$[\alpha]_{290}$ +800 (pk), $[\alpha]_{240}$ −2657°; λ_{max}^{EtOH} 228 nm, 281, and 299 sh (log ε 4.55, 4.36, and 4.06); mass spectrum *m/e* 606 (M^+), 400, 293, 236, 220, 206 (base), 192, and 91; high-resolution mass spectrum *m/e* 605.2992 (calcd for $M^+ - 1 = C_{38}H_{41}N_2O_5$, *m/e* 605.3014).

Acknowledgments. This research was supported by Grant CA11450 from the National Institutes of Health to M. S. The authors are grateful to Professor M. Tomita for a sample of authentic 2,10-dimethoxyaporphine. *B. baluchistanica* was identified by Dr. A. R. Beg, Plant Taxonomist, Forest Research Institute, Peshawar, Pakistan.

Biosynthesis of Ergosterol in Yeast. Evidence for Multiple Pathways[1]

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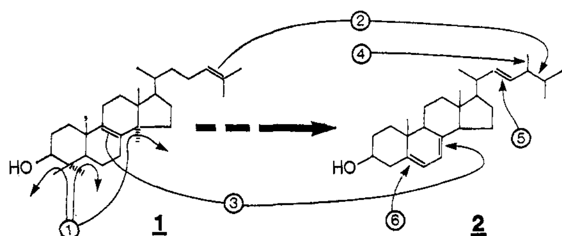
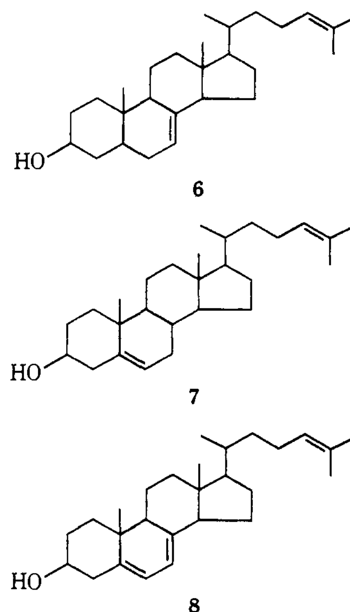
Abstract: The conversion of lanosterol to ergosterol in *Saccharomyces cerevisiae* has been investigated. Time-course analysis of the sterol content and feeding–trapping experiments with suspected intermediates led to the discovery of several alternative pathways in the latter stages of ergosterol biosynthesis. Maintenance of the yeast under anaerobic conditions depleted the sterol content of the organism. The sterols most rapidly consumed under these conditions were those possessing $\Delta^{5,7}$ unsaturation. During anaerobic maintenance squalene accumulated. A subsequent change to aerobic conditions was accompanied by accelerated sterol production. Time-course analysis of the changing sterol composition during aeration indicated that the initial structural modifications following the formation of lanosterol involved nuclear demethylation at C_4 and C_{14} as well as alkylation at C_{24}. In order to investigate subsequent modifications synthesis of suspected 4,14-desmethyl-24-alkyl sterol intermediates (unlabeled and ^{14}C or ^3H labeled) possessing varied unsaturation, *e.g.*, Δ^8, Δ^7, $\Delta^{5,7}$, and $\Delta^{24(28)}$, was carried out. Chromatographic separation and analysis of *S. cerevisiae* sterol mixtures led to the discovery of five previously unreported sterols (**14** and **16–19**) in this organism. Feeding and trapping experiments with suspected intermediates and previously reported yeast sterols revealed that several alternative pathways are operative in the latter stages of the lanosterol to ergosterol bioconversion. Based on relative incorporation efficiencies, the major route from fecosterol to ergosterol involves $\Delta^8 \rightarrow \Delta^7$ isomerization, introduction of unsaturation at C_{22}, then at C_5, and finally reduction of the 24-methylene. The first of these transformations was shown to be reversible while the last three were essentially nonreversible.

The enzymatic conversion of lanosterol (1) to ergosterol (2) in yeast (*Saccharomyces cerevisiae*) requires six general transformations:[2] (1) removal of the three methyl groups in lanosterol at C_4 and C_{14}; (2) alkylation at C_{24} with concomitant reduction at C_{25} and generation of a $\Delta^{24(28)}$-methylene; (3) isomerization of the Δ^8 double bond to Δ^7; (4) reduction of the $\Delta^{24(28)}$ double bond generating a C_{24}-methyl; (5) introduction of a Δ^{22} double bond; and (6) introduction of a Δ^5 double bond.





A priori, these transformations could occur in any order. A variety of studies, particularly those involving investigation of enzyme–substrate specifications, have provided an insight into the approximate order in which these transformations occur. By analogy with cholesterol biosynthesis, it has been assumed that nuclear demethylation is the initial step in the lanosterol to ergosterol conversion. Gaylor, *et al.*, have recently provided evidence that demethylation precedes $\Delta^8 \rightarrow \Delta^7$

(1) Preliminary communication: M. Fryberg, A. C. Oehlschlager, and A. M. Unrau, *Biochem. Biophys. Res. Commun.*, **48**, 593 (1972).
(2) L. J. Mulheirn and P. J. Ramm, *Chem. Soc. Rev.*, **27**, 259 (1972).

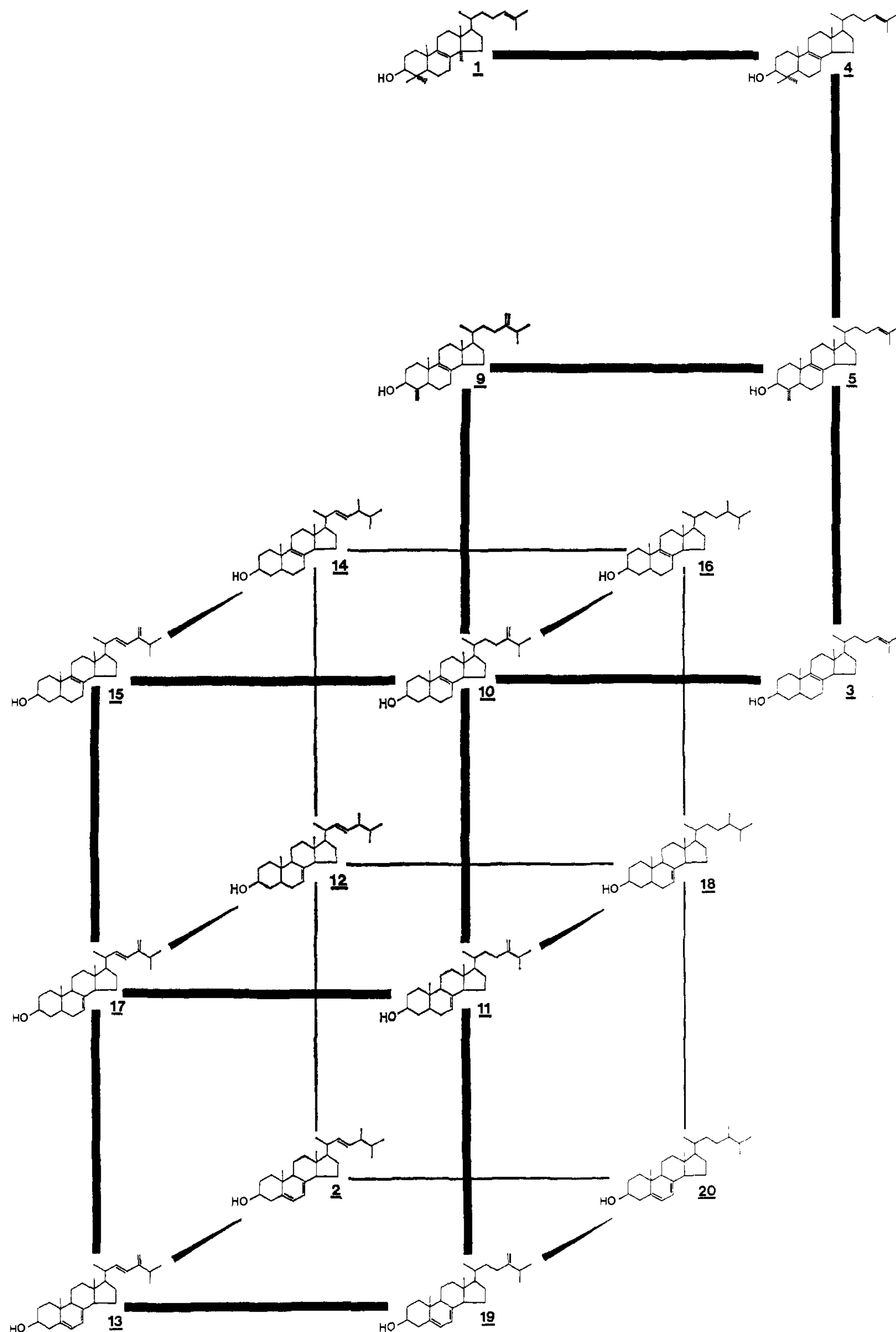


Figure 1. Model for proposed routes to ergosterol in yeast.

isomerization.[3] They[4] have also demonstrated that zymosterol (**3**) is superior to 4,4-dimethylzymosterol (**4**), 4α-methylzymosterol (**5**), cholesta-7,24-dien-3β-ol (**6**), cholesta-5,24-dien-3β-ol (**7**), and cholesta-5,7,-24-trien-3β-ol (**8**) as a substrate for a soluble Δ^{24}-sterol methyltransferase isolated from yeast (Figure 1). Assuming the relative rates observed in this system reflect *in vivo* substrate specificities, C_{24} alkylation most likely occurs after partial as well as complete nuclear demethylation, but prior to introduction of the Δ^{22}, Δ^{7}, and Δ^{5} unsaturation. Consistent with this sequence is the apparent absence in yeast sterol mixtures of **6–8** and the presence of **4**,[5] **5**,[5] **9**,[6] zymosterol,[7]

(3) (a) J. T. Moore, Jr., and J. L. Gaylor, *Arch. Biochem. Biophys.*, **124**, 167 (1968); (b) J. L. Gaylor, C. V. Delwiche, and A. C. Swindell, *Steroids*, **8**, 353 (1966).

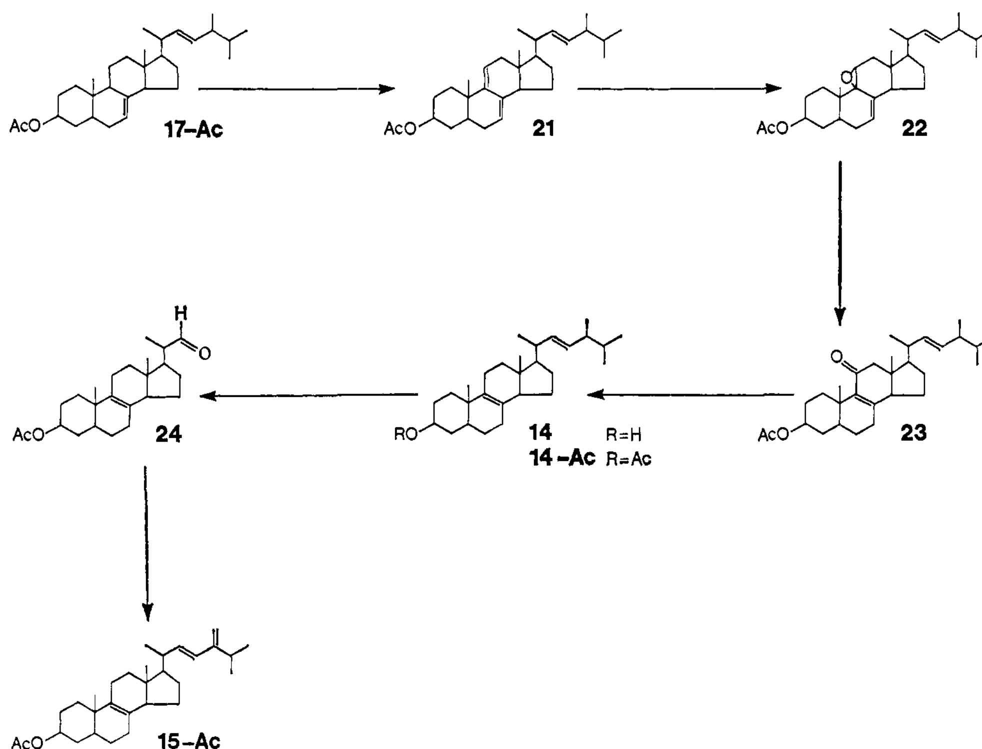
(4) J. T. Moore, Jr., and J. L. Gaylor, *J. Biol. Chem.*, **245**, 4684 (1970).

(5) G. Ponsinet and G. Ourisson, *Bull. Soc. Chim. Fr.*, 3682 (1965).

(6) D. H. R. Barton, D. M. Harrison, G. P. Moss, and D. A. Widdowson, *J. Chem. Soc.*, 775, (1970).

(7) I. Smedley-MacLean, *Biochem. J.*, **22**, 22 (1928).

Scheme I



fecosterol (**10**),[8] episterol (**11**),[8] 5,6-dihydroergosterol (**12**),[9] and ergosta-5,7,22,24(28)-tetraen-3β-ol (**13**).[10] Since zymosterol is the best substrate for the Δ^{24}-sterol methyltransferase and the immediate product of its enzymatic methylation is its C_{24}-methylene derivative (fecosterol), a rather well-defined pattern is evident to this point. Subsequent to this, $\Delta^8 \rightarrow \Delta^7$ isomerization, $\Delta^{24(28)}$ reduction, and Δ^5 and Δ^{22} unsaturation occur in an undefined order. Figure 1 shows the possible alternative sequences that could conceivably operate in the transformation of fecosterol to ergosterol.

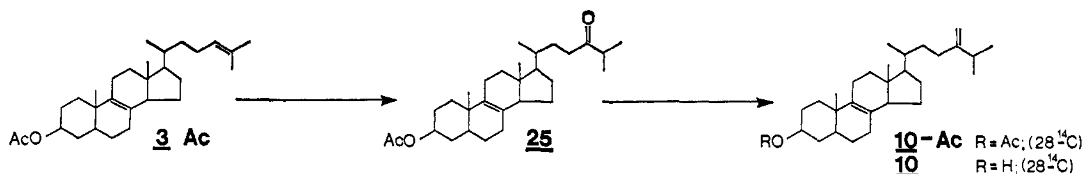
Using Figure 1 as a model, we approached the investigation of these alternative pathways from three complementary directions. Initially we synthesized the suspected sterol intermediates **10**–**20**. With the synthetic samples in hand, we proceeded with the isolation of most of the corresponding natural sterols from yeast sterol mixtures. In order to obtain information on possible *in vivo* precursor–product relationships, we investigated the time course change in sterol composition of anaerobically pretreated, aerobically growing yeast. Although these experiments were designed to minimize initial sterol content and maximize sterol production in the cultures under study, little information concerning the interconversions of the 4,14-desmethyl sterols was obtained. Significantly more light was shed on the operative *in vivo* interconversions by incubation of the synthetic intermediates with aerobically growing yeast. This was coupled with assays for radioactivity in suspected metabolites (nearest structural neighbors) as well as ergosterol. It was found that all but two of the suspected sterols, namely ergosta-8,22,24(28)-trien-3β-ol (**15**) and ergosta-5,7-dien-3β-ol (**20**), were present in growing yeast and involved in the production of ergosterol.

Chemical Synthesis of Intermediates. The preparation of 3β-acetoxyergosta-8,22-diene (**14**-Ac) and 3β-acetoxyergost-8-ene (**16**-Ac) was carried out according to previously reported procedures. Thus, oxidation of 3β-acetoxyergosta-7,22-diene (**17**-Ac) with mercuric acetate gave the 7,9(11),22-triene **21**[11] which was epoxidized[12] at the 9(11) unsaturation (**22**) and converted to 3β-acetoxyergosta-8,22-dien-11-one (**23**) with ferric chloride[13] (Scheme I). Reduction of **23** with lithium aluminum hydride gave the diol[14] which was converted to **14** with lithium in ethylamine. Acetylation of **14** gave **14**-Ac.

Ozonolysis of **14**-Ac followed by a Wittig reaction[15] of the resultant aldehyde (**24**) gave 3β-acetoxyergosta-8,22,24(28)-triene (**15**-Ac). Experiments[15] executed prior to this work have revealed that the ozonolysis–Wittig sequence used for conversion of **14**-Ac to **15**-Ac did not alter the configuration at C-20.

Although mixtures of Δ^{22} cis and trans isomers are undoubtedly formed in the Wittig reactions, the proportion of cis isomer can be kept to a minimum by use of nonpolar solvents such as hexane.[16] Nuclear magnetic resonance spectra and thin layer chromatographic analysis of the crystallized Wittig products revealed that substantially pure Δ^{22} trans isomers could be isolated by one or two crystallizations of the crude reaction product.[16]

(8) H. Wieland and G. Coutelle, *Justus Liebigs Ann. Chem.*, **548**, 270 (1941).
(9) R. K. Callow, *Biochem. J.*, **25**, 87 (1931).
(10) O. N. Breivik, O. L. Owades, and R. F. Light, *J. Org. Chem.*, **19**, 1734 (1954).
(11) G. Saucy, P. Geistlich, R. Helbling, and H. Heusser, *Helv. Chim. Acta*, 37, 250 (1954).
(12) H. Heusser, K. Eichenberger, P. Kurath, H. R. Dallenbach, and O. Jeger, *ibid.*, 34, 2106 (1951).
(13) H. Heusser, R. Anliker, K. Eichenberger, and O. Jeger, *ibid.*, 35, 936 (1952).
(14) A. S. Hallsworth, H. B. Henbest, and T. I. Wrigley, *J. Chem. Soc.*, 1969 (1957).
(15) M. Fryberg, A. C. Oehlschlager, and A. M. Unrau, *Tetrahedron*, 27, 1261 (1971).
(16) M. Fryberg, A. C. Oehlschlager, and A. M. Unrau, *Chem. Commun.*, 1194 (1971).

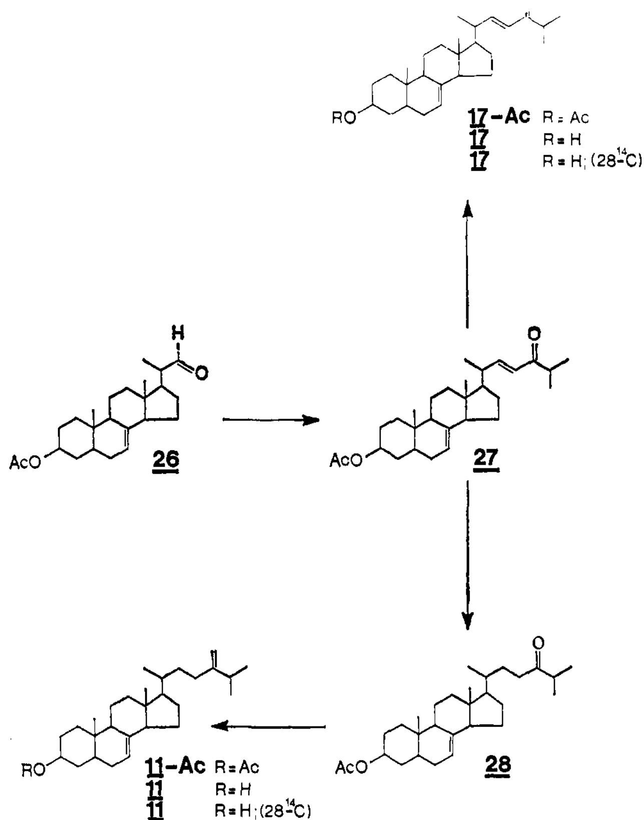


Fecosterol (**10**), with 28-^{14}C label, was synthesized from zymosteryl acetate (**3**-Ac) by a Wittig reaction of [*methylene*-^{14}*C*]triphenylphosphorane with 24-oxozymosteryl acetate (**25**). Unlabeled fecosterol was obtained from yeast sterol mother liquors.

Previous work[15] resulted in the development of efficient procedures for the synthesis of **26**,[15] which in turn was an efficient precursor of Δ^7 sterols having various side-chain structures.

Wittig reaction of **26** gave **27** which could be converted to labeled or unlabeled **17** by a second Wittig reaction with methylenetriphenylphosphorane. Partial reduction of **27** afforded **28** which gave labeled and unlabeled **11** upon the appropriate Wittig reaction (Scheme II). Unlabeled **18** was prepared by reduction

Scheme II



of ergosterol acetate. Tritium was incorporated into **18** at C_2 and C_4 by base-catalyzed exchange of the corresponding ketone in the presence of ^3H_2O followed by $NaBH_4$ reduction.

The $\Delta^{5,7}$ sterol intermediates were prepared by several routes. Ergosta-5,7,22,24(28)-tetraen-3β-ol (**13**) was prepared in labeled and unlabeled form as described earlier.[15] Labeled ergosterol was obtained from yeast cultures grown on [U-^{14}C]acetate. Ergosta-5,7-dien-3β-ol (**20**) was synthesized from labeled and unlabeled ergosterol *via* reduction of the ergosteryl acetate–maleic anhydride adduct.[17] Ergosta-5,7,24(28)-trien-3β-ol (**19**) was synthesized with and without label from **29**.[15] Partial reduction of **29** with palladium on barium sulfate gave **30**. Reaction of **30** with ethylene glycol in the presence of *p*-toluenesulfonic acid gave ketal **31**, which was converted to the corresponding benzoate, **32**, prior to conversion to **33**. The Δ^7 unsaturation was introduced by allylic bromination with NBS followed by dehydrohalogenation in refluxing trimethyl phosphite.[15] This latter reaction was carried out on the benzoate to reduce the proportion of $\Delta^{4,6}$ diene.[15] Deketalization and appropriate Wittig reaction gave **19**.

Isolation of Sterols from Yeast. In order to establish the presence, or absence, of the postulated and chemically synthesized sterol intermediates in yeast, we investigated two sources of natural sterol mixtures: mother liquors from commercial ergosterol production and the sterol fraction of laboratory grown yeast cultures. The latter source contained >80% ergosterol and tetraenol (**13**) with only minor amounts of lanosterol, zymosterol, and other intermediates. The first source proved to be suitable for the isolation of a greater variety of suspected intermediates.

Partially purified sterol mixtures obtained from mother liquors (major portion of ergosterol previously removed) were further chromatographically separated into five major fractions as illustrated in Figure 2.

Fraction 1 contained squalene, identified by comparison of its nmr spectrum with an authentic sample, and a clear oil consisting of a number of low-boiling compounds which had glpc retention times close to that of squalene.

Fraction 2 contained essentially pure lanosterol (**1**).

Fraction 3 contained a mixture of 4,4-dimethylzymosterol (**4**), 4α-methylzymosterol (**5**), and 4-methyl-24(28)-methylenezymosterol (**9**) whose structures were assigned by comparison of glpc retention times[18] and mass spectra[19] with published values.

Fraction 4 was a mixture of mono-, di-, and triunsaturated 4,14-desmethyl sterols.

Fraction 5 contained ergosterol (**2**), tetraenol (**13**), and a third sterol.

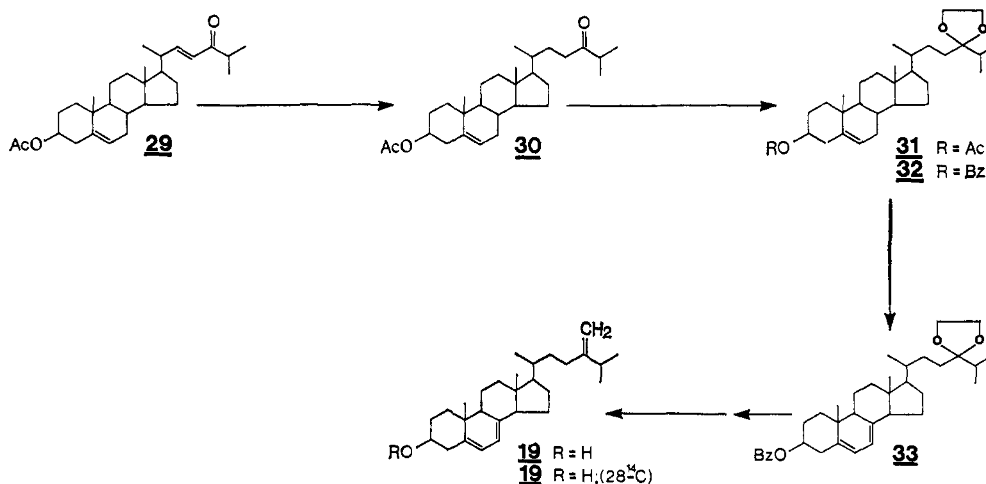
Fraction 4 was acetylated and further separated by chromatography. Three subfractions were collected and further separated by preparative thin layer chromatography as illustrated in Figure 3.

The acetates of **3**, **10**, **11**, **12**, **14**, **16**, **17**, and **18** were identified in this fraction. Separation of fraction 5 gave ergosterol (**2**), tetraenol (**13**), and $\Delta^{5,7,24(28)}$-trienol (**19**).

Thus, in addition to those sterols previously reported,[4-10] **14** and **16–19** also occur in *S. cerevisiae*. Barton, *et al.*,[20] have recently reported the isolation of **17–19** from the same source. The only sterols in Figure 1 not yet detected in this yeast are **15** and **20**.

(17) H. H. Inhoffen, *Justus Liebigs Ann. Chem.*, **508**, 81 (1954).

(18) G. W, Patterson, *Anal. Chem.*, **43**, 1165 (1971).
(19) T. J. Scallen, A. K. Dhar, and E. D. Loughran, *J. Biol. Chem.*, **246**, 3168 (1971).
(20) D. H. R. Barton, U. M. Kempe, and D. A. Widdowson, *J. Chem. Soc., Perkin Trans. 1*, 513 (1972).



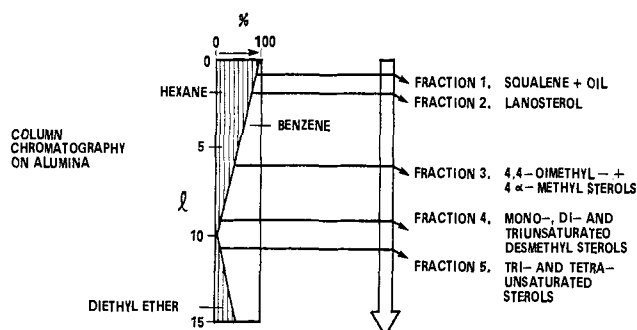


Figure 2. Column chromatography of yeast sterols.

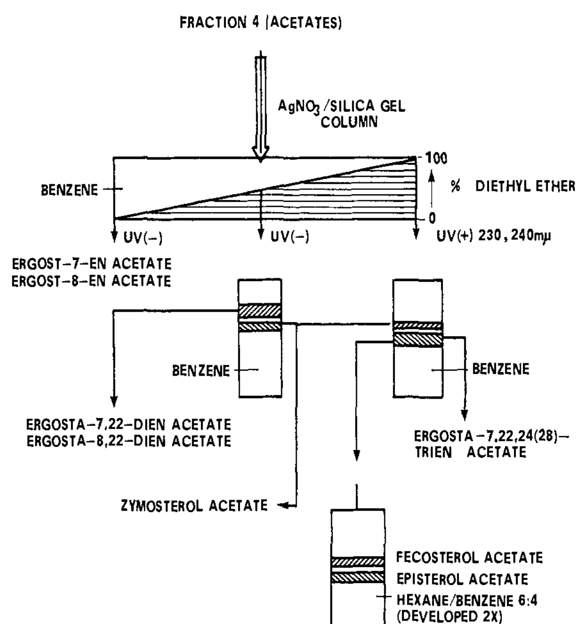


Figure 3. Fractionation of desmethyl sterol acetates.

Results and Discussion

Time-Course Study of Yeast Sterol Composition. As a prelude to the investigation of precursor–product relationships of the intermediates present in yeast sterol mixtures, we studied conditions under which vigorous sterol synthesis could be induced. Ideally a yeast culture was desired which was depleted of sterols and which upon initiation of sterol production would generate sequentially each intermediate. It was hoped that from such time course studies of the sterol composition would emerge a biogenetic sequence.

Several experiments indicated inoculation of liquid medium with yeast followed by aerobic growth gave cultures in which the amount of sterol on a dry weight basis remained relatively unchanged over several days. Time course analysis of the sterol composition of these cultures revealed rather insignificant variations that were not interpretable in terms of precursor–product relationships. Yeast harvested during this type of growth contained ergosterol (**2**) and tetraenol (**13**) as major (80%) sterols with minor amounts of zymosterol (**3**) and lanosterol (**1**) also present.

It was known from the experiments by Klein, *et al.*,[21] that sterol formation could be induced in *Saccharomyces cerevisiae* by vigorous aeration of cells that had previously been maintained under strictly anaerobic conditions. Under these conditions, *S. cerevisiae* becomes auxotropic for sterols and squalene accumulates.[22] This is considered to be due to the necessity of molecular oxygen for the formation of 2,3-oxidosqualene, the immediate precursor of lanosterol.[23,24]

We considered it likely that aeration of such a culture would be accompanied by an accumulation of primordial intermediates. Depending on the rates of interconversion, some intermediates could appear more prominantly in the sterol mixture during early growth rather than at later stages.

When the yeast was cultured under nitrogen for 80 hr, the major portion (95%) of the sterols disappeared. The composition of the small amount of sterol left had changed appreciably. Although there were traces of ergosterol, the major sterol was ergosta-7,22-dien-3β-ol (**12**) followed by decreasing amounts of ergosta-7,22,24(28)-trien-3β-ol (**17**), lanosterol (**1**), zymosterol (**3**), and ergosta-8,22-dien-3β-ol (**14**).

The anaerobically pretreated yeast was resuspended in fresh medium and aerated. Upon aeration the total sterol content increased rapidly (Figure 4). The

(21) H. P. Klein, N. R. Eaton, and J. C. Murphy, *Biochim. Biophys. Acta*, **13**, 591 (1954).
(22) H. P. Klein, *J. Bacteriol.*, 73, 530 (1957).
(23) E. J. Corey, W. E. Russey, and P. R. Ortiz De Montellano, *J. Amer. Chem. Soc.*, **88**, 4750 (1966).
(24) E. E. van Tamelen, J. D. Willett, R. B. Clayton, and K. E. Lord, *ibid.*, **88**, 4752 (1966).

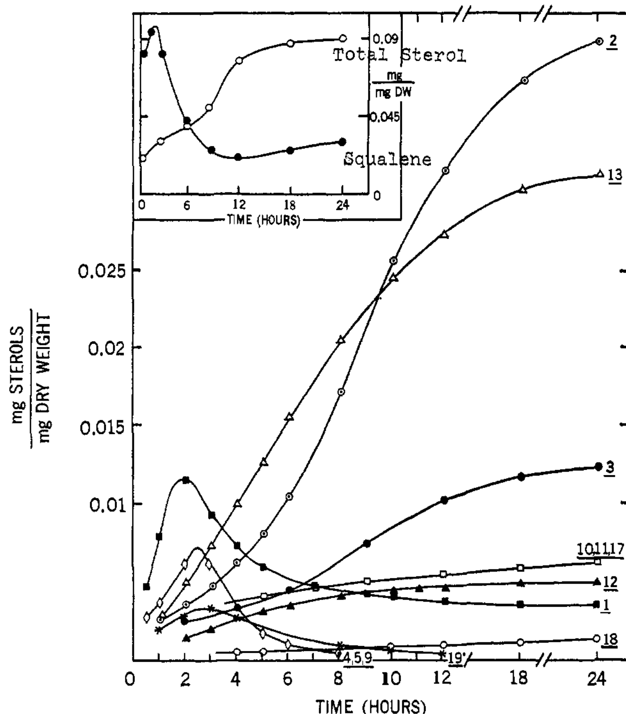


Figure 4. Variation of sterol composition in aerobically growing yeast.

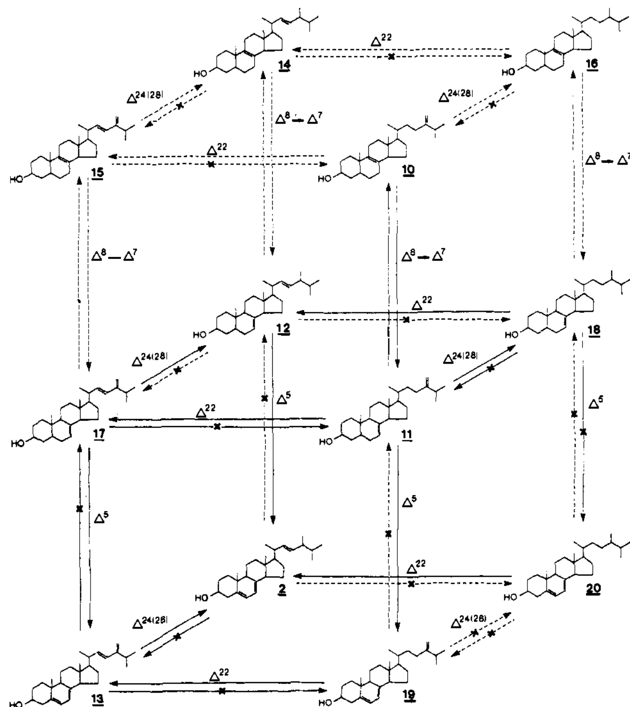


Figure 5. Biosynthetic pathways operative in yeast. Δ^{22}, $\Delta^{24(28)}$, Δ^{5}: introduction or removal of indicated unsaturation. $\Delta^{8} \rightarrow \Delta^{7}$: isomerization (——) operative transformations; (-----) transformations probably operating; (—X—) transformations not operating; (--X--) transformations probably not operating.

changing composition of the yeast sterol mixture during this period is illustrated in Figure 4.

These time-course studies revealed that squalene concentration increased briefly upon the commencement of aeration and then decreased sharply as the total sterol content increased. When sterol production became constant, squalene synthesis approximately paralleled sterol synthesis. Three types of behavior with respect to sterol pool size were revealed. The concentrations of lanosterol, 4-methylated and 4,4-dimethylated intermediates, and $\Delta^{5,7,24(28)}$-trienol **19** increase rapidly to attain early maxima, then subsequently decrease to low values. This confirms previous reports[25,26] concerned with the accumulation of lanosterol during fermentation.

The concentrations of the Δ^{8} and Δ^{7} 4,14-desmethyl sterols increase slowly toward their steady-state concentrations. Episterol (**11**), ergosta-7,22,24(28)-trien-3β-ol (**17**), and fecosterol (**10**) increase steadily with their relative concentrations deviating only slightly from a 4:2:1 ratio. Noteworthy is the relatively low concentration of ergost-7-en-3β-ol (**18**) compared with the other desmethyl sterols. The concentrations of ergosterol and tetraenol (**13**) increase slowly at first, then at increasing rates through the middle of the time course. Finally, these two sterols reach high concentrations in the sterol fraction as would be expected of final metabolic products.

The variation of concentration with time observed for the 4,14-dimethylated and 4-methylated intermediates is that expected of initial intermediates produced from squalene in a culture primed by squalene accumulation. Since these compounds are undoubtedly precursors of the desmethyl sterols they must ordinarily undergo rapid turnover.

(25) W. K. Bronn, Abstract of Papers, International Fermentation Symposium, London 1964, Paper D23.

(26) E. Kodicek and D. R. Ashby, *Biochem. J.*, 60, P-35 (1957).

The case of the $\Delta^{5,7,24(28)}$-trienol **19** is not as clear-cut. After reaching an early maximum, it decreases in concentration to a very low value. It is perhaps noteworthy that the rate of increase of concentration of tetraenol **13** decreases appreciably as the concentration of trienol **19** drops. A possible interpretation of the above is that tetraenol is initially formed from **19** as well as **17**. With time the net production of **19** decreases with a concomitant decrease in the rate of formation of tetraenol.

Although anaerobic priming induced rapid accumulation of some intermediates, no definitive information concerning the biointerconversions of the 4,14-desmethyl sterols present in *S. cerevisiae* emerged from this study. The results were, however, useful in the interpretation of subsequent incubation experiments.

Tracer Studies. Tracer experiments were designed to yield information concerning the bioconversions of the 4,14-desmethyl sterols found in *S. cerevisiae.* Labeled intermediates were incubated with aerobically maintained, anaerobically pretreated yeast. The incubations were stopped after 7–9 hr aerobic growth and the sterols isolated in the usual fashion (hydrolysis, extraction, acetylation, tlc separation). Analogous unlabeled sterol acetates were added to the proper tlc fraction (Table II, see Experimental Section). The individual sterol acetates separated in this manner were crystallized to constant activity and their purity was established by glpc. The incorporations are recorded in Table I and illustrated in Figure 5.

Since the most probable combinations of 4,14 demethylation and 24 alkylation proceeding from lanosterol result in production of fecosterol (**10**), our initial

Table I. Activity of Isolated Metabolites[a]

Labeled sterol added to whole cells	**10**	**14**	**11**	**17**	**12**	**18**	**13**	**19**	**20**	**2**	Total incorporation
10			8.2				12.6			13.1	46.2
11						0.21				5.1	29.0
				6.1		0.32	5.4			2.9	52.6
							17.6	0.4		4.6	84.6
	10.1	0.4								12.3	64.0
17			<0.001		0.23		9.7			3.7	83.3
18					10.45			<0.001	<0.01	16.4	32.3
			<0.001		11.6		<0.001			15.2	27.8
13				<0.001				<0.001		17.8	38.5
19							7.1			3.5	58.1
20					<0.001			<0.001		19.2	44.1
2							0.1[b]				

[a] The values are per cent incorporation based on total activity recovered in the nonsaponifiable fraction. [b] D. H. R. Barton, T. Shioiri, and D. W. Widdowson, *J. Chem. Soc.*, 1968 (1971).

experiments commenced with the incubation of labeled **10**. Trapping for episterol (**11**), tetraenol **13**, and ergosterol yielded activity in all three sterols. The several experiments involving incubation of episterol (**11**) revealed that it underwent four *in vivo* transformations: $\Delta^8 \rightarrow \Delta^7$ isomerization, introduction of unsaturation at C_{22} or C_5 or reduction of the 24(28)-methylene. The intermediates produced from each of these transformations were subsequently shown to be converted to ergosterol.

Of particular interest is the conversion of episterol (**11**) to fecosterol (**10**) and to the $\Delta^{8,22}$ dienol **14**. These transformations indicate that the $\Delta^8 \rightarrow \Delta^7$ isomerization responsible for the **10** → **11** conversion is reversible. This observation parallels that which was made for the $\Delta^8 \rightarrow \Delta^7$ isomerase present in liver microsomes.[27] This was the only transformation which in our system was found to be reversible. Once Δ^{22} or Δ^5 unsaturation is introduced they are not removed ($\Delta^{22} \nrightarrow$ 22H23H: **17** $\nrightarrow$ **11**, **13** $\nrightarrow$ **19**; $\Delta^5 \nrightarrow$ 5H,6H: **13** $\nrightarrow$ **17**). Neither does reintroduction of the 24(28)-methylene occur after it has been reduced to the corresponding 24-methyl (24-$CH_3 \nrightarrow \Delta^{24(28)}$: **18** $\nrightarrow$ **11**, **20** $\nrightarrow$ **19**, **2** $\nrightarrow$ **13**). Although we did not check the **12** → **17** transformation, we suspect it to be nonoperative since it involves reintroduction of a 24(28)-methylene. Since **18** is converted to **2** as well as **12**, the **12** → **2** conversion must also occur *in vivo*. This means that **18** is the only 5,6-dihydro sterol (of the four tested) not directly desaturated at C_5. The failure to find **20** in yeast sterol mixtures means that, in addition to the inefficiency of the **18** → **20** conversion, the **19** → **20** conversion must also be inefficient or nonoperative. Similar conclusions can be drawn for transformations producing **15** whose presence in yeast sterol mixtures could not be confirmed.

There were a higher number of isolable 4,14-desmethyl sterols compared to their 4,14-methylated precursors. This coupled with the many transformations revealed by the incubation experiments is consistent with the view that the enzymatic systems responsible for nuclear demethylation, C_{24} alkylation as well as modification of the 4,14-desmethyl sterol intermediates will each accept a variety of closely related substrates. For example, introduction of Δ^{22} (and Δ^5) unsaturation (as well as reduction of the 24(28)-methylene) occurs on substrates not possessing this functionality. These observations make it appear that these transformations can occur in any order. The notable absence of 4,14-desmethyl sterols not alkylated at C_{24}, sterols possessing Δ^5 but not Δ^7 unsaturation, and Δ^7 sterols possessing 4-methylation eliminates from present consideration a number of alternative sequences. The different pathways considered to be operative based on time-course and incubation studies are those illustrated in Figure 5.

There are two basic approaches to the analysis of the available data with a view toward deduction of the major pathway for ergosterol production. Since preceding arguments have pointed to fecosterol as the major product of initial demethylation and C_{24} alkylation, one approach would be to determine which intermediates formed from fecosterol and its metabolites were most efficiently transformed to ergosterol. The rationale for the use of this approach is based on the assumption that the individual labeled intermediates administered exogenously diffused into the cellular sterol pools at comparable rates. The rate of the respective conversions to ergosterol should therefore be manifested in radioactivity accumulated in this final metabolite. Since independent experiments[28] have shown that within a single experiment exogenous labeled sterols of such widely divergent structure as lanosterol and zymosterol diffuse into cellular sterol pools at nearly identical rates, this approach appears valid. Unfortunately, there is considerable variance from one experiment to another in the incorporation of the same intermediate into ergosterol (*e.g.*, **11** → **2**, Table I), making incorporation into ergosterol noncomparable between individual experiments. This variation is most likely due to difficulty in reproducing exact growth conditions and sterol production rates.

Finally, this analysis suffers drawbacks for quite a different reason. It was found that high incorporations of intermediates into ergosterol could be obtained even for those sterols, such as **20**, not present in detectable amounts.

A second and more fruitful approach is to couple the

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information gained from the time-course studies of sterol pool size with that from the incubation experiments on incorporation efficiencies. The rationale in this instance is that the observation of a high pool size coupled with high incorporation from a precursor is interpretable in terms of an efficient transformation. The high efficiency of the fecosterol (**10**) to episterol (**11**) conversion coupled with the moderate pool size of each suggests that this is a major transformation. Episterol, in turn, is converted to all its structural nearest neighbors **17**, **18**, and **19** (and **10** as discussed earlier). The most efficient of these transformations is that giving the $\Delta^{7,22,24(28)}$-trienol **17**. Since **17** has a smaller pool size than **18** or **19**, the higher incorporation of label into **17** certainly suggests that it is the major metabolite of episterol. The transformation of **17** to ergosterol occurs *via* both dienol **12** and tetraenol **13**. Based on incorporation efficiencies of **17** into **12** and **13** as well as the efficiency of the **13** $\rightarrow$ **2** transformation, it appears that **17** is converted to ergosterol primarily *via* tetraenol **13**.

The major sequence from fecosterol to ergosterol is thus deduced to be: fecosterol (**10**) $\rightarrow$ episterol (**11**) $\rightarrow$ ergosta-7,22,24(28)-trien-3β-ol (**17**) $\rightarrow$ ergosta-5,7,22,-24(28)-tetraen-3β-ol (**13**) $\rightarrow$ ergosterol (**2**).

Experimental Section

Melting points were measured on a Fisher-Johns apparatus and are uncorrected. Ultraviolet spectra were measured on a Unicam SP800 with ethanol as the solvent. Infrared spectra were measured on a Perkin-Elmer 457 instrument. Nmr spectra were recorded on Varian A56/60 or XL-100 spectrometers and reported as δ *vs.* an internal TMS (δ 0) standard. Mass spectra were obtained on a Perkin-Elmer Hitachi RMU-7 instrument using an ionization voltage of 80 eV. Glpc analyses were carried out on a Varian Model 2100 instrument equipped with an all glass system and a flame ionization detector. The columns used were (3.4 m $\times$ 25 mm) packed with either 3% QF-1 or 3% XF-60 on Chromosorb G-AW-DMCS (80–100 mesh). Quantitative determinations were carried out according to the method of Rozanski.[29]

Plates for preparative tlc were made by spreading a mixture of 18 g of $AgNO_3$, 40 g of silica gel G (Merck), 0.1 g of Rhodamine 6G, 65–70 ml of H_2O, and 20 ml of ethanol over four plates (20 $\times$ 20 cm). 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 was extracted four times with diethyl ether by resuspension and centrifugation. The ether was evaporated; the residue was taken up in 10 ml of hexane-ether (4:1, v/v) and percolated through a column (10 $\times$ 1 cm) of Celite-silica gel (1:1, w/w) to remove the extracted Rhodamine. The column was washed with an additional 50 ml of the same solvent mixture. The solvent was evaporated giving the crude sterol acetates.

Control experiments with several radioactive compounds showed a recovery of at least 85% using the above method. For analytical plates the Rhodamine 6G was omitted and the plates were developed 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°.

Radioactivity was determined with a Beckman Model LS-200B liquid scintillation spectrometer. The sample to be assayed was dissolved in a cocktail containing 4 g of PPO and 50 mg of POPOP per liter of toluene. Counting was carried out for 50–100 min, depending on the activity of the sample.

Preparation of 3β-Acetoxyergosta-8,22-diene (14-Ac). Ergosta-8,22-dien-3β,11β-diol[14] was reduced by the method of Hallsworth,[14] *et al.* Acetylation gave **14**-Ac, mp 168–169° (lit.[14] mp 166–169°).

Preparation of 3β-Acetoxyergost-8-ene (16-Ac). Hydrogenation of **14**-Ac in ethyl acetate in the presence of Adams catalyst[14] yielded **16**-Ac, mp 158–159° (lit.[14] mp 156–158°). Glpc and nmr comparison with 3β-acetoxyergost-8(14)-ene[30] revealed significant differences indicating $\Delta^8 \rightarrow \Delta^{8(14)}$ isomerization did not occur during this reaction.

Preparation of 3β-Acetoxyergosta-8,22,24(28)-triene (15-Ac). Ozonolysis of **14**-Ac at $-70°$ in CH_2Cl_2 containing 2% pyridine followed by zinc-acetic acid work-up[15] gave 3β-acetoxy-23,24-dinor-5α-chol-8(14)-en-22-al which was isomeric with the desired Δ^8 compound.

Ozonolysis was repeated using the method of Pappas, *et al.*,[31] to give a 50% yield of 3β-acetoxy-23,24-dinor-5α-chol-8-en-22-al (**24**) *ca.* 93% pure by glpc: nmr ($CDCl_3$) δ 0.61 ($C_{18}HC_3$, s), 0.95 (C_{19}-CH_3, s), and 9.57 (O=CH, d, J = 3 Hz).

This aldehyde (250 mg) was reacted with an excess of 2-methylene-3-methylbutane-1-triphenylphosphorane[15] in THF. The reaction mixture was stirred for 1 hr at room temperature and refluxed for 2.5 hr. Excess reagent was decomposed with wet THF. The mixture was then poured into 500 ml of MeOH-H_2O (1:5) and the product extracted with ether. The ether extract was washed several times with saturated NaCl solution, dried over anhydrous Na_2SO_4, and evaporated. The crude product was reacetylated in pyridine-acetic anhydride (2:1) at room temperature for 12 hr and worked up by rapid filtering through a short silica gel column using hexane-ether (9:1) as solvent. Final purification by preparative tlc gave 3β-acetoxyergosta-8,22,24(28)-triene (**15**-Ac, 45%): mp 131–133°; ν_{max} (KBr) 1722 and 890 cm^{-1}; λ_{max} 230 nm (ϵ 28,500) and 240 nm sh (16,800); nmr ($CDCl_3$) δ 0.57 ($C_{18}CH_3$, s), 0.93 (C_9CH_3, s), 1.05 ($C_{25}(CH_3)_2$, $C_{20}CH_3$, d, J = 7 Hz), 2.10 (CH_3COO, s), 4.8 ($C_{24}CH_2$, d, J = 2 Hz), 5.51 ($C_{22}H$, d of d, J = 16 Hz, J = 8 Hz) and 5.90 ($C_{23}H$, d, J = 16 Hz).

Anal. Calcd for $C_{28}H_{44}O$: C, 84.79; H, 11.18. Found: C, 84.84; H, 11.14.

Preparation of [28-^{14}C]Ergosta-8,24(28)-dien-3β-ol (10). To 500 mg of zymosterol acetate (**3**-Ac, 1.17 mmol) in 15 ml of THF and 3 ml of water at 0°, NBS (250 mg, 1.4 mmol) was added with stirring. The mixture was stirred for 2 hr, 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 (**25**), pure by glpc: nmr ($CDCl_3$) δ 0.62 ($C_{18}CH_3$, s), 1.0 ($C_{19}CH_3$, s), and 1.06 ($C_{20}CH_3$, d, J = 7 Hz). The ketone (100 mg) was reacted with the ylide prepared from [^{14}C]methyltriphenylphosphonium iodide and BuLi in THF analogous to methods described earlier.[15] After work-up, acetylation, and purification by tlc as described above, fecosterol acetate (**10**-Ac) was crystallized from methanol in a pure (glpc) state. Comparison of the physical data of this sample with those of **10**-Ac isolated from yeast revealed no significant differences.

Fecosterol acetate (**10**-Ac) was hydrolyzed by refluxing in 2% K_2CO_3-10% H_2O in methanol for 1 hr. Fecosterol (**10**) was isolated with an activity of 6.9 $\times$ 10^5 cpm/mg.

Preparation of 3β-Acetoxy-5α-chol-7-en-22-al (26). Ergosterol acetate (15 g, 0.034 mol) in 400 ml of thiophene-free benzene was hydrogenated at 40 psig with *ca.* 10 g of Raney Ni. After 2 hr 1.1 mol equiv of H_2 was taken up. The catalyst was filtered through a short column of Celite 535 and the solvent evaporated. The residue was crystallized from ethyl acetate-methanol to give **12**-Ac (92%), mp 184.5–187° (lit.[32] mp 182–186.5).°

Aldehyde **26** was prepared from **12**-Ac by ozonolysis,[33] mp 144–145° (lit.[33] mp 144–145°), and nmr spectrum with prominent signals identical with those reported[34] for this compound.

Preparation of 3β-Acetoxy-5α-cholesta-7,22-dien-24-one (27). The aldehyde **26** was converted to ketone **27** by reaction with 3-methylbutan-2-one-1-triphenylphosphorane as described elsewhere.[34] The product was purified on a silica gel column using 10% ethyl ether in hexane as the eluting solvent to give **27** (67%), mp 143–144° (lit.[34] mp 143–144°). The nmr spectrum of **27** contained prominent absorptions at positions identical with those reported[34] for this compound.

Preparation of 3β-Acetoxy-5α-cholest-7-en-24-one (28). A solu-

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tion of 1.2 g (2.73 mmol) of ketone **27** in 50 ml of ethyl acetate was stirred at room temperature at 50-psig H_2 over 10% Pd–$BaSO_4$.[35] The suspension was filtered through a column of Celite 535 and the product recrystallized from methanol to give mp 97–98° (lit.[31] mp 96–99°).

Preparation of 3β-Acetoxyergosta-7,24(28)-diene (11-Ac). Methylenetriphenylphosphorane was prepared by addition of BuLi (2.2 mmol) in heptane to a suspension of methyltriphenylphosphonium iodide (2.4 g, 10 mmol) in 50 ml of dry THF. The mixture was allowed to react under N_2 for 1 hr at room temperature. Ketone **28** (0.9 g, 2.05 mmol) in THF was added and the reaction allowed to proceed at room temperature for 1 hr and at reflux for 2.5 hr. Work-up as described above followed by acetylation and crystallation from ethanol gave 0.55 g (60.5%) of **11**-Ac, mp 136–137°. Two recrystallizations gave a melting point of 140–140.5° (lit.[36] mp 140°). Hydrolysis of episterol acetate gave the free alcohol, mp 130–130.5° (lit.[36] mp 131°).

Anal. 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)-dien-3β-ol (11). This sterol was prepared as above from ketone **28** using [^{14}C]methyltriphenylphosphonium iodide. The compound had nmr and mass spectra identical with **11** prepared above and showed an activity of 6.2 × 10^5 cpm/mg.

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

Preparation of 3β-Acetoxyergosta-5α-7,22,24(28)-triene (17). Ketone **27** (1.0 g, 2.28 mmol) was reacted in THF with a fivefold excess of the ylide generated from methyltriphenylphosphonium iodide and BuLi in THF, in the same manner as for ketone **28**. The same work-up and reacetylation gave 652 mg (65%) of **17**-Ac: mp 134.5–136.5; ν_{max} (KBr) 1640, 975, 965, and 890 cm^{-1}; λ_{max} 232 nm (ε 33,100); nmr ($CDCl_3$) δ 0.54 ($C_{18}CH_3$, s), 0.90 (C_{19}-CH_3, s), 1.06 ($C_{25}(CH_3)_2$, $C_{20}CH_3$, d, J = 7 Hz), 2.01 (CH_3COO, s), 2.48 ($C_{25}H$, sept., J = 7 Hz), 4.8 ($C_{24}CH_2$, d, J = 2 Hz), 5.14 (C_7H, broad s), 5.52 ($C_{22}H$, d of d, J = 16 Hz, J = 8 Hz), and 5.92 ($C_{23}H$, d, J = 16 Hz). Hydrolysis of **12**-Ac gave the free alcohol **17**, mp 121–124°.

Anal. Calcd for $C_{28}H_{44}O$: C, 84.79; H, 11.18. Found: C, 84.55; H, 11.01.

Preparation of [28-^{14}C]Ergosta-5α-7,22,24(28)-trien-3β-ol (17). This compound was prepared as above from ketone **27** using [^{14}C]methyltriphenylphosphonium iodide. The trienol had the same spectral properties as **17** above and had an activity of 4.52 × 10^5 cpm/mg.

Anal. Calcd for $C_{28}H_{46}O$: C, 84.35; H, 11.63. Found: C, 84.51; H, 11.48.

Preparation of Ergosta-7-en-3β-ol (18). Ergosterol acetate (2.0 g) dissolved in thiophene-free benzene (70 ml) was hydrogenated at room temperature and 45 psig using freshly prepared Raney Ni until 2.2 mol equiv as absorbed. The mixture was filtered over Celite 535, the solvent evaporated, and the product recrystallized from ethanol. The product, **18**-Ac, was separated from unreacted starting material (~6% by glpc) by chromatography on $AgNO_3$-coated silica gel, mp 162–164° (lit. mp 162–164°;[37] 153°;[38] 158–160°[39]).

The acetate was hydrolyzed with 2% K_2CO_3–10% H_2O in methanol to give the alcohol, mp 145–146° (lit. mp 141–145°;[38] 148°[39]).

Preparation of [2,4-^{3}H]Ergost-7-en-3β-ol (18). A solution of ergost-7-en-3β-ol (**18**, 750 mg) in 100 ml of acetone was cooled to 10° and treated for 15 min with 1.2 ml of Jones reagent. Excess reagent was decomposed with isobutyl alcohol and the mixture poured over ice. The product was extracted with ether and the extract washed with water, dried over anhydrous Na_2SO_4, and evaporated. Crystallization from ethanol gave 610 mg of ergost-7-en-3-one: mp 164.5–165.5°; ν_{max}^{KBr} 1708 C=O cm^{-1}; nmr ($CDCl_3$) δ 0.56 ($C_{18}CH_3$, s), 0.78 (d, J = 6.5 Hz), 1.0 ($C_{19}CH_3$, s), and 5.1–5.3 (C_7H, m).

The ketone (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 ml) were added. The mixture was kept at room temperature for 44 hr, then added to an excess of water and extracted with ether. The ether extract was washed several times with water, dried over anhydrous Na_2SO_4, and evaporated, and the residue was crystallized from methanol; 82 mg, mp 162.5–164.5°. No depression of the melting point was observed when mixed with the original ketone.

The tritiated ketone (80 mg) was dissolved in 25 ml of methanol and 20 mg of $NaBH_4$ added. The mixture was stirred at room temperature 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 **18** with an activity of 3.2 × 10^5 cpm/mg.

Preparation of [28-^{14}C]Ergosta-5,7-22,24(28)-tetraen-3β-ol (13). The tetraenol **13** was prepared *via* Wittig reaction of 3β-benzoxyergosta-5,7,22-trien-24-one and [^{14}C]methyltriphenylphosphonium iodide as described earlier.[15] The compound had an activity of 7.2 × 10^5 cpm/mg.

Preparation of [U-^{14}C]Ergosterol. Labeled ergosterol was obtained biosynthetically by incubating yeast aerobically in he presence of [U-^{14}C]acetate. The harvested cells were hydrolyzed as described below and ergosterol was recovered as the acetate. Hydrolysis, purification by tlc, and six crystallizations gave the pure compound (by glpc) with an activity of 3.0 × 10^4 cpm/mg.

Preparation of Ergosta-5,7-dien-3β-ol (20). This compound was prepared from ergosterol acetate *via* the maleic anhydride adduct and hydrogenation over Adam's catalyst as described;[17] melting point of the acetate was 155–156° (lit.[17] mp 157–158°). The labeled compound was prepared in an analogous manner using [U-^{14}C]ergosterol obtained biosynthetically as described above. The activity was 2.9 × 10^4 cpm/mg.

Preparation of 3β-Acetoxycholest-5-en-24-one (30). A solution of 1.2 g (2.7 mmol) of 3β-acetoxycholesta-5,22-dien-24-one (**29**)[15] in 50 ml of ethyl acetate was hydrogenated at room temperature and 50 psig pressure for 35 min, using 10% Pd–$BaSO_4$[35] as catalyst. The mixture was filtered through a Celite 535 column and the solvent evaporated. Recrystallization from methanol gave 1.14 g of **30** (95%), mp 128–129.5° (lit.[40] mp 129–130°).

Preparation of 3β-Acetoxycholest-5-en-24-one Ethylene Ketal (31). A solvent of 1.0 g (2.2 mmol) of ketone **30**, 3 ml of ethylene glycol, and 150 mg of *p*-toluenesulfonic acid in 800 ml of dry benzene was refluxed for 1 hr. Benzene was then slowly removed by distillation until 500 ml of distillate was collected. The benzene solution containing the product was washed with concentrated $NaHCO_3$ solution, then with H_2O until neutral, dried over anhydrous $CaCl_2$, and evaporated. The crystalline residue (0.97 g, 88.5%) was recrystallized from acetone to give **31**: mp 134.5–135°; ν_{max} (KBr) 1728 (acetate C=O), 1040, and 798 (Δ5) cm^{-1}; nmr ($CDCl_3$) δ 0.69 ($C_{18}CH_3$, s), 0.925 ($C_{21}CH_3$, d, J = 6.5 Hz), 1.02 ($C_{19}CH_3$, s), 2.02 (CH_3COO, s), 3.95 (-OCH_2CH_2O-, s), 4.3–4.9 (C_3H, m), and 5.3–5.55 (C_6H, m).

Preparation of 3β-Benzoxycholest-5-en-24-one Ethylene Ketal (32). The acetate **31** was hydrolyzed by refluxing in 2% K_2CO_3 in 10% aqueous methanol for 1 hr and the alcohol obtained was benzoylated with benzyl chloride in pyridine. After the usual work-up purification over a short alumina column, and recrystallization from acetone, the benzoate, **31**, had mp 146–148°: ν_{max} (KBr) 1710 (C=O, benzoate), 1250, 1100 (CO), 1040, and 798 (Δ5) cm^{-1}; nmr ($CDCl_3$) δ 0.695 ($C_{18}CH_3$, s), 0.92 ($C_{25}(CH_3)_2$, $C_{20}CH_3$, d, J = 6.5 Hz), 3.96 (-OCH_2CH_2O-, s), 4.6–5.2 (C_3H, m), 5.4–5.6 (C_5H, m), and 7.3–8.25 (aromatic H, m).

Preparation of 3β-Benzoxycholesta-5,7-diene-24-one Ethylene Ketal (33). A solution of 0.9 g (1.8 mmol) of 3β-benzoxycholest-5-en-24-one ethylene ketal (**32**) in 50 ml of CCl_4 was heated to reflux and 0.34 g of NBS (2.2 mol) 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 bromo sterol, was added dropwise to a vigorously boiling solution of 0.7 g of trimethyl phosphite in 20 ml of xylene. After the solution was refluxed for 90 min, the xylene was removed at 75° under vacuum, and the crystalline residue recrystallized from acetone to give 0.55 g (61%) of ketal **33** with mp 140–142°: ν_{max} (KBr) 1710 (C=O, benzoate), 1250, 1110 (-CO-), 1600, 1580, 1065, 1025, 832, and 800 (Δ^5, 7) cm^{-1}; nmr ($CDCl_3$) δ 0.64 ($C_{18}CH_3$, s), 0.93 ($C_{25}(CH_3)_2$, d, J = 6.8 Hz), 1.05 ($C_{19}CH_3$, s), 1.10 ($C_{20}CH_3$, d, J = 6.8 Hz), 1.05 ($C_{19}CH_3$, s), 1.10 ($C_{20}CH_3$, d, J = 7.0 Hz), 3.96 (-OCH_2CH_2O-, s), 4.75–5.3 (C_3H, m), 5.3–5.75 (C_5H, C_6H, m), and 7.3–8.25 (aromatic H, m).

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Preparation of 3β-Benzoxycholesta-5,7-dien-24-one. To a solution of 0.9 g (1.65 mmol) of ketal **33** in THF, 5% aqueous H_2SO_4 was added until the solution became turbid. The solution was clarified with additional THF, stirred at room temperature for 90 min, and neutralized with Na_2CO_3 solution and the THF evaporated. The product (0.71 g, 86%) was extracted with ether and recrystallized from methanol: mp 140–141°; nmr ($CDCl_3$) δ 0.64 ($C_{18}CH_3$, s), 1.0 ($C_{19}CH_3$, s), 1.10 ($C_{21}CH_3$, $C_{25}(CH_3)_2$, d, J = 7.0 Hz), 4.75–5.3 (C_3H, m), 5.3–5.8 (C_5H, C_6H, m), and 7.3–8.25 (aromatic H, m).

Preparation of Ergosta-5,7,24(28)-trien-3β-ol (19). A solution of 3β-benzoxycholesta-5,7-dien-24-one (0.4 g)i n THF was added to a THF solution of the ylide prepared from 1.2 g (5 mmol) of methyltriphenylphosphonium iodide and 4.5 mmol of butyllithium. The reaction mixture was kept 1 hr at room temperature and 3 hr at 65°. The usual work-up followed by reacetylation and recrystallization from methanol gave **19**-Ac (84 mg, 24%): mp 133–134°; ν_{max} (KBr) 1732 (C=O, acetate), 1645, 887 (terminal methylene), 1600, 1580, 1025, 832, 800 (Δ5, 7) cm^{-1}; nmr (in $CDCl_3$) δ 0.625 ($C_{18}CH_3$, s), 0.970 ($C_{19}CH_3$, s), 1.03 ($C_{25}(CH_3)_2$, $C_{20}CH_3$, d, J = 7 Hz), 2.03 (CH_3COO, s), 4.7 (CH_2=C<, m) and 5.3–5.65 (C_5H, C_6H, m).

The acetate (**19**-Ac) was hydrolyzed by refluxing in 2% K_2CO_3–10% H_2O in methanol for 1 hr to give **19**, mp 129–130.5°.

No melting point was reported for the natural product:[41] λ_{max} (hexane) 262.5 nm (ε 9800), 271.8 nm (13,780), 282.0 nm (8550); ν_{max} (KBr) 1650, 887 (CH_2=C<), 1600, 1580, 832, and 800 (Δ5, 7) cm^{-1}; nmr ($CDCl_3$) δ 0.64 ($C_{18}CH_3$, s), 0.95 ($C_{19}CH_3$, s), 1.04 ($C_{25}(CH_3)_2$, C-20, d, J = 7 Hz), 1.265 (HOC, s), and 4.7 (CH_2=C<, broad m).

Anal. Calcd for $C_{28}H_{44}O$: C, 84.79; H, 11.18. Found: C, 84.61; H, 11.25.

Preparation of [28-^{14}C]Ergosta-5,7,24(28)-trien-3β-ol (19). This sterol was prepared from ketone **33** *via* acetate **19**-Ac using [^{14}C]-methyltriphenylphosphonium iodide. The product showed melting point and nmr identical with **19** prepared above and had an activity of 7.1×10^5 cpm/mg.

Separation of Yeast Sterols. The mother liquors from ergosterol production as supplied by Mycofarm-Delft were evaporated and the residue was partially purified by acetylation, crystallization, and hydrolysis. The semicrystalline mixture of sterols obtained (~10 g) was dissolved in a minimum amount of benzene and added to a column (200–250 g of Activity II alumina) made up in hexane. Elution was started with hexane, changed to benzene, and then to benzene–ethyl ether (1:1). In a typical run *ca.* 15 l. of solvent as used. Fractions of 10 ml were collected and analyzed by tlc, glpc, and/or uv and those of like composition combined into the five fractions illustrated in Figure 2.

Fractions 1, 2, and 3 were analyzed by glpc–ms and nmr.

Fraction 4 was acetylated and separated as per Figure 3 on a silica gel column containing 15% $AgNO_3$. Further resolution of the resultant subfractions by preparative tlc (silica gel with 45% $AgNO_3$ and 0.25% Rhodamine 6G) gave the acetates of **3**, **10**, **11**, **12**, **17**, and **18** in sufficient quantities for comparison of their melting points, nmr, and mass spectra and glpc retention times with synthetic samples in hand.

A compound which cochromatographed with 5,6-dihydroergosteryl acetate (**12**-Ac) could not be isolated in pure form but had a glpc retention time and mass spectrum (glpc–ms) which corresponded to that of 3β-acetoxyergosta-8,22-diene (**14**-Ac). Similarly, a compound which cochromatographed with 3β-acetoxyergost-7-ene (**18**-Ac) was revealed in the glpc trace of this fraction (4-1) as a small peak with the relative retention time corresponding to 3β-acetoxyergost-8-ene (**16**-Ac).

A glpc and tlc search for 3β-acetoxyergosta-8,22,24(28)-triene (**15**-Ac) and 3β-acetoxyergosta-5,7-diene (**20**-Ac) in the fractions into which they would have separated revealed no detectable amounts of these compounds.

Preparative tlc separation of fraction 5 gave, in order of their descending R_f on benzene developed plates, **2**, **13**, and **19** identified as above.

Yeast Growth Conditions. *Saccharomyces cerevisiae* of an unknown strain (ale brewers yeast), obtained fresh from a local brewery, was maintained under N_2, then resuspended for aerobic growth. For anaerobic maintenance, the medium (M-1) had the following composition:[42] (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 *M* in phosphate pH 6.4); (5) K_2PHO_4, 0.78% (0.1 *M* in phosphate pH 6.4); (6) glucose, 2.5%. Components 1–5 and glucose were dissolved in 500 ml of water in separate 1-l. flasks and sterilized for 15 min at 120°. Immediately after removal from the autoclave the solutions were combined and cooled to 30°. The flask was fitted with a Bunsen valve and gas inlet tube. The flask was flushed with N_2 for 10 min, then 50 ml of yeast cream (*ca.* 8 g dry weight) in phosphate buffer 0.1 *M*, pH 6.4, was added to each. The flask was filled with sterilized buffer, flushed with N_2 for 10 min, and kept at 30° for 80 hr during which it was flushed every 12 hr with N_2 for 5 min. The yeast was harvested by centrifugation, washed twice with buffer, resuspended in buffer, refrigerated for 10–15 hr, and then used for the *aerobic growth phase*. The yeast cells (~5.0 g) 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 at 30° with aeration of 4–5 l. min^{-1} and 400 rpm stirring. Dow Corning Antifoam A spray was used to prevent foaming.

Base Hydrolysis and Extraction of the Nonsaponifiable Fraction. Cells, harvested by centrifugation, were weighed wet and hydrolyzed. For each 2-g wet weight portion a solution of 15 g of KOH in 20 ml of H_2O and sufficient ethanol to give a total of 100 ml was used. The solution was refluxed under N_2 for 3 hr, diluted with an equivalent amount of water, and extracted with 4 vol equiv of heptane. The heptane extract was washed with water until neutral, dried over anhydrous Na_2SO_4, and evaporated to give the crude nonsaponifiable material.

Time-Course Studies. The sterol content and composition (Figure 4) of yeast cultures were determined by analysis of the product of acetylation of the crude nonsaponifiable fraction by tlc and glpc. Total sterol was determined by integration of the area associated with the glpc trace of the crude sterol acetates *vs.* an internal standard (cholestanol). Amounts of the individual components were determined by tlc separation of the crude sterol acetates as given in column A of Table II. Each fraction was then subfractionated by rechromatography as designated in column B. Finally, these subfractions were analyzed by glpc. The presence of individual sterol acetates was determined by mixed injection with authentic samples and their amounts were determined by comparison of peak areas with an internal standard of 3β-cholestanol.

General Procedure for Incorporation Studies. 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 distilled water (~3 ml) and added to the incubation flask. The acetone was removed with a stream of N_2. Sterilized growth medium (1.5 l.) M-2 was added and the solution stirred vigorously for 10 min. Anaerobically pretreated yeast cells (~5.0 g wet weight) were added and the culture was grown as described. At the end of the incubation time (7 hr) the cells were collected by centrifugation (20 min at 2500 rpm), washed three to four times with phosphate buffer,[43] centrifuged, hydrolyzed, and extracted as above.

The crude nonsaponifiable material was dried under vacuum, weighed, and dissolved in heptane. This solution was made up to 50 ml and a 0.5 ml aliquot removed to determine the total incorporation (Table I). 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 were dissolved in 50 ml of heptane. Total activity of the acetates was determined. In all cases 95% or more of the activity of the sterol fraction was found in the acetates. The heptane was evaporated and the acetates were separated by tlc into the single fractions as described in Table II.

The single fractions were dissolved in 25 ml of heptane and a 0.5-ml aliquot was removed for counting to give the total activity in the band. A known amount was analyzed by glpc and the total content of the component in question determined.

If the sterol was subject to investigation in that particular experiment, unlabeled material was added and the sterol purified and crystallized to constant activity. The reported per cent incorporation into the individual sterols was based on the actual

(41) G. Goulstone and E. I. Mercer, *Phytochemistry*, **8**, 1949 (1969).

(42) J. R. Turner and L. W. Parks, *Biochim. Biophys. Acta*, **98**, 394 (1965).

(43) This washing was necessary to remove exogenous sterol. After each wash the cells were centrifuged before resuspension. Monitoring of the supernatant after each wash revealed the bulk of activity associated with exogenous sterol was removed after a single wash. No activity was usually encountered in the supernatant after the third wash.

Table II. Tlc Separation of Sterol Acetates ($AgNO_3$–Silica Gel)

A, benzene			B, solvent as indicated			
Band	R_f	Glc analyses rel retention times[a]	Solvent system[c]	Band	Glc analyses	RRT
1	Origin	b				
2	0.2–0.25	2 peaks	B–EA	2-1	Ergosta-5,7,24(28)-dien-3β-ol	2.2
			9:1	2-2	Ergosta-5,7,22,24(28)-tetraen-3β-ol	2.1
3	0.25–0.4	1 peak	B	3-1	Ergosterol	1.88
4	0.4–0.55	2 peaks	B–H	4-1	Fecosterol	1.99
			2:3		Episterol	2.15
			2×			
5	0.55–0.6	1 peak	B–H 4:1	5-1	Ergosta-7,22,24(28)-trien-3β-ol	2.05
6	0.6–0.65	1 peak	B–H 1:1	6-1	Zymosterol	1.64
7	0.65–0.85	4 peaks	B–H	7-1[d]	Ergosta-7,22-dien-3β-ol	1.83
			1:5		Ergosta-8,22-dien-3β-ol	1.68
			2×	7-2[d]	Ergost-7-en-3β-ol	2.17
					Ergost-8-en-3β-ol	1.97
8	0.85–front	Mixture of squalene, lanosterol, and 4,14-methylated sterols			Lanosterol	2.4
					4α-Methylzymosterol	1.82
					4,4-Dimethylzymosterol	2.28

[a] Relative retention times are relative to 3β-cholestanol on QF-1. [b] Complex mixture of polar material. [c] B, benzene; H, hexane; EA, ethyl acetate. [d] The components could be further separated by continuous tlc (24–48 hr) using 1:9 benzene–hexane as solvent.

amount of the particular sterol present in the yeast as determined by glpc above.

The incubation of labeled episterol with growing yeast is described in detail as an example.

Labeled episterol (28-^{14}C), 1.78 × 10^5 cpm, was incubated with growing yeast. After 7 hr 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. Since 100 × 940 ± 5 cpm = 9.4 × 10^4 ± 500 cpm, the total incorporation = 52.6%. The free alcohols were acetylated and the total activity in the acetates was determined (9.0 × 10^4 ± 500 cpm).

The acetates were separated by tlc (five plates 20 × 20 cm), authentic sterol acetates were cochromatographed 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.

To the recovered ergost-7-enyl acetate (1 mg) 30 mg of unlabeled sterol acetate was added and the mixture crystallized and rechromatographed two times (benzene–hexane, 1:1). The compound was not pure by glpc as judged by the presence of a small peak that had the retention time of the $\Delta^{7,22}$-diene acetate (**12**-Ac). The mixture was therefore submitted to continuous tlc for 48 hr (10% benzene in hexane). The recovered material was pure by glpc and after two crystallizations had a constant activity of 9.9 cpm/mg (based on carrier sterol) equivalent to 0.32% incorporation. Total activity recovered from the tlc band containing ergosta-7,22,24(28)-trienyl acetate was 1.60 × 10^4 cpm. Glpc showed small amounts of episterol to be present. Inactive material (100 mg) was added and the mixture recrystallized, rechromatographed on tlc, and recrystallized. The resulting material was pure by glpc and had an activity of 55.5 cpm/mg (based on carrier sterol) corresponding to 6.1% incorporation.

To the recovered ergosta-5,7,22,24(28)-tetraenyl acetate (~1 mg) 100 mg of unlabeled material was added. The material was purified two times by tlc and crystallized to constant activity: fifth recrystallization 48.5 cpm/mg, mp 142.5–144°. Based on added carrier sterol 4.85 × 10^3 cpm is equivalent to 6.1% incorporation. Unlabeled ergosterol acetate (100 mg) was added to the fraction containing this sterol (~1 mg) and the mixture purified by tlc on time and crystallization: fifth recrystallization 26.4 cpm/mg corresponding to an incorporation of 2.93%.

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Communications to the Editor

Binding of Dioxygen to Iron(II). Reversible Behavior in Solution

Sir:

The binding of dioxygen to hemoglobin has been the source of considerable interest and speculation.[1] In all known iron(II) complexes reaction with oxygen *in solution* is irreversible and leads, through autoxidation, to iron(III) species,[2] whereas hemoglobin exhibits the well-known reversible behavior with 1:1 stoichiometry, *per* iron atom.[3] The nature of iron–dioxygen complexes is also of relevence with regard to biological hydroxylation, and, in the hope of shedding light on these problems of reversibility and activation of dioxygen, we

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