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# Biosynthesis of Ergosterol in Yeast - Studies with Mutants

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**Abstract** : The biosynthesis of ergosterol from lanosterol in yeast is reviewed with special emphasis on the use of mutants for the elucidation of the sequence of reactions. A multiple pathway model for the biosynthetic routes from zymosterol to ergosterol in yeast is proposed and the synthetic uses of the yeast mutants discussed.

## 1. Introduction

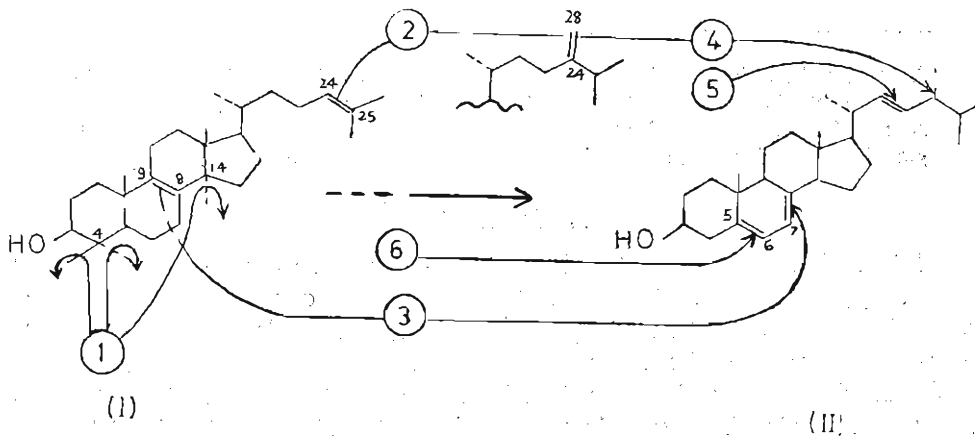
Sterol formation in both plant and animal systems occur through four principal stages : (*a*) conversion of acetate to mevalonate, (*b*) conversion of mevalonate to squalene, (*c*) cyclisation of squalene, and (*d*) conversion of the first cyclic intermediate (cycloartenol or lanosterol) to the 4-desmethyl sterol products. Cholesterol is considered the major sterol in animal tissues. $C_{29}$ sterols such as sitosterol are predominent in photosynthetic plants and ergosterol is the major sterol in most fungi such as yeast.

During the past two decades numerous research publications have appeared concerning the biosynthesis of ergosterol in yeast.[2-7] As a result of these investigations, the route to lanosterol (I), the first steroidal intermediate in yeast from mevalonic acid (MVA) via squalene was realised. However, the major pathway from lanosterol (I) to ergosterol (II) is not yet fully understood. In recent years, in addition to the conventionally employed methods, due recognition has been given to the use of mutants in elucidating the biosynthetic sequences in lower organisms.[1-3] These studies have contributed largely to the understanding of this complex sequence of biosynthetic reactions operating in yeast. This article reviews the main areas of recent investigations with particular reference to the use of mutants in the elucidation of the biosynthetic pathways leading to ergosterol (II) in yeast (*Saccharomyces cerevisiae*).

## 2. Biosynthetic conversion of lanosterol to ergosterol

The enzymatic conversion of lanosterol (I) to ergosterol (II) in yeast requires six general transformations[2-7] (enzyme or enzyme complex involved in each transformation is given in parenthesis) : (1) removal of the three methyl groups in lanosterol at $C_4$ and $C_{14}$ (demethylase) ; (2) alkylation at $C_{24}$ with concomitant reduction at $C_{25}$ and generation of a $\Delta^{24(28)}$—methylene (methyl transferase) ; (3) isomerisation

of the $\Delta^8$ double bond to $\Delta^7$ ($\Delta^8 \rightarrow \Delta^7$ isomerase); (4) reduction of the $\Delta^{24(28)}$ double bond generating a $C_{24}$—methyl (24, 28—reductase); (5) introduction of a $\Delta^{22}$ double bond (22, 23—dehydrogenase); and (6) introduction of a $\Delta^5$ double bond (5, 6– dehydrogenase) (see Scheme 1). *A priori*, these transformations could occur in any order. Several groups have carried out investigations to determine the probable order of this sequence.[5,15,21]



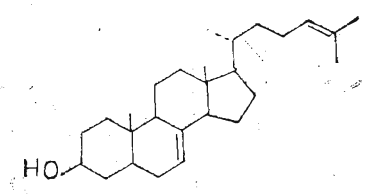

Scheme 1. The enzymatic transformation of lanosterol (I) to ergosterol (II).

### 3. Methods of study and results

In general, five approaches have been recognised for the study of biosynthesis; namely, chemical speculation,[10] isolation of intermediates, use of isotopically labeled precursors,[14] examination of enzyme systems *in vitro*[9] and the use of organisms with blocked biosynthetic pathways.[1] Out of these the first three methods are the most widely used. The employment of *in vitro* enzyme systems was found to suffer from one disadvantage; although this method is eventually required for the confirmation of a biosynthetic sequence, it could hardly be used for exploration purposes. Of these approaches the use of organisms *in vitro* with blocked biosynthetic pathways is known to be the most informative method.[27]

During the transformation of lanosterol to ergosterol, by analogy with cholesterol biosynthesis nuclear demethylation to produce desmethyl sterols i.e. sterols without $C_4$ and $C_{14}$ methyl groups was assumed to be the initial step. Recent work of Gaylor and his co-workers,[16,24] with yeast enzyme systems have provided evidence that demethylation precedes $\Delta^8 \rightarrow \Delta^7$ isomerisation. They were also able to demonstrate that in the biosynthetic pathway zymosterol (VI) is superior to 4, 4—dimethylzymosterol (III), 4$\alpha$—methylzymosterol (IV), cholesta—7,24—dien– 3$\beta$—ol (XVIII) and cholesta—5,24—dien—3$\beta$—ol (XIX). Cholesta—5,7,24– trien —3$\beta$—ol (XX) was shown to be a substrate for a soluble $\Delta^{24}$—sterol methyltransferase

enzyme isolated from yeast.[26] Assuming that the relative rates observed in this reflect *in vivo* substrate specificities, $C_{24}$, alkylation most likely occurs prior to introduction of the $\Delta^{22}$, $\Delta^{7}$ and $\Delta^{5}$ unsaturations. Consistent with this sequence is the apparent absence in wild type yeast sterol mixtures of cholesta—7, 24—dien—3β—ol (XVIII) and cholesta—5, 7, 22, 24—tetraen—3*β*—ol (XXI) and the presence of zymosterol,[29] fecosterol (VII),[28] episterol (XI),[31] ergosta—7,22—dien—3β—ol (XIV)[12] and ergosta—5, 7, 22, 24(28)—tetraen—3*β*—ol (ergostatetraenol, XVI).[11] Since zymosterol (VI) is the most suitable substrate for the $\Delta^{24}$—sterol methyltransferase[25] and the immediate product of its enzymatic methylation is its $C_{24}$—methylene derivative (fecosterol, VII), a rather well defined pattern is evident up to this point. Subsequent to this $\Delta^{8} \rightarrow \Delta^{7}$ isomerisation, $\Delta^{24(28)}$ reduction, and $\Delta^{5}$ and $\Delta^{22}$ unsaturation could occur in an undefined order.

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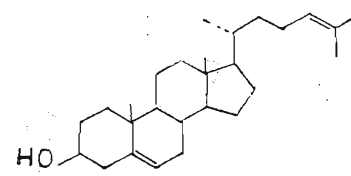


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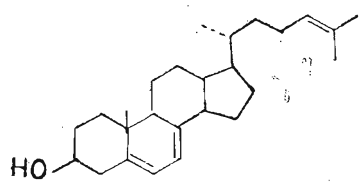


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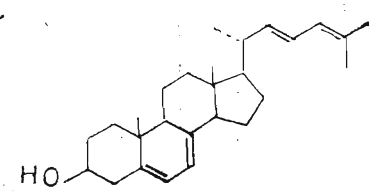
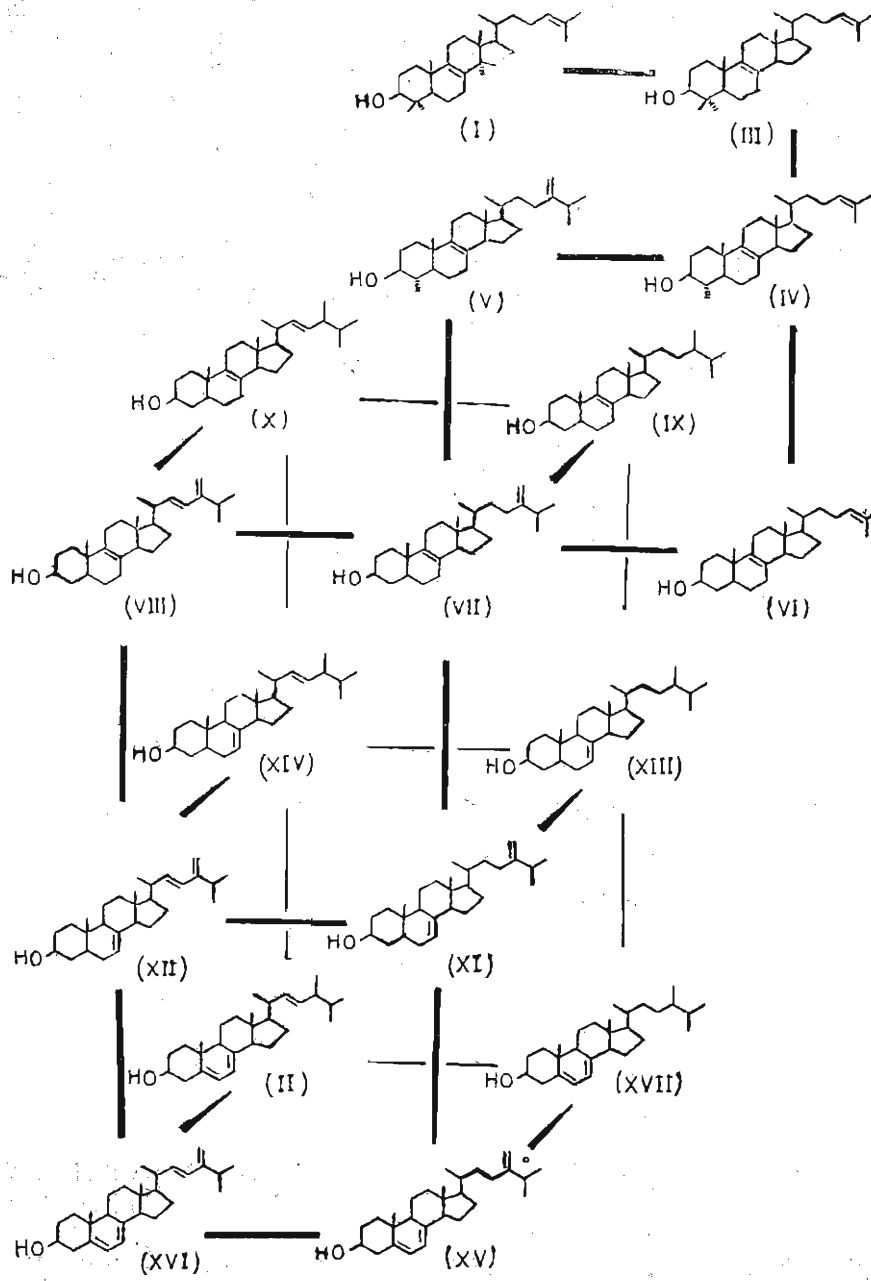



(XXI)

### 3.1. Multiple pathway hypothesis

The work of Barton,[5] Bloch,[21] Oehlschlager[15] and their co-workers with isotopically labeled precursors have led to the conclusion that there is no specific route to ergosterol in yeast but that the transformations occur by a multiple pathway. This pathway is depicted in Scheme 2. One feature incorporated into this scheme is that, at least under normal growth conditions, there appears to be essentially no movement of metabolites backwards on the biosynthetic path.




Scheme 2. The model for proposed routes to ergosterol (II) in yeast (major pathways are shown in thick lines).

Using Scheme 2 as a model, Oehlschlager and co-workers[15] have recently approached the investigation to these alternative pathways from three complementary directions. These are (1) isolation of natural sterols, (2) investigation of time course change in sterol compositions of anaerobically pretreated, aerobically growing yeast, and (3) coupling of this with assays for radioactivity in suspected metabolites as well as ergosterol. It was found that all but two of the suspected sterols, namely ergosta—8, 22,24(28)—trien—3$\beta$—ol (VIII) and ergosta—5,7—dien—3$\beta$—ol (XVII) were present in growing yeast and involved in the production of ergosterol.

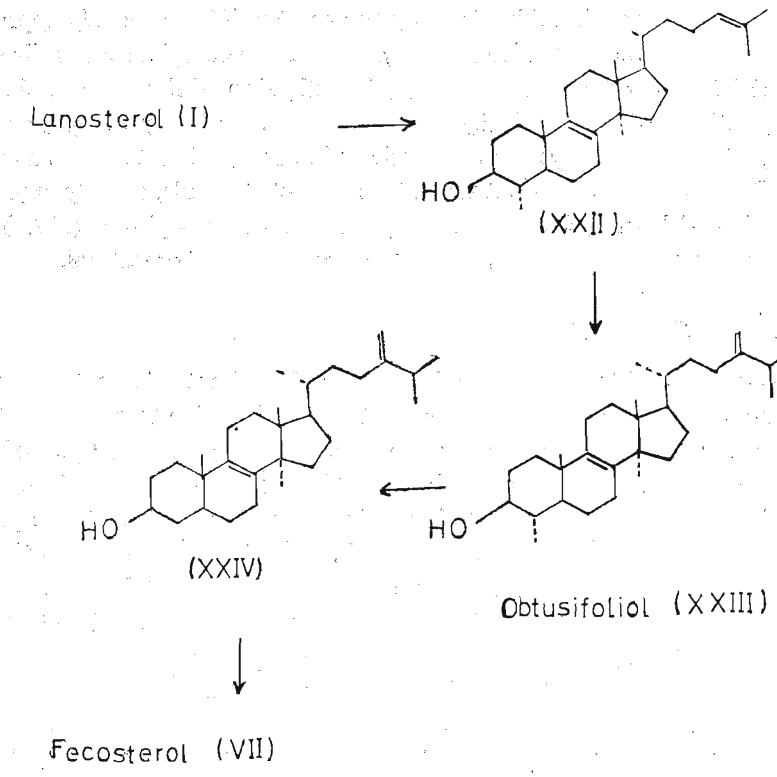
### 3.2. Mutants of *S. cerevisiae*

Recently, in order to obtain more detailed information about this multiple pathway hypothesis some workers have considered that it was desirable to use mutant organisms.[4,6,7,23] Two kinds of organisms with blocked biosynthetic pathways are known. They are the growth inhibiting metabolic analogues and auxotropic mutants. Blocking of an enzyme reaction catalysing a single step in the biosynthetic pathway will produce all the intermediates upto the block. The intermediates upto the point of blocking would accumulate since they cannot be further metabolised. Identification of such accumulated intermediates from mutants blocked at different points on the biosynthetic pathway should reveal the reaction sequence. This could be confirmed by enzyme studies and isotopic labelling methods.

Early attempts to isolate sterol-requiring mutants of *S. cerevisiae* assuming that ergosterol was essential for growth have been unsuccessful.[2,17] An alternative approach for the isolation of yeast mutants has been to select varieties resistant to polyene antibiotics such as nystatin. These antibiotics act by complexing with sterols in the cell membrane, thus causing loss of selective permeability and eventual death.[22] Polyene-resistant mutants have been prepared for a number of microorganisms.[3,18,19,33]

### 3.3. Studies with single yeast mutants

Recently, two groups were successful in obtaining sterol mutants of *S. cerevisiae*.[23,30] Trocha's group have reported a series of nystatin resistant and ergosterol requiring mutants.[30] The results of their sterol analysis have indicated a block in the removal of the methyl group at $C_{14}$ of lanosterol (I). Isolation of 4, 14—dimethylzymosterol (XXII), obtusifoliol (XXIII), and 14—methylfecosterol (XXIV) suggested an alternative route for the conversion of lanosterol to ergosterol in yeast. This alternative pathway depicted in Scheme 3 was also supported by *in vivo* conversion of obtusifoliol to ergosterol.[5]




Scheme 3. An alternative pathway from lanosterol (1) to fecosterol (VII)

Genetic analysis of the nystatin resistant mutants of *S. cerevisiae* prepared by Woods and Ahmed have resulted in the characterisation of four genes;[32] *pol* 1, *pol* 2, *pol* 3, and *pol* 5 (abbreviation *pol* stands for polyene resistance). None of these mutants were auxotropic. Preliminary studies involving *UV* spectrophotometric analysis of their sterols had shown altered patterns and hence were suspected to be defective in the terminal steps of the biosynthetic pathway ; namely, the conversion of zymosterol (VI) to ergosterol (II).[23]

Chemical[6] and *GLC*[4] analyses of these four mutants have revealed the presence of some sterols new to yeast. The results of these analyses are given in Table 1. In each mutant a clear implication of a biosynthetic block was shown in one of the transformations between zymosterol and ergosterol. Four such metabolic blocks have been found. They were at $C_{24}$ methylation (*pol* 1), at $\Delta^8 \rightarrow \Delta^7$ isomerisation (*pol* 2), at 5, 6-dehydrogenation (*pol* 3), and at 22,23—dehydrogenation (*pol* 5) (refer Scheme 1). Since these were single mutants and the blocks were complete, only one enzyme (or enzyme complex) can be involved in each transformation,

whatever the substrate. A mutant lacking the $\Delta^{24(28)}$ reductase (step 4, Scheme 1) has not been isolated, possibly because such a mutant would not have exhibited sufficient nystatin resistance to emerge from the initial screening procedure applied, during the selection of these mutants.[23]

TABLE 1. Chemical and *GLC* analyses of sterols in single *pol* mutants.

| Mutant strain (*pol*) | Sterols found by chemical analysis | Sterols found by *GLC* analysis |
|---|---|---|
| 1 | Cholesta-8, 24-dien-3β-ol (VI) | (VI) |
| | Cholesta-5, 7, 24-trien-3β-ol (XX)† | |
| | Cholesta-5, 7, 22, 24-tetraen-3β-ol (XXI)† | (XXI) and 1 unidentified sterol |
| 2 | Ergosta-8-en-3β-ol (IX)† | (IX) |
| | Fecosterol (VII) | (VII) |
| | Ergosta-8, 22-dien-3β-ol (X)† | (X) and/or |
| | Ergosta-5, 8, 22-trien-3β-ol (XXV)† | (XXV) |
| 3 | (VII) | (VII) and/or (VIII) |
| | (X) | (X) |
| | Ergosta-8, 22, 24 (28)-trien-3β-ol (VIII)† | |
| | Episterol (XI) | |
| | Ergosta-7, 22-dien-3β-ol (XIV) | (XIV) |
| | Ergosta-7, 22, 24 (28)-trien-3β-ol (XII) | |
| 5 | (XI) | (XI) |
| | Ergosta-5, 7-dien-3β-ol (XVII)† | (XVII) |
| | Ergosta-5, 7, 24 (28)-trien-3β-ol (XV) | |
| | Ergosta-8, 14, 24 (28)-trien-3β-ol (XXVI)† | (VI) and 2 unidentified sterols |

### 3.4. Studies with double yeast mutants

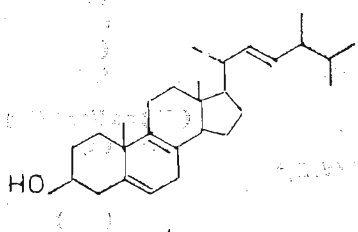
Sexual reproduction of *pol* single mutants had given rise to *pol-pol* double mutants.[23] All the possible double mutants have been prepared. The viability of these yeast mutants have been found to lessen with increasing mutation and the double mutants were shown to be less vigorous in growth than the single mutants. Their sterols have been analysed by the chemical[7] and the *GLC*[4] methods. The results of these analyses are presented in Table 2. These results have indicated that each mutant complied with the requirements of the transmission of two enzymatic blocks. In no case had a double mutant effected a chemical step which would have been genetically orbidden.[7]

† sterols new to yeast

TABLE 2. Chemical and *GLC* analyses of sterols in double *pol* mutants.

| Mutant strain (*pol-pol*) | Sterols found by chemical analysis | Sterols found by *GLC* analysis |
|---|---|---|
| 1—2 | (VI) | (VI) and 1 unidentified sterol |
| 1—3 | (VI)<br>Cholesta-7, 24-dien-3β-ol (XVIII)† | (VI) and 5 unidentified sterols |
| 1—5 | (VI)<br>Cholesta-5, 7, 24-trien-3β-ol (XX) | (VI) and 2 unidentified sterols |
| 2—3 | (VII) | (VII), (IX), (X) and 1 unidentified sterol |
| 2—5 | (VII)<br>(IX)<br>Ergosta-5, 8-dien-3β-ol (XXVII)† | (VII) and 2 unidentified sterols |
| 3—5 | (VI), (VII), (XI),<br>Ergosta-7-en-3β-ol (XIII) | (VI), (VII), (XI) (XIII) and (IX) |

†Sterols new to yeast.

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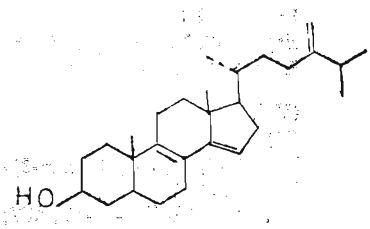


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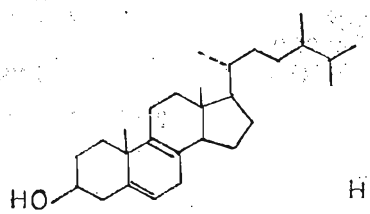


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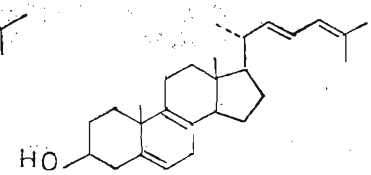


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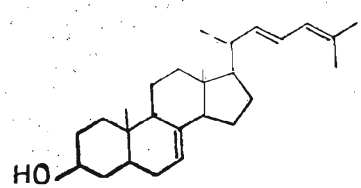



(XXIX)

The skeletal patterns of the final products in these mutants have been predicted with the knowledge of the biosynthetic sequence. These are presented in Table 3 along with the ultimate skeletal patterns found. The mutant blocked at the methyl-transferase and $\Delta^8 \rightarrow \Delta^7$ isomerase steps (*pol* 1—*pol* 2) had given cholesta—8, 24—dien—3$\beta$—ol (VI) as the most developed sterol whereas it should have produced cholesta—5, 8, 22, 24—tetraen—3$\beta$—ol (XXVIII) according to the random pathway hypothesis.[5,15,21] Similarly, *pol* 1—*pol* 3 and *pol* 2—*pol* 3 have not produced detectable quantities of the predicted sterols ( Table 3 ). However, the mutants *pol* 1—*pol* 5, *pol* 2—*pol* 5 and *pol* 3—*pol* 5 have given rise to the products predicted by the multiple pathway hypothesis. These results had confirmed that the enzymes involved in the final transformations (zymosterol $\rightarrow$ ergosterol) were not substrate specific.

### 3.5. Feeding experiments with yeast mutants

The single mutant, *pol* 2 which lacked $\Delta^8 \rightarrow \Delta^7$ isomerase enzyme has been shown to be able to metabolise $\Delta^7$—sterols by feeding a culture with radio-labelled ergosta—7, 22—dien—3$\beta$—ol (XIV).[6] An incorporation of 7.96% of it into ergosterol had confirmed that if *pol* 2 had contained any endogenous $\Delta^7$—sterols it would have almost certainly contained ergosterol as well.

Recently, *pol-pol* double mutants have been employed to provide some evidence indicating an inefficient reduction of episterol (XI) and fecosterol (VII) into their 24—methyl derivatives.[20] Thus fecosterol was the only major sterol isolated from *pol* 2—*pol* 5 and episterol and fecosterol were found in far greater abundance than ergosta—7—en—3$\beta$—ol (XIII) in *pol* 3—*pol* 5 although these mutants should possess $\Delta^{24(28)}$ sterol reductase activity.[7] To clarify these conflicting results the ability of whole-cell cultures to reduce 24—methylene group of various sterols has been studied using *pol-pol* double mutants.[20] By choice of an appropriate double mutant for each substrate it has been possible to obtain comparative data on 24—methylene sterol and ergostatetraenol (XVI) reduction without the concurrent competitive reactions. The results had indicated that fecosterol (VII) was reduced to ergosta—8—en—3$\beta$—ol (IX) by *pol* 2—*pol* 5, episterol (XI) was reduced to ergosta—7—en—3$\beta$—ol (XIII) and ergosta—7, 22, 24(28)—trien—3$\beta$—ol (XII) to ergosta—7, 22—dien—3$\beta$—ol (XIV) by *pol* 3—*pol* 5. In each case the amount transformed has been very much less than the ergosterol formed from ergostatetraenol (XVI) fed in parallel experiments. The results were in general agreement with those from similar experiments with wild-type strains[5,15] and confirmed that although *pol-pol* double mutants had the ability to reduce ergostatetraenol efficiently, other 24—methylene sterols were reduced much less readily. These results had also shown that in contrast to previous studies with whole-cells which have indicated the possibility of multiple pathways of ergosterol biosynthesis in *S. cerevisiae*,[5,6] the strict specificity of ergosta-tetraenol reductase enzyme provides evidence for a major pathway. In addition ergostatetraenol (XVI) has been implied to be the only important immediate precursor of ergosterol.

TABLE 3. Enzyme blocks of first and second generation *pol* mutants. Ultimate skeletal patterns predicted and found in second generation mutants.

| First generation mutant (*pol*) | Block | Second generation double mutant (*pol-pol*) | Transmitted blocks expected | Ultimate skeletal pattern | |
|---|---|---|---|---|---|
| | | | | Predicted | Found |
| 1 | Methyl transferase | 1—2 | Methyl transferase<br>$\Delta^8 \rightarrow \Delta^7$ isomerase | Cholesta-5, 8, 22, 24-tetraene (XXVIII) | Cholesta-8, 24-diene (IV) |
| | | 1—3 | Methyl transferase<br>5, 6-dehydrogenase | Cholesta-7, 22, 24-triene (XXIX) | Cholesta-7, 24-diene (XVII) |
| | | 1—5 | Methyl transferase<br>22, 23-dehydrogenase | Cholesta-5, 7, 24-triene (XX) | Cholesta-5, 7, 24-triene (XX) |
| 2 | Isomerase | 2—3 | $\Delta^8 \rightarrow \Delta^7$I somerase<br>5, 6-dehydrogenase | Ergosta-8, 22-diene (X) | Ergosta-8, 24(28)-diene (VII) |
| | | 2—5 | $\Delta^8 \rightarrow \Delta^7$ Isomerase<br>22, 23-dehydrogenase | Ergosta-5, 8-diene (XXVII) | Ergosta-5, 8-diene (XXVII) |
| 3 | 5, 6-Dehydrogenase | 3—5 | 5, 6-dehydrogenase<br>22, 23-dehydrogenase | Ergosta-7-ene (XII) | Ergosta-7-ene (XII) |
| 5 | 22, 23-Dehydrogenase | | See above | | |

### 3.6. Synthetic uses of yeast mutants

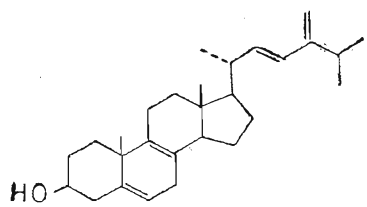
It has been pointed out that the additional limitations on the spectrum of sterols found in the double mutants as compared to the wild-type is compensated for by their usefulness as a source of pure normally inaccessible sterols.[7] Notably, *pol*- 2-*pol* 3 has produced fecosterol as the sole detectable product. This sterol could be considered as a starting material for the synthesis of steroidal hormones.

Mutants could also be employed to obtain sterols which are difficult to synthesise chemically. One such example has been the use of *pol* 2—*pol* 5 for the synthesis of ergosta—5, 7—dien—3$\beta$—ol (XVII).[8] The precursor ergosta—7—en—3$\beta$—ol (XIII) was introduced to the anaerobically pretreated and aerobically growing mutant organism and the total sterols analysed periodically. It has been observed that the maximum conversion (ca. 17%) of this precursor to the required ergosta—5, 7—dien—3$\beta$—ol occurred in 20 hours.

## 4. Conclusion

The results obtained with yeast mutants show that the biosynthesis of ergosterol in yeast involves a very complex system of metabolic pathways.[4,6,7,20,30] Only five enzymes can be involved in the later (sterol) stages. The enzymes must be operating essentially randomly on a variety of substrates. The concept of unit (enzymatic) transformations as revealed by blocked mutants permits many routes to ergosterol. It allows a precise definition of the significant operations in the biosynthetic pathway and can be considered a better approach to the problem than the previously employed methods.

The model depicted in Scheme 1 can be elaborated to include all the sterols encountered in mutant yeasts. The new model for proposed routes from zymosterol (VI) to ergosterol (II) in yeast is presented in Scheme 4. It depicts all the possible biosynthetic pathways operating in yeast during this conversion. Although major pathways undoubtedly occur (see above), until more is known about the control mechanisms operating on the enzymes, determination of them is an illusory exercise.

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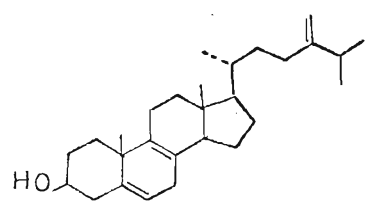


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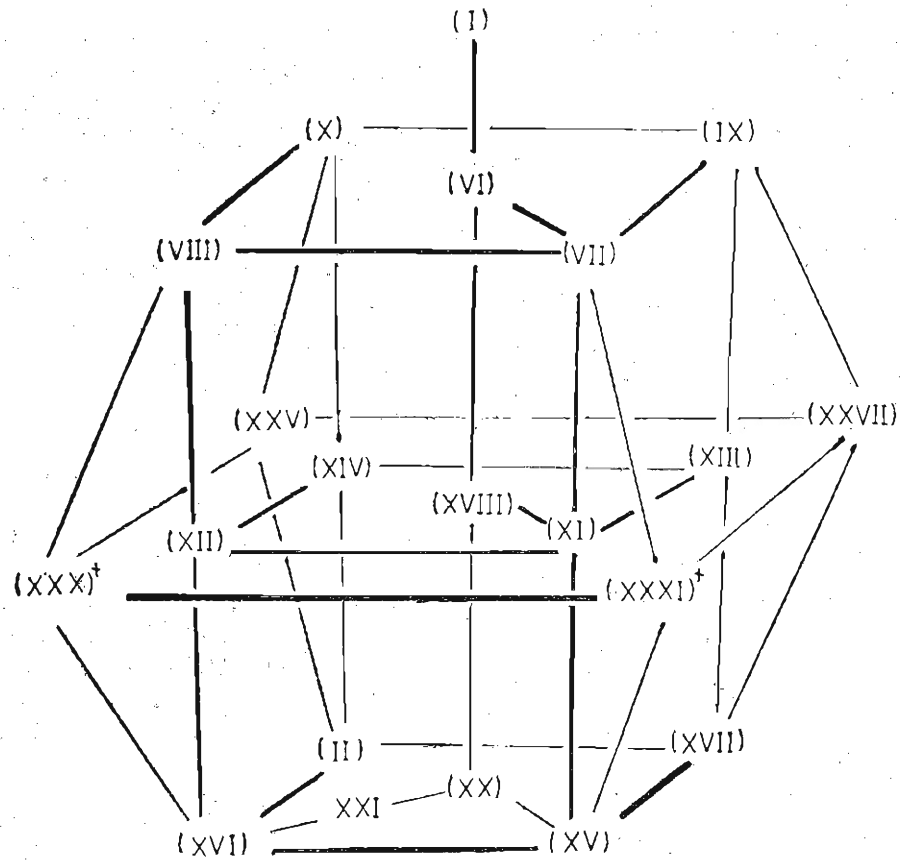


Scheme 4. A multiple pathway model for the biosynthetic routes from zymosterol (VI) to ergosterol (II) in yeast.

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