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STEREOCHEMICALLY DISTINCT ROLES FOR STEROL IN SACCHAROMYCES CEREVISIAE*

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SUMMARY: Cholesterol, (E)- but not (Z)-17(20)-dehydrocholesterol, 5α-cholestan-3β-ol, sitosterol, and certain other sterols lacking a 24β-methyl group will replace (spare) most but not all of the 24β-methylsterol which has recently been found to be absolutely necessary for growth of oxygen-deprived wild type Saccharomyces cerevisiae in the presence of 2,3-iminosqualene. The results imply the existence of two stereochemically distinct roles for sterol in this organism. One of them (perhaps regulatory) requires, whereas the other (probably playing the so-called "bulk" membranous role) does not require the presence of the 24β-methyl group. The latter function, for which most of the sterol is needed, can be performed by various 24-alkyl- and 24-desalkylsterols.

A number of sterols, e.g., cholesterol and sitosterol, with side chains differing stereochemically from that of ergosterol have been reported to support the growth of both wild type Saccharomyces cerevisiae (5-9) and yeast mutants (10-15). Nevertheless, the 24β-methyl group of ergosterol has been known for several years to have at least some importance in wild type yeast (5,6) and mutants (11,16). More recently we have been able to show that it is absolutely critical (1,17). When endogenous synthesis of sterol in wild type yeast was blocked simultaneously by oxygen-deprivation and the presence of 2,3-iminosqualene, a 2,3-oxidosqualene cyclase inhibitor (18),

*The work in this paper forms a part of the doctoral dissertation of three of us (1-3) and a preliminary discussion of some aspects was given by one of us (4) at the Symposium on the Biochemistry and Function of Isopentenoids at the Western Regional Research Laboratory of the USDA, Berkeley, CA, March, 1982.

Abbreviations: RRT = Relative retention time; TLC = Thin layer chromatography; GLC = Gas-liquid chromatography; RPLC = Reversed phase liquid chromatography; MS = Mass spectrometry; NMR = Nuclear magnetic resonance.

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no growth at all occurred with sterols such as cholesterol and sitosterol. Growth only occurred with ergosterol or with sterols bearing the 24β-methyl group. The alkyl substituent on C-24 could not be removed or enlarged, nor could the chirality of C-24 be inverted without complete loss of the ability to support growth (1,17). We now wish to report that a large portion of the 24β-methylsterol can be replaced or "spared" (19) by cholesterol as well as by a variety of other sterols which do not possess the exact spatial qualities of the 24β-methyl group. This leads us to believe that among the multiple functions for sterol in yeast (1,4,20) there must be two stereochemically distinct kinds, one of which has a higher degree of stereospecificity than the other.

Materials and Methods

Yeast culture - Wild type diploid S. cerevisiae (ATCC 18790) was cultured for 68 hrs as described previously (5) unless otherwise noted in the presence of 50 μM 2,3-iminosqualene which was prepared as earlier reported (21). The concentration of sterol was 5.0 mg/l and that of Tween 80 was 15 ml/l except where indicated. The term "old cells" implies a culture well adapted over several years to oxygen-deprivation in the presence of ergosterol (5). "New cells" were derived from the ATCC culture by oxygen-deprivation in continuous culture with ergosterol for four weeks or less (5).

In order to examine the influence of structure on the ability of sterols to spare ergosterol, an inoculum of old cells was injected through a septum into medium containing the appropriate sterol and 2,3-iminosqualene. We then waited 68 hrs while the culture was stirred magnetically to establish a "base line" for any growth which the sterol in question might produce alone. In the case of the sterols discussed in this paper which lacked a 24β-methyl group, none gave any growth. We then permitted endogenous biosynthesis of ergosterol (see subsequently for data) to occur by injecting 10 ml of air. The headspace above the medium amounted to about 100 ml. After another 68 hrs the cells were counted and harvested in a centrifuge.

Source and analysis of sterols - The origin and purity of the sterols used are given elsewhere (17,22). The harvested cells from the sparing experiments (Table II) were hydrolyzed with refluxing (1 hr) 10% KOH in 95% ethanol and the 4-desmethylsterols isolated from the neutral lipids by TLC (silica gel G, 10% ether in benzene). RPLC was performed on a Zorbax ODS column using 20% isopropanol in acetonitrile at 60°. Instrumentation for "stop-flow" UV-analysis during RPLC is described in one of our recent papers (17) as are the techniques for GLC, TLC, MS, and ^{1}H-NMR. A QF-1 GLC column was used to distinguish between cholesterol and 5α-cholestan-3β-ol.

Results and Discussion

In the presence of 2,3-iminosqualene, as shown in Table I, growth of an oxygen-deprived old culture reached 10^8 cells/ml on ergosterol. Similar results have been obtained with other 24β-methylsterols, e.g., 24β-methyl-

Table I. Sparing of Ergosterol by Cholesterol in the Growth of Oxygen-Deprived S. cerevisiae in the Presence of 2,3-Iminosqualene

Ergosterol Alone		Mixture of Sterols		
Conc. (mg/l)	Cell-Count* (millions of cells/ml)	Ergosterol conc. (mg/l)	Cholesterol conc. (mg/l)	Cell-Count* (millions of cells/ml)
0	0.2	0	5.00	0.2
0.20	0.2	0.05	4.95	0.2
0.40	0.4	0.10	4.90	12
0.50	0.8	0.15	4.85	45
0.60	21	0.20	4.80	48
0.70	30	0.25	4.75	68
0.80	43	0.30	4.70	71
1.00	54	0.40	4.60	95
1.20	78	0.50	4.50	107
1.50	101	0.70	4.30	106
2.00	104	1.00	4.00	108

*Average cell-count of two experiments

cholesterol (17). A study of the dependence of growth on sterol concentration (Table I) revealed that 1.5 mg/l of ergosterol was sufficient to give the maximal cell-count, but no growth would occur unless at least 0.5 mg/l was present. Under the same conditions we have shown previously (17) that neither cholesterol nor any of the sterols listed in Table II would allow any significant growth to occur (< 0.5 million cells/ml) at 5.0 mg/l. However, when we combined 4.9 mg/l of cholesterol with as little as 0.1 mg/l of ergosterol, some growth now became evident. Furthermore, when we raised the ratio of ergosterol to cholesterol to, for instance, 0.5/4.5 (in mg/l) the growth actually became maximal even though neither of these sterols alone at the respective concentrations was supportive of growth. Clearly cholesterol was able to spare (19) ergosterol and ergosterol was able to permit growth on cholesterol.

In the above experiments we used old cells, but it was of interest to know that adaptation to oxygen-deprivation (new to old cells) was not itself

Table II. Sparing Ability of Various 24-Desalkyl- and 24-Alkylsterols

Sterol (5.0 mg/l)	Cell-count* (millions of cells/ml)	% Relative to cholesterol†
Cholesterol	112 (3)	100
(E)-17(20)-Dehydrocholesterol	80 (3)	64
(Z)-17(20)-Dehydrocholesterol	16 (2)	0
7-Dehydrocholesterol	100 (2)	86
Lathosterol	97 (3)	83
21-Norcholesterol	97 (3)	83
5α-Cholestan-3β-ol	85 (3)	69
24α-Methylcholesterol (campesterol)	107 (3)	94
24α-Ethylcholesterol (sitosterol)	89 (3)	74
24α-Ethyl-22-trans-dehydrocholesterol (stigmasterol)	104 (2)	91
24β-Ethylcholesterol (clionasterol)	103 (3)	90
Control (no sterol)	24 (3)	0

*Average cell-count obtained after the addition of 10 ml of air as described in the text. Number of experiments is given in parentheses.

†Cell-count less that of control divided by same for cholesterol.

responsible for the results. Similarly, we wanted to exclude 2,3-iminosqualene as a participant beyond inhibition of sterol biosynthesis. This was achieved in a second set of experiments (Fig. 1) which were analogous to the first (Table I) except for substitution of new cells for old ones, elimination of the 2,3-iminosqualene, and extreme efforts (very long times for flushing with purified nitrogen, extra care in sealing openings, etc.) to approximate anaerobiosis. The results (Fig. 1) were in principle the same as before (Table I). When 3.0 mg/l of cholesterol was added to the medium, about 3 million cells/ml were observed and 8 million cells/ml with 0.6 mg/l of ergosterol alone. However, combination of the 0.6 mg/l of ergosterol with 2.4 mg/l of cholesterol led to a cell-count of 31 million cells/ml representing an increase of 23 million cells/ml or a 4-fold enhancement in the growth produced by the 0.6 mg/l of ergosterol alone. Again cholesterol and ergosterol acted synergistically (cf. 23 for related

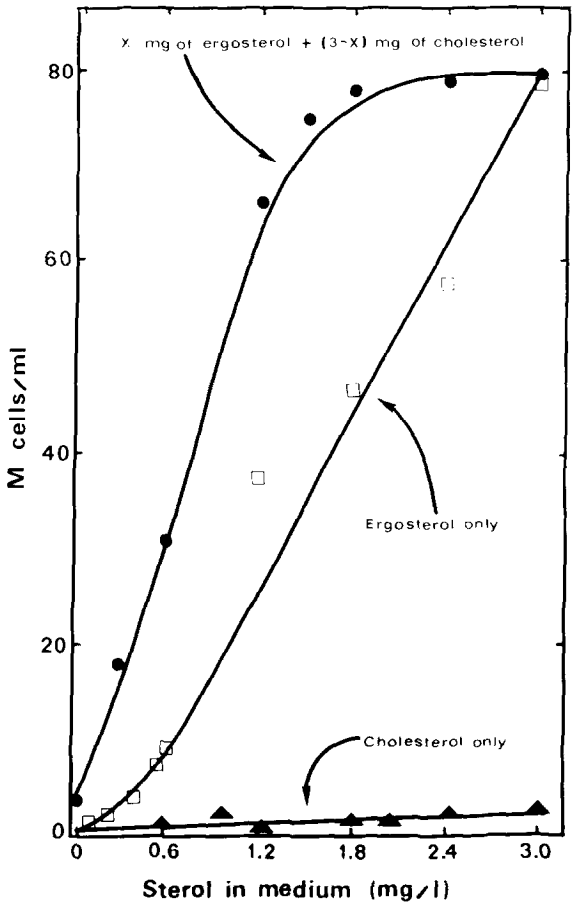

Fig. 1. Sparing of Ergosterol by Cholesterol in S. cerevisiae. New cells were used. The total amount of sterol was 3.0 mg for each point on the sparing curve for mixtures (●); the concentration of ergosterol (x) is given as the abscissa, and the concentration of cholesterol is 3 - x. On the ordinate, M = millions.

results in a prokaryote) showing that adaptation to oxygen-deprivation and the presence of inhibitor, viz., 2,3-iminosqualene, had nothing fundamental to do with the sparing phenomenon. The small growth which occurred on cholesterol by itself is, incidentally, explained if traces of endogenously synthesized ergosterol were present as we have shown for old cells lacking 2,3-iminosqualene (17). Quantitative differences in growth-response between old and new cells have been found to be unrelated to the sparing phenomenon per se and will form the subject of a separate communication.

To study the influence of structure on sparing ability (Table II), ergosterol was generated in situ by the addition of air to the culture (see

Materials and Methods). The endogenously formed sterols were examined in cultures lacking added sterol. Several experiments were combined. RPLC with stop-flow UV-analysis of the 4-desmethylsterol fraction showed three sterols were present. Each had the typical spectrum (λ_{max} 272, 282, 294 nm) of the $\Delta^{5,7}$-system, and from the rates of movement the three sterols were ergosterol, 22-dihydroergosterol, and 24-methylene-7-dehydrocholesterol (5-dehydroepisterol) (22). Quantitation by peak-height at 282 nm gave molar ratios of 10:2:1, respectively. GLC confirmed the identifications except that, as expected, the two minor components eluted in a single peak on XE-60.

When cholesterol or various other sterols were added (Table II), a substantial increase in growth occurred beyond that induced simply by the endogenously synthesized 24ß-methylsterols, even though none of the added sterols (Table II) induced growth in the absence of air. In each of the cases shown in Table II where sterol induced growth, the unchanged substrate was identified by GLC in the 4-desmethyl fraction derived from the cells. Representative cases were selected for more thorough examination and the physical properties obtained (Table III) confirmed that the substrates had indeed been recovered. In no case did more than a very minor component (a few %) accompany the sterol, and no attempt was made to identify these substances which appeared to include ergosterol and 22-dihydroergosterol.

As will be seen from Table II, the following structural features were consistent with sparing ability although not with equal efficacy: the absence of an alkyl group at C-24, the presence of a $\Delta^{17(20)}$-bond, the presence of a Δ^{7}-bond alone or in conjugation with a Δ^{5}-bond, the absence of C-21, the absence of a Δ^{5}-bond, the presence of either a C_1- or C_2-group at C-24, and inversion of the configuration at C-24. It will also be seen, as we found earlier without the inhibitor (5), that C-22 must be oriented to the right (trans with respect to C-13), because the (E)- but not the (Z)-isomer of 17(20)-dehydrocholesterol was active. This requirement for C-22 to lie to the right has similarly been noted in Tetrahymena pyriformis (24) and Phytophthora cactorum (25). It is interesting that the trans-Δ^{22}-bond seems

Table III. Physical Properties of Sterols Reisolated from S. cerevisiae

Sterol	RRT[a] in GLC (XE-60)		α_c[b] in RPLC		MS[c]
	YS[d]	AS[e]	YS[d]	AS[e]	(M^+)
Cholesterol[h]	1.00[f] 1.01[f]	1.00[f] 1.00[f]	1.00	1.00	386
5α-Cholestan-3β-ol	1.00[f] 1.09[f]	1.00[f] 1.09[f]	NM[g]	NM[g]	388
(E)-17(20)-Dehydrocholesterol	0.94	0.92	0.68	0.68	384
7-Dehydrocholesterol	1.22	1.22	0.76	0.77	384
Campesterol[h]	1.28	1.28	1.11	1.10	400
Sitosterol	1.63	1.65	1.23	1.21	414

a. Retention time relative to that of cholesterol.
b. The α_c is k for test sterol/k for cholesterol (22).
c. The fragmentation patterns agreed with expectation and the only M^+ values found, as listed, were the ones agreeing with the substrate.
d. YS = sterol reisolated from the yeast.
e. AS = authentic sterol.
f. On QF-1 which permits separation of cholesterol and cholestanol with nearly base line resolution.
g. NM = not measured, since the Δ^0 has no UV absorption.
h. The ^{1}H-NMR spectrum of the recovered sterol also confirmed its structure.

to enhance growth (see also 9,11), that addition of a 24α-methyl group to cholesterol appears to have at most a small (negative) effect, and that extension of the size of the group to 24α-ethyl seems to depress activity.

In conclusion, the ability of a sterol to spare ergosterol was independent of the chirality of C-24 as well as of the size of a substituent at this position within the limits of C_0 to C_2 (Table II). This lack of stereospecificity contrasts sharply with the very precise requirement for a 24β-methyl group when growth depends on the presence of a single sterol (17). Two kinds of function must therefore exist. One of these is satisfied by but does not require the 24β-methyl group, while the other has a strict requirement for it. The former has been shown (Table I and Fig. 1) to involve most of the sterol and may coincide with what has been called the "bulk" membranous function (26). The other role may be a regulatory one. Bulk and regulatory functions for sterol have also recently been identified in Mycoplasma capricolum (26).

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