na_2CO_3 solution and the THF evaporated. The product (0.71 g, 86%) was extracted with ether and recrystallized from methanol, m. p. 140 - 141°.

N. m. r. ($CDCl_3$): δ 0.64 (CH_3-C-18, s), 1.0 (CH_3-C-19, s), 1.10 (CH_3-C-21, (CH_3)$_2$-C-25, d, J = 7.0 Hz), 4.75 - 5.3 (H-C-3, m), 5.3 - 5.8 (H-C-5, H-C-C-6, m) and 7.3 - 8.25 (aromatic H, m).

Preparation of 3β-Acetoxy-ergosta-5,7,24(28)-trien, 51.

A solution of 3β-benzoxy-cholesta-5,7-dien24-one, 50, (0.4

g) in THF was added to a THF solution of the ylide, prepared from 1.2 g (5 mmole) of methyltriphenylphosphonium iodide and 4.5 mmole of butyllithium. The reaction mixture was kept 1 hr at r. t. and 3 hrs at 65°. After the usual work up (see previous prep.), reacetylation with acetic anhydride in pyridine, purification on a silica gel column and recrystallization from methanol, the product (84 mg, 24%) had m. p. 133 - 134°. (No m. p. was reported for the natural product by Goulstone et al.[59]

υ_{max} (KBr): 1732 (C=O, acetate) 1645, 887 (terminal methylene), 1600, 1580, 1025, 832, 800 (Δ5, 7) cm^{-1}. N. m. r. (in $CDCl_3$): δ 0.625 (CH_3-C-18, s), 0.970 (CH_3-C-19, s), 1.03 ((CH_3)$_2$-C-25, CH_3-C-20, d, J = 7 Hz), 2.03 (CH_3-CO-O-, s), 4.7 (CH_2 = C <, m), and 5.3 - 5.65 (H-C-5, H-C-6, m).

Preparation of Ergosta-5,7,24(28)-trien -3β-ol, 23.

The acetate 51 was hydrolyzed by refluxing in 2% K_2CO_3/ 10% H_2O in methanol for 1 hr. M. p. 129 - 130.5°, λ_{max} (hexane) 262.5 mμ (ε=9800), 271.8 mμ (ε=13780), 282.0 mμ (ε=8550).

υ_{max} (KBr) 1650, 887 (CH_2=C <), 1600, 1580, 832, and 800 (Δ5,7) cm^{-1}; n. m. r. ($CDCl_3$): δ 0.64 (CH_3-C-18, s), 0.95 (CH_3-C-19, s), 1.04 ((CH_3)$_2$-C-25, C-20, d, J = 7 Hz), 1.265 (H-O-C, s) and 4.7 (CH_2=C< , broad m).

Analysis calcd. for $C_{28}H_{44}O$: C 84.79, H 11.18

Found: C 84.61, H 11.25.

The synthetic sterol had physical properties identical to the isolated naturally occurring compound .

Preparation of [28-^{14}C)-Ergosta-5,7,24(28)-trien -3β-ol, 23.

This sterol was prepared from ketone 50 via acetate 51 using [^{14}C]-methyltriphenylphosphonium iodide. The product showed m. p. and n. m. r. identical to 23 prepared above and had an activity of 7.1 x 10^5 cpm/mg.

Preparation of [26,27-^{14}C]-Lanosterol, 6.

This labelled sterol was prepared as described by Akhtar et al.[14]. After purification of the acetate on T. L. C., reduction with $LiAlH_4$, the product was crystallized to constant activity (4x). Lanosterol had an activity of 1.75x10^5 cpm/mg. The product had physical properties identical to authentic lanosterol.

Preparation of [2,4 ^{3}H]-Zymosterol, 14.

A mixture of 200 mg of zymosterol, chromium trioxide (290 mg), water (4 ml), glacial acetic acid (6 ml) and 15 ml of benzene was shaken for 2 hrs at r. t. Benzene (40 ml) was added and the aqueous layer discarded. The benzene fraction was washed with $NaHCO_3$ soln., then with water until neutral and dried over Na_2SO_4. The solvent was evaporated and the residue crystallized from methanol. The product melted over the range 85 - 91° and was used without further purification.

Base catalyzed ^{3}H-exchange was carried out in an analogous fashion to that described for ergost-7,22-dien-3-one. After reduction with $NaBH_4$, purification by T. L. C. and crystallization from methanol, the product was pure by g. l. c. and had a m. p.

of 113-114°. No depression of the m. p. was observed when mixed with unlabelled material. Zymosterol had an activity of 2.75×10^5 cpm/mg.

BIOSYNTHETIC EXPERIMENTS

All feeding and trapping experiments using labelled precursors were carried out in an analogous manner. The results are reported in tabular form. The activities are in most cases calculated based only on the added carrier. If the weight of the recovered material could be determined it was taken into account. The % incorporation is based on actually incorporated activity. Experiment #1 is described in detail as an example

Experiment #1

Labelled episterol ([28-^{14}C]-ergosta-7,24(28)-diene-3β-ol) 1.78×10^5 cpm was incubated with growing yeast (~500 mg wet weight). After 7 hours, the cells were collected by centrifugation and washed with phosphate buffer. Base hydrolysis and extraction gave the crude free sterol fraction which was dissolved in 50 ml of heptane. An aliquot of 0.5 ml was counted and had an activity of 940±5 cpm ∴ 100 x 940±5 cpm = $9.4 \times 10^4 \pm$ 500 cpm. Total incorporation = 52.6%. The free alcohols were acetylated and the total activity in the acetates determined analogously: $9.0 \times 10^4 \pm 500$ cpm.

The acetates were separated on T. L. C. (5 plates 20 x 20); authentic sterols were co-chromatographed as markers) and the bands moving with ergost-7-enyl acetate, ergosta-7,22,24(28)-trienyl-acetate, ergosta,5,7,22,24(28)-tetraenyl-acetate and ergosterol acetate were recovered.

1.) Ergost-7-enyl acetate. To the recovered material (> 1 mg) 30 mg of unlabelled sterol was added and the mixture

crystallized and rechromatographed (2x, benzene/hexane 1:1). The compound was not pure by g. l. c. The small peak observed had the rel. ret. time of $\Delta^{7,22}$-diene. The mixture was therefore submitted to continuous T. L. C. for 48 hrs (10% benzene in hexane). The recovered material was pure by g. l. c. and after two crystallizations had a constant activity of 9.9 cpm/mg (based on carrier sterol) equivalent to 0.32% incorporation.

2.) Ergosta-7,22,24(28)-trienyl acetate. Total activity in band recovered from T. L. C. containing the trien was 1.60 x 10^4 cpm. G. l. c. showed small amounts of episterol to be present. Inactive material (100 mg) was added and the mixture recrystallized, rechromatographed and recrystallized. The obtained material was pure by g. l. c. and had an activity of 55.5 cpm/mg based on carrier sterol, corresponding to 6.1% incorporation.

3.) Ergosta-5,7,22(28)-tetraenyl acetate. To the recovered material ($\sim$ 1 mg) 100 mg of unlabelled material was added. The material was purified 2x by T. L. C. and crystallized to constant activity.

1st recryst. 30.3 cpm/mg
2nd recryst. 49.0 cpm/mg
3rd recryst. 50.4 cpm/mg
4th recryst. 50.2 cpm/mg
5th recryst. 48.5 cpm/mg

M. p. of 5th cryst. 142.5 - 144°.

Based on added carrier sterol, 4.85 x 10^3 cpm are equivalent to 5.4 incorporation.

4.) Ergosterol acetate. Unlabelled ergosterol acetate (100 mg) was added to the recovered material and the sterol purified by T. L. C. (1x) and crystallization:

1st recrystallization 60.3 cpm/mg

2nd recrystallization 34.1 cpm/mg

3rd recrystallization 31.1 cpm/mg

4th recrystallization 26.8 cpm/mg

5th recrystallization 26.4 cpm/mg

2.64×10^3 cpm/mg corresponding to an incorporation of 2.93%.

EXPERIMENT # 1

Incubation of

[28-^{14}C]-ergosta-7,24(28)-diene-3β-ol

Total activity added: 1.78×10^5 cpm

Incubation time: 7 hours

Activity recovered from nonsaponifiable material: 0.94×10^5 cpm

Total activity in acetates: 0.90×10^5 cpm

Incorporation: 52.6%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergost-7-en-3β-ol	0.32	2.7×10^2
(2) ergosta-7,22,24(28)-trien-3β-ol	6.1	5.55×10^3
(3) ergosta-5,7,22,24(28)-tetraen-3β-ol	5.4	5.05×10^3
(4) ergosterol	2.93	2.76×10^3

EXPERIMENT # 2

Incubation of

[28-^{14}C]-ergosta-7,24(28)-dien-3β-ol

Total activity added: 8.88×10^4 cpm

Incubation time: 7 hours

Activity recovered from nonsaponifiable material: 7.518×10^4 cpm

Total activity in acetates: 7.30×10^4 cpm

Incorporation: 84.6%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergosta-5,7,24(28)-trien-3β-ol	0.4	0.7×10^3
(2) ergosta-5,7,22,24(28)-tetraen-3β-ol	17.6	9.2×10^3
(3) ergosterol	4.6	2.1×10^3

EXPERIMENT # 3

Incubation of

[28-^{14}C]-ergosta-7,24(28-dien-3β-ol

Total activity added: 1.24×10^5 cpm

Incubation time: 7 hours

Activity recovered from nonsaponifiable material: 0.36×10^5 cpm

Total activity in acetates: 0.31×10^5 cpm

Incorporation: 29.0%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergost-7-en-3β-ol	0.21	290
(2) ergosterol	5.1	1.85×10^3

EXPERIMENT # 4

Incubation of

[2,4-^{3}H]-ergost-7-en-3β-ol

Total activity added: 7.926×10^{5} cpm

Incubation time: 7 hours

Activity recovered from nonsaponifiable material: 2.56×10^{5} cpm

Total activity in acetates: 1.41×10^{5} cpm

Incorporation: 32.35%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergosta-7,22-dien-3β-ol	10.45	2.7×10^{4}
(2) ergosta-5,7,22,24(28)-tetraen-3β-ol	>0.001	--
(3) ergosta-5,7-dien-3β-ol	>0.01	--
(4) ergosterol	16.43	4.24×10^{4}

EXPERIMENT # 5

Incubation of

[2,4-^{3}H]-ergost-7-en-3β-ol

Total activity added: 9.3×10^{5} cpm

Incubation time: 7 hours

Activity recovered from nonsaponifiable material: 2.58×10^{5} cpm

Total activity in acetates: 2.10×10^{5} cpm

Incorporation: 27.8%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergosta-7,24(28)-dien-3β-ol	0	0
(2) ergosta-5,7,22,24(28)-tetraen-3β-ol	0	0
(3) ergosta-7,22-dien-3β-ol	11.6	3.01×10^{4}
(4) ergosterol	15.2	3.92×10^{4}

EXPERIMENT # 6

Incubation of

[U-^{14}C]-ergosta-5,7-dien-3β-ol

Total activity added: 8.23×10^5 decomp./m

Incubation time: 7 hours

Activity recovered from nonsaponifiable material: 3.92×10^5 decomp./m

Total activity in acetates: 3.63×10^5 decomp./m

Incorporation: 44.1%

Recovered material: 421 mg

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergosta-5,7,24(28)-trien-3β-ol	0	--
(2) ergost-7-en-3β-ol	0	--
(3) ergosterol	19.2	7.54×10^4

EXPERIMENT # 7

Incubation of

[28-^{14}C]-ergosta-7,22,24(28)-trien-3β-ol

Total activity added: 9.90×10^5 cpm

Incubation time: 7 hours

Activity recovered from nonsaponifiable material: 8.25×10^5 cpm

Total activity in acetates: 7.9×10^5 cpm

Incorporation: 83.3%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergosta-7,22-dien-3β-ol	0.23	1.9×10^3
(2) ergosta-5,7,22,24(28)-tetraen-3β-ol	9.7	7.9×10^4
(3) ergosta-7,24(28)-diene-3β-ol (episterol)	>0.001	-
(4) ergosterol	3.7	3.10×10^3

EXPERIMENT # 8

Incubation of

[28-^{14}C]-ergosta-5,7,24(28)-trien-3β-ol

Total activity added: 1.02×10^5 cpm

Incubation time: 7 hours

Activity recovered from nonsaponifiable material: 5.92×10^4 cpm

Total activity in acetates: 5.4×10^4 cpm

Incorporation: 58.1%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergosta-5,7,22,24(28)-tetraen-3β-ol	7.1	4.2×10^3
(2) ergosterol	3.5	2.07×10^3

EXPERIMENT # 9

Incubation of

[28-^{14}C]-ergosta,5,7,22,24(28)-tetraen-3β-ol

Total activity added: 6.95×10^5 cpm

Incubation time: 7 hours

Activity recovered from nonsaponifiable material: 2.68×10^5 cpm

Total activity in acetates: 2.25×10^5 cpm

Incorporation: 38.5%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergosta-7,22,24(28)-trien-3β-ol	0	-
(2) ergosta-5,7,24(28)-trien-3β-ol	0	-
(3) ergosterol	17.8	4.78×10^4

EXPERIMENT #10

Incubation of

[28-^{14}C]-ergosta-8,24(28)-dien-3β-ol

Total activity added: 1.3×10^5 cpm

Incubation time: 9 hours

Activity recovered from nonsaponifiable material: 0.6×10^5 cpm

Total activity in acetates: 0.58×10^5 cpm

Incorporation: 46.2%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) episterol	8.2	2.1×10^3
(2) ergosta-5,7,22,24(28)-trien-3β-ol	12.6	3.1×10^3
(3) ergosterol	13.1	2.9×10^3

EXPERIMENT #11*

Incubation of

[28-^{14}C]-ergosta,7,24(28)-dien-3β-ol (Episterol)

Total activity added: 1.04×10^6 cpm

Incubation time: 8 hours

Activity recovered from nonsaponifiable material: 5.48×10^5 cpm

Total activity in acetates: 5.27×10^5 cpm

Incorporation: 52.5%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergosta-8,22-dien-3β-ol	2.1	750
(2) ergosta-7,22,24(28)-trien-3β-ol	6.3	600
(3) ergost-7-en-3β-ol	2.7	580
(4) ergosta-7,22-dien-3β-ol	12.1	1260
(5) ergosta-8,24(28)-dien-3β-ol	16.1	615
(6) ergosta-5,7,22-trien-3β-ol	0	0
(7) episterol	34.1	1.8×10^4

*nys-3

EXPERIMENT # 12*

Incubation of

[28-^{14}C]-ergosta-8,24(28)-dien-3β-ol (Fecosterol)

Total activity added: 1.04×10^6 cpm

Incubation time: 8 hours

Activity recovered from nonsaponifiable material:

Total activity in acetates: 5.27×10^5 cpm

Incorporation: 50.6%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergosta-8,22,24(28)-trien -3β-ol	1.8	510
(2) ergosta-7,24(28)-dien -3β-ol	4.5	610
(3) ergost-7-en-3β-ol	2.1	490
(4) ergosta-7,22-dien -3β-ol	11.8	1130
(5) ergosterol	0	0

*nys-3

EXPERIMENT # 13

Incubation of

[28-^{14}C]-ergosta-7,24(28)-dien-3β-ol

Total activity added: 1.25×10^5 cpm

Incubation time: 8.5 hours

Activity recovered from nonsaponifiable material: 0.80×10^5 cpm

Total activity in acetates: 0.80×10^5 cpm

Incorporation: 64%

Activity determined in the acetates of	Found	
	% Incorp.	cpm/mg
(1) ergosta-8,24(28)-dien-3β-ol	10.1	1.4×10^3
(2) ergosta-8,22-dien-3β-ol	0.4	510
(3) ergosterol	12.3	1.2×10^3

EXPERIMENT #14

Trapping of Ergosta-7,22-dien-3β,5α-diol

[28-^{14}C]- rgosta-7,2,24(28)-dienol(1.5×10^5cpm) was added to a growing yeast culture as usual. After 10 hrs the cells were harvested, 100 mg of unlabelled 3β,5α-diol added and the mixture hydrolyzed as usual. The crude sterols were acetylated. (Total activity in acetates: 0.85×10^5 cpm) The mixture was separated on a small alumina column (activity II). Hexane-benzene (1:1 υ/υ) removed sterol acetate, benzene-ether (3:1 υ/υ) gave 3β-acetoxy-ergosta-7,22-dien-5α-ol. The compound was crystallized from ethyl acetate-methanol.

1st recrystallization	173 cpm/mg
2nd recrystallization	58 cpm/mg
3rd recrystallization	41 cpm/mg
4th recrystallization	15 cpm/mg
5th recrystallization	16 cpm/mg
6th recrystallization	14 cpm/mg
7th recrystallization	18 cpm/mg
8th recrystallization	17 cpm/mg

A total incorporation of 170 cpm/mg corresponding to 0.2% incorporation is calculated based on added carrier and on the activity recovered in the total acetates.

Double label feeding experiment:

A mixture of [26,27-^{14}C]-lanosterol (3.4 mg, 1.72x10^{5} cpm/mg) and [2,4-^{3}H]-zymosterol (3.7 mg, 1.75x10^{5} cpm/mg) was fed to yeast cells depleted from sterol by anaerobic growth. Aliquots were removed at set time intervals and the cells collected by centrifugation. The cells were thoroughly washed 3 times by resuspension in buffer and centrifugation. Unlabelled zymosterol (50 mg) and ergosterol (50 mg) were added to the cells and the mixture hydrolyzed and extracted with hexane as usual. The hexane extract was made up to exactly 50 ml and an aliquot removed for counting. The $^{14}C/^{3}H$ ratio was determined for the total sterol fraction. After evaporation of the solvent the crude sterol fraction was separated on T. L. C. (10% ethyl acetate in benzene) and the zymosterol and ergosterol bands collected. Both sterols were purified by T. L. C. and crystallization until pure by g. l. c. and showed constant activity. The $^{14}C/^{3}H$ ratios were determined. The results are shown in Fig. 16.

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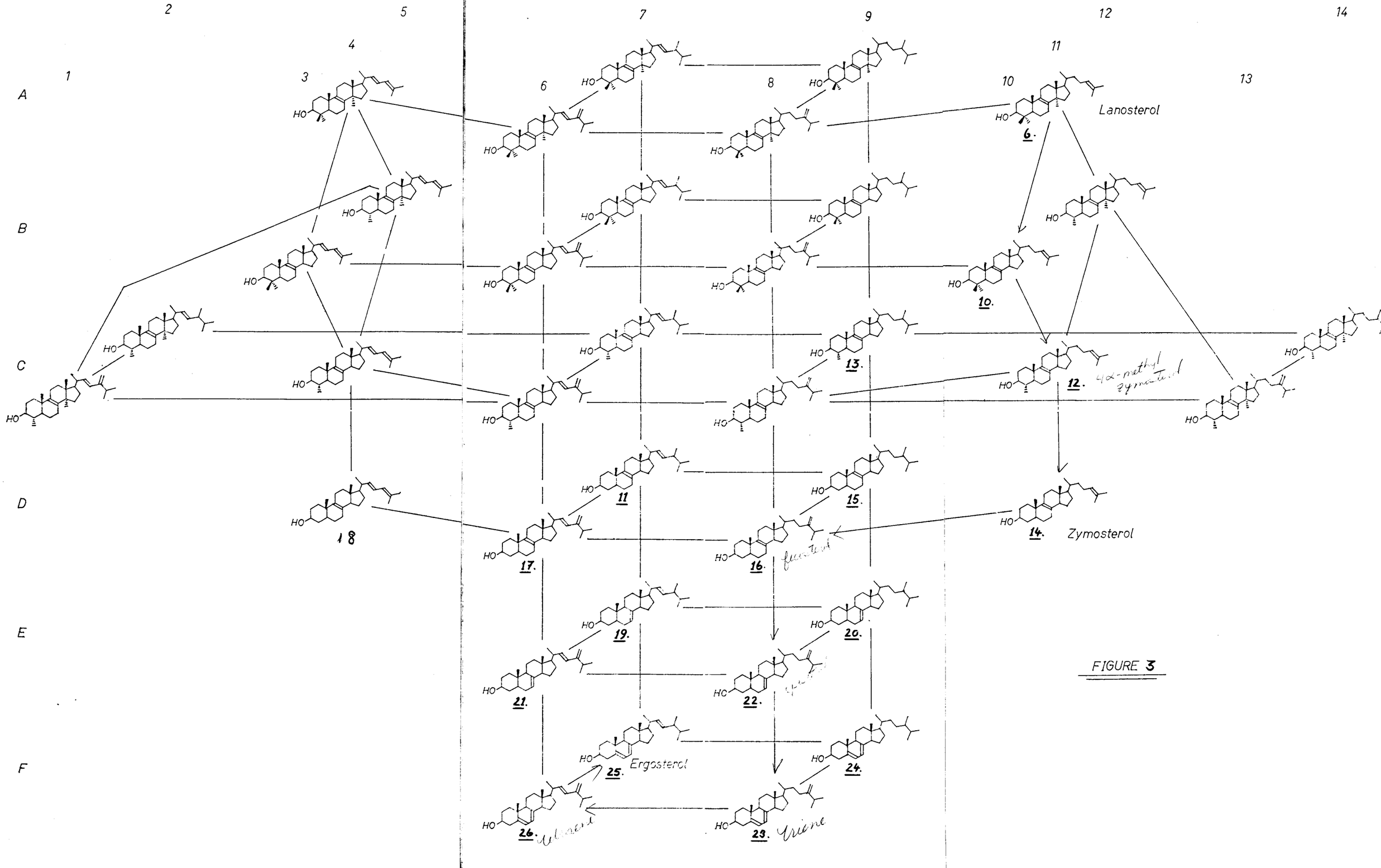
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16. fecosterol
19.
20.
21.
22. episterol
25. Ergosterol
24.
26. ullucosterol
23. triene

FIGURE 3