Utilization and Metabolism of Methyl-Sterol Derivatives in the Yeast Mutant Strain GL7†

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ABSTRACT: Sterols modified at various positions of the tetracyclic nucleus were tested as growth supplements for *Saccharomyces cerevisiae* strain GL7 *erg*12 *heme*3. Derivatives of 3β-cholestanol or Δ^7-3β-cholestenol bearing either a single α-oriented methyl group or a *gem*-dimethyl group at C-4 supported the growth of the mutant whereas 4β-methyl sterols did not. The nutritionally active alkyl derivatives were metabolized to 4-demethyl sterols while 4β-methyl derivatives were incorporated unchanged, indicating that the C-4 demethylase of yeast is specific for α-oriented methyl groups. It appears that 4-demethyl sterols are obligatory for growth of this organism. C-4 methyl derivatives of cholesterol did not support growth, suggesting that the Δ^5 double bond blocks demethylation at the adjacent C-4. In other experiments, 14α-methyl sterols were effective growth supplements, while 3α-methylcholesterol was totally inactive. Removal of the C-19 methyl group of cholesterol (19-norcholesterol) rendered the sterol somewhat less effective as a sterol source. The sterol specificity for yeast appears to be particularly strict with regard to substituents that add bulk to the A ring of the steroid nucleus.

Saccharomyces cerevisiae, cultured in the absence of oxygen, displays an absolute requirement for exogenous sterol and unsaturated fatty acid (Andreasen & Stier, 1954). While a wide variety of sterols have been tested as growth supplements (Nes et al., 1978; Proudlock et al., 1968; Hossack & Rose, 1976; Masters, 1963), the extreme technical difficulty in obtaining strict anaerobicity has led to conflicting results regarding the specificity of the yeast sterol requirement (Nes et al., 1978). For example, according to one report, lanosterol satisfies the requirement of anaerobic yeast (Proudlock et al., 1968), while in several other studies, this sterol was found to be ineffective (Nes et al., 1978; Masters, 1963; M. T. Sobus and K. Bloch, unpublished experiments). Conflicting results have also been reported for the efficacy of cholesterol as a sterol source for anaerobic yeast (Nes et al., 1978; Proudlock et al., 1968; Hossack & Rose, 1976), possibly for the same reason.

An approach for studying the sterol requirements of yeast which obviates the need for anaerobicity is to use mutants blocked in sterol biosynthesis. In most of the sterol mutants described, the defect occurs relatively late in the biosynthetic pathway, causing accumulation of abnormal sterols (Barton et al., 1974; Bailey et al., 1976; Trocha et al., 1977; Pierce et al., 1978a). These late-stage mutants have been useful for delineating the sequence of events during yeast sterol biosynthesis (Fryburg et al., 1975; Pierce et al., 1978b) and the effects of altered sterol composition on the properties of yeast membranes (Bard et al., 1978; Lees et al., 1979). In this category are two C-14 demethylase mutants isolated in the laboratories of Sprinson (Trocha et al., 1974, 1977) and Oehlschlager (Pierce et al., 1978a). The mutants accumulate a variety of sterols, all containing the lanosterol-derived 14α-methyl group. Since both C-14 demethylase mutants are able to grow without exogenous sterol supplements (Trocha et al., 1977; Pierce et al., 1978a), 14α-methyl sterols must be able to satisfy the sterol requirements of *S. cerevisiae*. This is of considerable interest for two reasons. First, sterols bearing a 14α-methyl group are rather ineffective in changing the fluidity of model membranes (Lala et al., 1978; Dahl et al., 1980a,b), and second, several antimycotic agents are believed to inhibit fungal growth by blocking C-14 demethylation (Ragsdale, 1975; Kato & Kawase, 1976; VandenBossche et al., 1978, 1980).

Strain GL7, isolated by Gollub et al. (1977), is blocked in the cyclization of 2,3-oxidosqualene to lanosterol as well as in heme biosynthesis and is strictly dependent upon a source of exogenous sterol. Strain GL7 appeared ideal for extending our studies on alkylated sterol utilization (Lala et al., 1978; Dahl et al., 1980a,b; Yeagle et al., 1977) to an eukaryotic organism. We have now tested several sterols modified at different positions of the tetracyclic nucleus (Figure 1) for their ability to support the growth of the mutant. We find that the presence or absence of alkyl substituents in the A ring is a major determinant in the adequacy of sterols for supporting growth of the mutant. The same studies allow the conclusion that strain GL7, despite its heme deficiency, is capable of demethylating a number of C-4 alkylated sterols as well as introducing a C-5(6) double bond.

Materials and Methods

Growth Conditions. Saccharomyces cerevisiae strain GL7 (*erg*12 *heme*3) (Gollub et al., 1977) was kindly supplied by D. B. Sprinson. The mutant was grown on a synthetic medium supplemented with methionine (Gollub et al., 1977). Sterols were added to the medium as 0.2% solutions in either Tween 80/ethanol (1:4 w/w) or Brij 58/ethanol (1:4 w/w) to a final concentration of 10 μg/mL. Palmitoleic and oleic acids (1:4 w/w) were included in the sterol–Brij 58 solutions to a final concentration of 50 μg/mL of medium. Cultures were grown with shaking at 30 °C, and growth was monitored by measuring absorbances at 540 nm. For routine growth experiments, log-phase cells were centrifuged, washed twice with minimal media, and resuspended in the medium to an optical density of 1.0 at 540 nm (10^8 cells/mL). The cell suspension was diluted into fresh media (1:100 to 1:500) and distributed among flasks containing the various sterol supplements to be tested.

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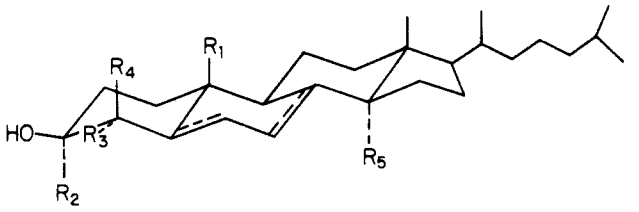
Table I: Retention Times and Mass Spectral Data Used for Identifying Sterol Metabolites

	retention time (min)	rel retention time[a]	major ions in mass spectrum[b]	lit. ref.[c]
cholestanol	9.2	2.30	388 (M^+), 373, 355, 233, 215	i
cholesterol	9.3	2.32	386 (M^+), 368, 353, 301, 275, 235, 231, 213	i
Δ^7-cholestenol	10.6	2.65	386 (M^+), 368, 255, 229, 213	ii
7-dehydrocholesterol	10.7	2.67	384 (M^+), 366, 351, 325, 253, 211	ii
4α-methylcholestanol	10.2	2.55		
4α-methyl-Δ^7-cholestenol	11.8	2.95	400 (M^+), 382, 267, 269, 243, 227	ii
4β-methylcholestanol	12.3	3.07		
4,4-dimethylcholestanol	12.8	3.20		
4,4-dimethyl-Δ^7-cholestenol	15.0	3.75		
dihydrolanosterol	12.6	3.15	428 (M^+), 413, 395	iii

[a] Retention times relative to 5α-cholestane (4.0 min). [b] Only the major or characteristic peaks are given. [c] (i) Ryhage & Stenhagen (1960); (ii) Galli & Maroni (1967); (iii) Brooks et al. (1968).



19-norcholesterol: $R_1 - R_5 = H$; Δ^5

3α-methylcholesterol: $R_1, R_2 = CH_3$; $R_3 - R_5 = H$; Δ^5

4α-methylcholestanol: $R_1, R_3 = CH_3$; $R_2, R_4, R_5 = H$

4β-methylcholestanol: $R_1, R_4 = CH_3$; $R_2, R_3, R_5 = H$

4,4-dimethylcholestanol: $R_1, R_3, R_4 = CH_3$; $R_2, R_5 = H$

14α-methyl-Δ^7-cholestenol: $R_1, R_5 = CH_3$; $R_2 - R_4 = H$; Δ^7

FIGURE 1: Position of nuclear methyl groups in alkylated sterols tested.

So that cells of strain GL7 growing on dihydrolanosterol could be obtained, cells were first grown into mid log phase with ergosterol (10 μg/mL). Thereafter, the culture was diluted 1:10 into media containing dihydrolanosterol (10 μg/mL), resulting in an ergosterol/dihydrolanosterol ratio of 1:10 in the fresh medium. After the diluted culture reached mid log phase (24 h), the culture was diluted 1:20 into media containing dihydrolanosterol (10 μg/mL). After three to four such dilutions, the residual medium ergosterol was considered negligible. No ergosterol was detected in the adapted cells.

Chemicals. Cholesterol and cholestanol were obtained from Sigma and were recrystallized before use. Dihydrolanosterol was purified from crude lanosterol (Sigma) by the procedure of Rodewald & Jagodzinski (1978). T. A. Spencer kindly provided 4α-methyl- and 4β-methylcholestanol, while 4α-methylcholesterol, 4β-methylcholesterol, 14α-methyl-Δ^7-cholestenol, and 4β-methyl-Δ^7-cholestenol were generously supplied by G. J. Schroepfer, Jr. The Δ^7-cholestenol was the gift of R. B. Clayton, and the 4α-methyl-Δ^7-cholestenol was donated by C. Djerassi. 3α-Methylcholesterol and all of the 4,4-dimethyl derivatives were synthesized in this laboratory (Lala et al., 1978; Gautschi & Bloch, 1958). J. Mathieu provided the 19-norcholesterol. Except for 4,4-dimethyl-Δ^7-cholestenol (93%) and 4α-methyl-Δ^7-cholestenol (80%; contains double-bond isomers), all sterols tested were >95% pure. Tween 80, Brij 58, oleic acid, and palmitoleic acid were obtained from Sigma.

Sterol Analysis. Cells to be analyzed for their sterol content were washed twice with distilled water and saponified (20% KOH/90% MeOH at 70 °C for 1 h). Nonsaponifiable lipids were extracted into petroleum ether and the extracts brought to dryness. The sterol samples were dissolved in hexane and analyzed on a Perkin-Elmer Model 900 gas chromatograph equipped with a 6-ft column of 3% SP 2250 on 100/120 Supelcoport (Supelco) (column temperature = 275 °C). Positive identifications of sterols were obtained with the gas chromatography–mass spectrometry facility at M.I.T., using a Perkin-Elmer Model 990 gas chromatograph interfaced by a jet separator to a Hitachi Model RMVGL mass spectrometer. Table I lists the gas–liquid chromatography (GLC) retention times for the various sterols tested as well as mass spectral data obtained from the literature.

Identification of Sterol Metabolites. Cells grown with the various supplements were saponified and extracted with petroleum ether as described above. Total nonsaponifiable lipids were analyzed by GLC; all cells contained 2,3-oxidosqualene in addition to sterols as expected from the cyclase deficiency.

1. Cholestanol cells contained a single sterol, t_R 9.2 min; GC–MS *m/e* 388 (M^+), 373, 355, 233, 215.

2. Δ^7-Cholestenol cells contained a single sterol by GLC, t_R 10.6 min. GC–MS analysis, however, demonstrated the presence of two sterols with molecular ion peaks at *m/e* 386 and 384, indicating desaturation. Ions at *m/e* 386 (M^+), 368, 255, 229, 213 and the corresponding M − 2 series at *m/e* 384 (M^+), 366, 351, 325, 253, 211 indicated the presence of both Δ^7-cholestenol and 7-dehydrocholesterol. The presence of 7-dehydrocholesterol was further confirmed by UV analysis; an aliquot of the nonsaponifiable fraction gave the expected maxima at 262, 271, 282, and 293 nm.

3. 4α-Methylcholestanol cells contained two sterols, t_R 9.2 and 10.2 min; GC–MS (major peak) *m/e* 388 (M^+), 373, 355, 233, 215; (minor peak) *m/e* 402 (M^+), 387, 369, 262, 247, 229. The two sterols were identified as cholestanol (80%) and 4α-methylcholestanol (20%).

4. 4β-Methylcholestanol cells contained one major sterol (>95%), t_R 12.3 min. GC–MS confirmed identity as 4β-methylcholestanol: *m/e* 402 (M^+), 387, 369, 289, 262, 247, 229.

5. 4,4-Dimethylcholestanol cells contained a major sterol (83%), t_R 9.2 min, identified as cholestanol, and a minor sterol (17%), t_R 12.8 min, identified as 4,4-dimethylcholestanol by GC–MS: *m/e* 416 (M^+), 398, 383, 355, 262, 261, 243.

6. 4α-Methyl-Δ^7-cholestenol cells contained one sterol by GLC, t_R 10.7 min. GC–MS demonstrated the presence of Δ^7-cholestenol and 7-dehydrocholesterol. UV analysis confirmed the presence of the latter.

7. 4,4-Dimethyl-Δ^7-cholestenol cells contained a major sterol (>95%), t_R 10.7 min (7-dehydrocholesterol), and a trace peak at 15.0 min (4,4-dimethyl-Δ^7-cholestenol).

8. Adapted dihydrolanosterol cells contained in the nonsaponifiable fraction three sterols with retention times of 9.2, 10.2, and 12.6 min. TLC on silica gel G plates developed with benzene–ethyl acetate (4:1) gave three sterols bands (R_f 0.37,

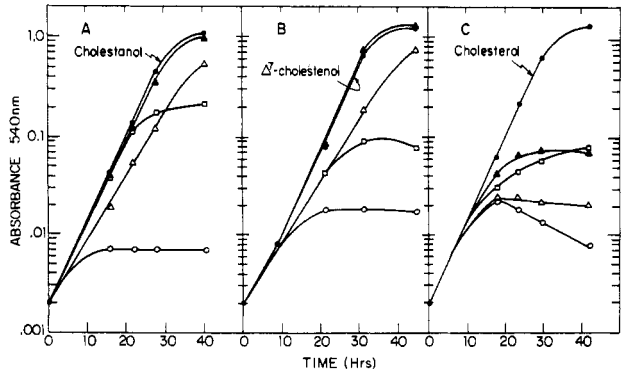


FIGURE 2: Growth of *S. cerevisiae* strain GL7 with C-4 methylated sterols. (A) Derivatives of cholestanol. (B) Derivatives of Δ^7-cholestenol. (C) Derivatives of cholesterol. (●) 4-Demethyl; (▲) 4α-methyl; (□) 4β-methyl; (△) 4,4-dimethyl; (○) no sterol.

0.50, and 0.57). The relative retention times on GLC and mobilities on thin-layer chromatography (TLC) suggested that the three sterols differed in their degree of methylation at C-4. GC–MS: sterol I (t_R 12.6 min; R_f 0.57) *m/e* 428 (M^+), 413 (base peak), 395; sterol II (t_R 10.2 min; R_f 0.50) *m/e* 414 (M^+), 399 (base peak), 381; sterol III (t_R 9.2 min; R_f 0.37) *m/e* 400 (M^+), 385 (base peak), 367. Sterol I (68%) was identified as dihydrolanosterol based upon its retention time on GLC, its molecular ion peak at 428, and its fragmentation pattern (Brooks et al., 1968). Further, the presence of a base peak at M − 15 is characteristic for 14α-methyl sterols (Ragsdale, 1975). Sterol II (18%) was identified as 4α,14α-dimethyl-Δ^8-cholestenol based upon its behavior on GLC, its molecular ion at 414, and its fragmentation pattern (base peak at 399). For similar reasons, sterol III (14%) was identified as 14α-methyl-Δ^8-cholestenol.

Results

Effectiveness of C-4 Methyl Sterols. Growth curves obtained with 4-methylcholestanols as supplements for strain GL7 are shown in Figure 2A. In the B-ring saturated series, both cholestanol and 4α-methylcholestanol supported excellent growth, while with 4,4-dimethylcholestanol, growth was somewhat slower. With 4β-methylcholestanol, cells grew initially but ceased to grow early and at a much lower optical density ($\sim 1/5$) than observed for cholestanol and 4α-methylcholestanol. Strain GL7 failed to grow in the absence of sterol supplements.

In Figure 2B are shown the growth curves obtained when strain GL7 was incubated with 4-alkyl derivatives of Δ^7-cholestenol. Again, the 4-demethyl and 4α-methyl sterols were very effective, the 4,4-dimethyl derivative was slightly less so, and the 4β-methyl-Δ^7-cholestenol was inactive. Finally, the C-4 methyl or dimethyl derivatives of cholesterol were similarly tested (Figure 2C). In contrast to the results obtained with the alkylated cholestanols and Δ^7-cholestenols, none of the Δ^5-stenols bearing a C-4 methyl group supported growth of the mutant. Cholesterol was an effective supplement.

The sterols of yeast cells obtained from the experiments described above were identified on the basis of their retention time on gas–liquid chromatography and their fragmentation pattern following mass spectroscopy (Table I). When strain GL7 was grown with either cholestanol, cholesterol, or 4β-methylcholestanol, only the added sterol or stanol was present in the cells (Table II). In contrast, after growth on Δ^7-cholestenol, the nonsaponifiable fraction contained primarily 7-dehydrocholesterol. Thus the mutant can desaturate the Δ^7-stenol to the $\Delta^{5,7}$-diene but does not further desaturate cholesterol.

Table II: Sterol Composition of *S. cerevisiae* Strain GL7 Grown with Various Alkylated Sterols

sterol supplement	sterols in nonsaponifiable fraction (%)[a]
cholestanol	cholestanol (100)
4α-methylcholestanol	cholestanol (80); 4α-methylcholestenol (20)
4β-methylcholestanol	4β-methylcholestanol (100)
4,4-dimethylcholestanol	cholestanol (83); 4,4-dimethylholestanol (17)
Δ^7-cholestenol	Δ^7-cholestenol and 7-dehydrocholesterol[b]
4α-methyl-Δ^7-cholestenol	Δ^7-cholestenol and 7-dehydrocholesterol (>95); 4α-methyl-Δ^7-cholestenol (trace)
4,4-dimethyl-Δ^7-cholestenol	Δ^7-cholestenol and 7-dehydrocholesterol (>95); 4,4-dimethyl-Δ^7-cholestenol (trace)
cholesterol	cholesterol (100)
dihydrolanosterol	dihydrolanosterol (68); 4α,14α-dimethyl-Δ^8-cholestenol (18); 14α-methyl-Δ^8-cholestenol (14)

[a] For identification of sterols see Materials and Methods. [b] Δ^7- and $\Delta^{5,7}$-sterols were not separated from each other by GLC.

Cells grown with 4α-methylcholestanol, 4,4-dimethylcholestanol, 4α-methyl-Δ^7-cholestenol, and 4,4-dimethyl-Δ^7-cholestenol contained almost exclusively 4-demethyl stanols or stenols, indicative of effective demethylation at C-4 (Table II). In cells grown with 4α-methyl-Δ^7-cholestenol and 4,4-dimethyl-Δ^7-cholestenol, the 4-demethyl sterol was primarily 7-dehydrocholesterol; i.e., demethylation is followed by desaturation at C-5, as in the case of added Δ^7-cholestenol. The cell yields obtained with 4β-methyl-Δ^7-cholestenol and with the C-4 methylated Δ^5-cholestenol (cholesterol) derivatives were not sufficient for analysis of the nonsaponifiable fractions.

Effectiveness of 14-Methylcholestenols. We have recently reported that neither lanosterol nor dihydrolanosterol supports aerobic growth of strain GL7 (Buttke & Bloch, 1980). In subsequent experiments, we found, however, that the mutant can be adapted to grow quite well with dihydrolanosterol[1] (see Materials and Methods). Clearly this occurred as the result of metabolic modification since GLC analysis revealed the presence of at least two sterols in addition to dihydrolanosterol in the nonsaponifiable fraction (Table II). TLC and GC–MS demonstrated that the mutant had demethylated dihydrolanosterol at C-4 to 4α,14α-dimethyl-Δ^8-cholestenol and 14α-methyl-Δ^8-cholestenol. The retention of the 14α-methyl group in these metabolites is expected since the heme-deficient strain GL7 lacks cytochrome P-450, known to be required for C-14 demethylation (Ohba et al., 1978).

Dihydrolanosterol-adapted cells were also tested for their ability to grow with the three 4,4-dimethyl derivatives tested previously (Figure 2). The results are shown in Figure 3A. Whereas dihydrolanosterol, 4,4-dimethylcholestanol, and 4,4-dimethyl-Δ^7-cholestenol supported growth of the adapted cells, 4,4-dimethyl-Δ^5-cholestenol was once again ineffective, in agreement with the results shown in Figure 2. As in unadapted cells, supplements that actively support growth were demethylated to cholestanol and 7-dehydrocholesterol, respectively.

Our results with dihydrolanosterol suggest that the presence of a 14α-methyl group, nonmetabolizable in strain GL7, does

[1] Strain GL7 could also be adapted to grow on lanosterol. GLC analysis demonstrated that this sterol was similarly demethylated at C-4. The possibility that lanosterol might be methylated at C-24, however, made dihydrolanosterol the preferred sterol for our experiments.

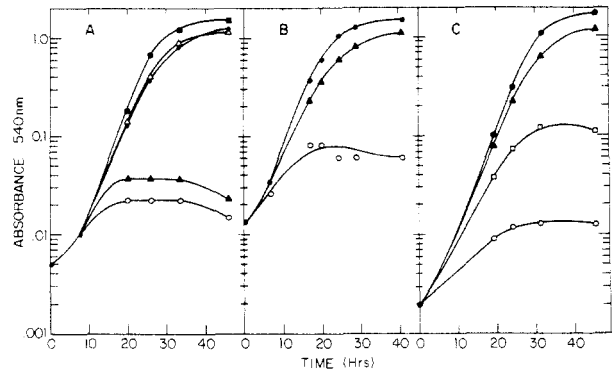


FIGURE 3: Growth of *S. cerevisiae* strain GL7 with various alkylated sterols. (A) Growth of dihydrolanosterol-adapted cells with (■) 4,4-dimethyl-Δ^7-cholestenol, (▲) 4,4-dimethylcholesterol, (△) 4,4-dimethylcholestanol, (●) dihydrolanosterol, (○) no sterol. (B) Growth of strain GL7 with (●) Δ^7-cholestenol, (▲) 14α-methyl-Δ^7-cholestenol, (○) no sterol. (C) Growth of strain GL7 with (●) cholesterol, (▲) 19-norcholesterol, (□) 3α-methylcholesterol, (○) no sterol.

not render an otherwise suitable sterol ineffective. In support of this conclusion, 14α-methyl-Δ^7-cholestenol as a sterol supplement was only slightly less effective than Δ^7-cholestenol (Figure 3B).

Effectiveness of 3α-Methylcholesterol and 19-Norcholesterol. The results described so far suggest that for strain GL7, nuclear methyl substituents near the polar hydroxyl group affect membrane function more adversely than the methyl substituent at C-14 which is located in a more hydrophobic region. Such regional effects were also seen with 3α-methylcholesterol. This synthetic cholesterol derivative did not support growth of the mutant (Figure 3C).

Finally we tested 19-norcholesterol, a cholesterol derivative that lacks the methyl group normally present at C-10. Compared to cholesterol, the 19-norsterol is significantly less effective in reducing the fluidity of model membranes (Lala et al., 1978). As a sterol source for the yeast mutant, 19-norcholesterol was also inferior to cholesterol (Figure 3C). Apparently the presence of a methyl group at C-10 contributes to optimal sterol–phospholipid interactions.

Discussion

In a recent speculative paper from this laboratory (Bloch, 1978), it was proposed that the nuclear demethylations involved in the biosynthetic conversion of lanosterol to cholesterol occur in a logical sequence that can be rationalized in terms of the membrane role of cholesterol. The driving force for these demethylations, it was suggested, is to render the sterol α face planar, thus optimizing van der Waals contacts with adjacent phospholipid acyl chains. Subsequent reports from this laboratory have provided experimental support for this hypothesis. In both model membranes (Lala et al., 1978; Dahl et al., 1980a; Yeagle et al., 1977) and *Mycoplasma capricolum* (Dahl et al., 1980a,b), sterols bearing nuclear methyl groups at C-4 and C-14 raise membrane microviscosity less than the corresponding demethyl sterols. Since the effectiveness of alkylated sterols as growth supplements for *Mycoplasma capricolum* parallels the efficacy of these sterols in condensing model membranes (Dahl et al., 1980a,b), it appears that sterols containing a planar α face permit optimal sterol–phospholipid interactions in both natural and model membranes.

The experiments we now describe were designed to determine whether the sterol requirement of *S. cerevisiae* is governed by similar factors. In principle, this information can be obtained by growing yeast anaerobically, conditions which impose an absolute requirement for external sterol.[2] For example, provided strict anaerobicity is maintained, wild-type *S. cerevisiae* fails to substitute lanosterol for ergosterol or cholesterol as the sterol supplement (Nes et al., 1978; Masters, 1963; M. T. Sobus and K. Bloch, unpublished experiments). Similar studies with yeast sterol auxotrophs have been limited, probably because few of the reported mutants lend themselves to manipulation of sterol content. However, in the recently isolated strain GL7, a mutant deficient in 2,3-oxidosqualene lanosterol-cyclase (Gollub et al., 1977), sterol composition can be tightly controlled. Testing C-4 methylated derivatives as sterol sources for strain GL7, we find that the suitability of the alkylated supplements depends on the orientation of the C-4 methyl group as well as the location of the B-ring double bond. Derivatives not demethylated by the cells did not support growth, demonstrating that yeast requires sterols unsubstituted at C-4. This is in contrast to *Mycoplasma capricolum* which can grow reasonably well with C-4 alkylated sterols without demethylating these molecules (Dahl et al., 1980a). Interestingly, the sterol-requiring protozoan *Tetrahymena paravorax* likewise utilizes only 4-demethyl sterols for growth (Hutner & Holz, 1962), suggesting that the sterol requirement of eukaryotic cells is more narrowly specific for an unalkylated A ring than that of the prokaryotic *M. capricolum*.

The ability of strain GL7 to use 14α-methyl-Δ^7-cholestenol as a growth supplement was unexpected and in contrast to results obtained with other systems (Lala et al., 1978; Dahl et al., 1980a,b; Yeagle et al., 1977). The membrane "incompetence" of this sterol had been attributed to the protruding bulky 14α-methyl group from an otherwise planar α face. On the basis of the finding that several antifungal agents block C-14 demethylation (Ragsdale, 1975; Kato & Kawase, 1976; VandenBossche et al., 1978, 1980), we assumed that a planar α face would also be required for functional yeast membranes. Judging from the fact that strain GL7 grew nearly as well with 14α-methyl-Δ^7-cholestenol as with Δ^7-cholestenol, it is clear that the exposed C-14 methyl group did not greatly interfere. In any event, growth was much better with the 14α-methyl-4-demethyl derivative than with the nonmetabolizable 4-monomethyl-14-demethyl sterols, implying that bulky additions to the sterol nucleus in the vicinity of the C-3 hydroxyl are the more deleterious for yeast. Similarly, the ability of the mutant to grow on dihydrolanosterol following metabolic demethylation which in this case occurs only at C-4 provides additional evidence that a methyl group at C-14 does not render the sterol molecule inactive for yeast. Although our findings with strain GL7 seemed anomalous at first sight, the ability of two *S. cerevisiae* mutants defective in C-14 demethylation to grow without exogenous sterol supplements (Trocha et al., 1977; Pierce et al., 1978a) is consistent with the present results and interpretations. The tolerance of yeast for 14α-methyl but not for 4-methyl or 4,4-dimethyl derivatives is, however, at variance with spatial relationships between sterol and phospholipid acyl chains in the membrane bilayer as specified by current models (Rothman & Engelman, 1972).

The results obtained with 3α-methylcholesterol and 19-norcholesterol provide additional evidence that the presence or absence of bulky substituents in the α-ring region of the sterol nucleus is especially adverse for the growth of *S. cerevisiae*. As space-filling models show, addition of an axial methyl group at C-3 or the removal of the C-19 methyl group

[2] Apart from the technical problem of maintaining strict anaerobiosis, it might also be argued that such conditions are abnormal for yeast and therefore are not representative of the requirements of the normal yeast cells.

significantly increases in one case, and reduces in the other, the thickness of the sterol nucleus in the region of the C-3 hydroxyl. Moreover, replacement of the 3α-hydrogen by a methyl group makes the sterol α face less planar while the loss of the C-19 methyl group creates a pocket between the sterol β face and the adjacent fatty acyl chain. Stimulated by a model for sterol–phospholipid interactions proposed by Huang (1977), we have previously suggested that retention of the C-18 and C-19 methyl groups in the course of sterol biosynthesis is favorable for van der Waals contacts between the β face of the sterol molecule and adjacent fatty acyl chains (Bloch, 1978). Thus the growth responses of the yeast mutant support the notion that evolutionary pressure led to the selective loss of the α-face methyl groups of lanosterol while preserving methyl groups at the β face.

Following the fate of the various alkyl derivatives during growth, we have obtained information, albeit indirect, on some properties of strain GL7 enzymes which catalyze C-5(6) desaturation and C-4 demethylation. It should be recalled that this mutant is heme deficient (Gollub et al., 1977) and therefore unable to synthesize cytochromes. Nevertheless, strain GL7 introduces a C-5 double bond into Δ^7-cholestenol, implying that C-5 desaturation in yeast is not cytochrome dependent. Previous workers have suggested that cytochrome b_5 may be involved (Osumi et al., 1977).

Cytochrome P-450 is known to be essential for C-14 demethylation in yeast (Ohba et al., 1978; Aoyama & Yoshida, 1978). Therefore the mutant should not, and indeed does not, remove the 14α-methyl group of either dihydrolanosterol or 14α-methyl-Δ^7-cholestenol. Demethylation at C-4 does not depend upon cytochrome P-450 (Ohba et al., 1978), and other cytochromes have so far not been implicated in this process, at least in yeast. Our results clearly show that heme proteins are not essential for oxidative demethylation at C-4. The possibility that strain GL7 synthesizes low levels of cytochromes appears unlikely since unsaturated fatty acid synthesis in the absence of exogenous heme is not detectable in the mutant (Buttke et al., 1980).[3]

The failure of strain GL7 to demethylate 4β-methyl sterols implies that the C-4 demethylating enzymes are specific for an α-oriented methyl group, a property shared with the analogous enzymes of rat liver (Sharpless et al., 1968, 1969; Rahman et al., 1970). Moreover, a double bond is not required in either system, as shown by the demethylation of 4α-methyl- and 4,4-dimethylcholestanol (Sharpless et al., 1968, 1969). Similarly, both strain GL7 and rat liver homogenates demethylate 4-alkyl derivatives containing either a Δ^7 or a Δ^8 double bond (Gautschi & Bloch, 1957, 1958; Ahman et al., 1970; Fried et al., 1968; Moore & Gaylor, 1968) whereas Δ^5 derivatives are inactive in both systems (Gautschi & Bloch, 1957). The reason for the failure of C-4 alkyl Δ^5-stenols to be demethylated is not known. As Dreiding models show, the introduction of a double bond at C-5 changes the conformation of ring B slightly but does not perceptibly alter the orientation of the 4α-methyl. Our finding that the mutant is capable of demethylating alkylated Δ^7-cholestenols is at variance with a previous study reporting the failure of cell-free extracts of wild-type yeast to demethylate 4,4-dimethyl-Δ^7-cholestenol (Moore & Gaylor, 1968). The discrepancy may be due to the use of different strains of yeast.

While the yeast and rat liver demethylases share certain specificities, they differ in at least one respect. That yeast can demethylate C-4 alkyl sterols without prior removal of the C-14 methyl group has been documented here and reported by others (Trocha et al., 1977; Pierce et al., 1978a; Ragsdale, 1975; Kato & Kawase, 1976; VandenBossche et al., 1978, 1980).[4] In rat liver, on the other hand, removal of the C-14 methyl prior to C-4 dealkylation appears to be obligatory (Sharpless et al., 1968; Nes & McKean, 1977). Clearly the sequence of demethylations is not invariant in yeast. In wild-type *S. cerevisiae*, demethylation at C-14 precedes demethylation at C-4 as in animal tissues. When the former is blocked by the absence of P-450, as is the case in the heme-deficient mutant, demethylation at C-4 nevertheless proceeds.

Finally, we would like to comment on the growth response of strain GL7 to dihydrolanosterol. We observed previously (Buttke & Bloch, 1980) that neither lanosterol nor dihydrolanosterol satisfied the sterol requirement of the mutant. More recently, we have, however, been able to design a procedure permitting growth of strain GL7 on either of these sterols. This has been achieved by adaptation, i.e., by successive transfers of cells grown originally on ergosterol to media containing dihydrolanosterol as the sole sterol source. Since adapted cells extensively demethylated lanosterol and dihydrolanosterol at C-4, it is possible that the "adaptation" period is essential for a gradually accelerating metabolism of the exogenous trimethyl sterols. It would then follow that lanosterol and dihydrolanosterol per se are unsuitable for membrane function in strain GL7 as they are for anaerobic wild-type yeast. Still unexplained remains the fact that anaerobically, strain GL7 grows well on lanosterol per se, without adaptation and without sterol dealkylation (Buttke & Bloch, 1980).

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[3] In a previous study by Bard et al. (1974), it was shown that porphyrin mutants of *S. cerevisiae* (lacking cytochromes) accumulate lanosterol as well as two to three additional sterols. Although the additional sterols were not identified, their behavior on GLC suggests that they may be 4α,14α-dimethyl-$\Delta^{8,24}$-cholestadienol and 14α-methyl-$\Delta^{8,24}$-cholestadienol.

[4] Judging from the relative recovery of demethylation products (Table II), the rate of C-4 demethylation of substrates containing a 14α-methyl group is slower than that of the corresponding 32-norlanosterol derivatives. These observations could explain the necessity for adapting cells to grow on lanosterol or dihydrolanosterol in contrast to growth on 14-demethyl-4-alkyl sterols.

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