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# GROWTH OF A YEAST MUTANT ON RING A MODIFIED CHOLESTEROL DERIVATIVES

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**The ability of cholesterol derivatives without a hydroxyl group or a side-chain, to support the growth of heme and cyclase deficient *Saccharomyces cerevisiae* mutant GL 7 was tried and found to be in conformity with the results obtained using liposomes. On the other hand, results with other Ring A modified steroids involving saturation or movement of C5-C6 double bond, or isomeric 3-hydroxy-3-methyl cholestane derivatives, indicated that even minor structural variations can cause considerable changes in their growth supporting potential. The consequence of such structural variations need not be obviated by studies using liposomes or vesicles.**

## Introduction

Structure-function relationship studies on cholesterol have been carried out in artificial membranes [1]. Before extrapolating these results to natural membranes, there have been attempts to test various cholesterol analogs in sterol auxotrophs, e.g. *Mycoplasma* [1,2], *Tetrahymena* [3,4], and yeasts. The studies on yeast were carried out under anaerobic conditions [5,6] and with mutant strains [7–9].

We have used a double mutant of *Saccharomyces cerevisiae* GL 7, isolated by Gollub et al. [10], as a useful model system for studying structure function relationship of sterols in membranes. GL 7 is a double mutant which is deficient in cyclase and heme. Thus it accumulates squalene oxide on growth and does not grow on lanosterol till heme is added to the growth medium [10]. This is very useful from the point of view of a model sterol auxotroph since there would be less chances of metabolic transformation of added sterol specially since several enzymes involved in lanosterol to cholesterol conversion are heme dependent.

The effect of various analogs of cholesterol on growth of GL 7 is reported here. Some of these analogs have earlier been studied in artificial membranes and we attempt to correlate the two systems. Using methyl sterols, C-3 hydroxyl isomers and double bond isomers of cholesterol, we find that structural variations around Ring A of cholesterol are very critical and cannot be explained merely on the basis of steric considerations.

## Materials and Methods

*Chemicals.* Dehydroepiandrosterone, 5$\alpha$-cholestane and cholestan-3$\beta$-ol were obtained from Sigma and cholesterol from SRL. They gave the reported melting points and NMR Spectra, and were used as such. Androst-5-en-3$\beta$-ol (m.p. 129–131°C) was prepared from dehydroepiandrosterone by Huang-Minlon reduction [11]. The infrared spectrum of the product did not show a carbonyl peak.

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Cholestan-3α-ol (m.p. 179°C) was prepared from cholestanyl tosylate using alumina [12]. Epicholesterol (m.p. 135–136°C) was prepared from cholesterol by the method of Bose et al. [13], m.p. of benzoate 98–99°C. The NMR spectrum of epicholesterol clearly gave the olefinic proton C 5H at 5.40 ppm and equitorial proton C 3H at 3.99 ppm (half band width 9 Hz). Cholest-4-en-3β-ol (m.p. 131°C) was prepared by $LiAlH_4$ reduction of cholest-4-en-3-one. The NMR spectrum gave the C 4H at 5.26 ppm and C 3H at 4.15 ppm. Cholest-1-en-3β-ol (m.p. 131°C) was prepared by reduction of cholest-1-en-3-one using $LiAlH_4$ [14]. The NMR spectrum of cholest-1-en-3β-ol showed the olefinic protons C 1H and C 2H at 5.92 and 5.48 ppm, respectively, ($^3J = 10$ Hz) and C 3αH as a broad multiplet at 4.30 ppm (half band width: 16 Hz). 3β-Hydroxy-3α-methyl cholestane (m.p. 114–115°C) and 3β-methyl-3α-hydroxycholestane (m.p. 161–162°C) were prepared from cholestan-3-one using methyl magnesium iodide [15], their NMR spectrum showed the C-3 methyl proton at 1.26 and 1.22 ppm, respectively.

*Growth conditions.* The *S. cerevisiae* mutant strain GL 7 was kindly supplied by Dr. D.B. Sprinson [10]. It was grown on complex medium as described before [7] except that the sterol concentration in the medium was 20 μg/ml. Cells were grown at 30°C without shaking. Growth was followed by measuring the absorbance at 640 nm after diluting the medium 3-fold. Growth was also measured by cell dry weight method.

*Steroid analysis.* Cells were harvested at 36 h after innoculation. The washed cells were refluxed with methanolic potassium hydroxide (10%, 5 ml per gram wet weight of cells) for 1.5 h and extracted with solvent ether. The ether fraction was chromatographed on TLC plates (solvent system: 15% ethyl acetate in benzene, double run). The sterol band was cut off, extracted with chloroform/methanol and the material obtained was analysed. In case of thermally stable compounds like cholesterol cholestan-3β-ol and cholestan-3α-ol, gas chromatographic analysis was carried out on a 3% OV-17 column and mass spectral analysis on an MAT 112-S spectrometer. NMR analysis proved to be more useful with thermally labile compounds. The NMR spectra were obtained either on Varian XL-100A or Bruker 270 MHz instrument.

## Results and Discussion

Ergosterol was found to be slightly better than cholesterol in supporting growth of GL 7. This point has been dealt with in detail by Buttke et al. [16]. Since we are using GL 7 as a model to test cholesterol analogs, all compounds are compared with cholesterol. Growth on cholesterol was taken as 100%. Taylor and Parks [8] have recently reported a heme deficient mutant of *S. cerevisiae* and suggest that cells become adapted to cholesterol and thus sterols like cholestan-3β-ol support growth only for about four cycles after previous growth on cholesterol. However, we find that GL 7 grows to the same extent irrespective of the steroid on which it is previously grown, e.g. cholesterol, ergosterol, cholestan-3β-ol. Thus we see no indications of any temporary adaptation. That cells may adapt themselves by varying the saturated-unsaturated fatty acid ratio is quite possible [16]. But this question arises more when two steroids with different structures support growth to a similar extent.

The presence of isooctyl side chain at C-17 and the hydroxyl group at C-3 have been found to be essential for cholesterol to condense artificial membranes [1] and to support growth of, anaerobically grown *S. cerevisiae* and other sterol auxotrophs [6]. To see if this is valid in GL 7 we tried to grow GL 7 on androst-5-en-3β-ol and androst-5-en-3β-ol-17-one. Both compounds did not support growth at all. Similarly, 5α-cholestane could not replace cholesterol for supporting growth of GL 7. This parallelism between earlier data and GL 7 encouraged us to use it as a model and we tried out other compounds.

*Ring B double bond and C-3 hydroxyl group.* The configuration of the hydroxyl group at C-3 of cholesterol has been considered to be quite important and the 3α-isomer epicholesterol does not condense the artificial membranes [1,17,18]. Accordingly, we find that epicholesterol does not support growth at all. On the other hand, saturation of 5,6-double bond gave contrary results. The absence of the 5,6-double bond in cholesterol slightly affects the membrane associated cholesterol properties [1]. Cholestan-3β-ol was only 64% as effective (Fig. 1) as cholesterol in supporting growth and cholestan-3α-ol was 67% effective.

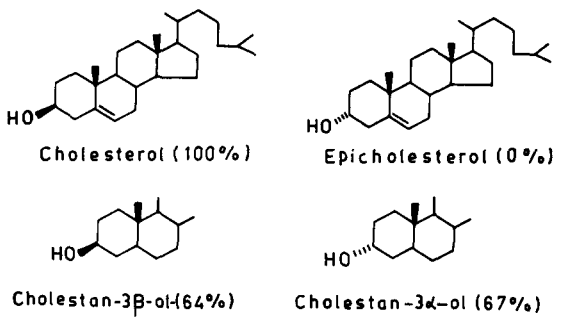
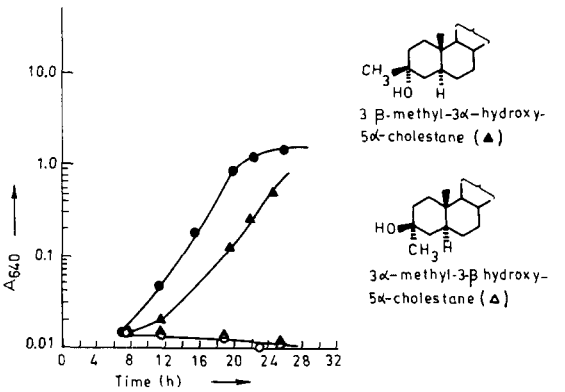



Fig. 1. Effect of the configuration around C3 and the 5,6-double bond of cholesterol. The percentages indicate the extent of growth supported by the compound compared to that with cholesterol as determined by cell dry weight method.




Fig, 3. Growth curves of GL 7 with cholesterol (●), 3β-methyl-3α-hydroxy-5α-cholestane (▲), 3α-methyl-3β-hydroxy-3α-cholestane (△), and control, no steroid, (○).

This suggests that the presence or absence of the 5,6-double bond does indeed make a difference (Fig. 2).

The introduction of the 5,6-double bond takes the C-3 carbon atom, and thereby it's substituents, slightly out of the plane of the steroid skeleton. Much as we wished to speculate on the effect of this on the growth of GL 7, it was not until we tried several other compounds that we felt the problem was far too complex to be clarified by trivial explanations. Thus 3β-hydroxy-3α-methyl-5α-cholestane did not support the growth at all. (Fig. 3). That the 3α-methyl group would lead to steric hinderance did not seem to be a reasonable argument. First because steric crowding on the α-face of the steroid by the 14α-methyl group does not seem to make much of a difference in GL 7, as was reported recently [9]. Secondly, 5α-cholestan-3α-ol supported growth nearly as well as 5α-cholestan-3β-ol. The steric argument was further countered by the observation that 3α-hydroxy-3β-methyl-5α-cholestane supports growth as well as 5α-cholestan-3β-ol.

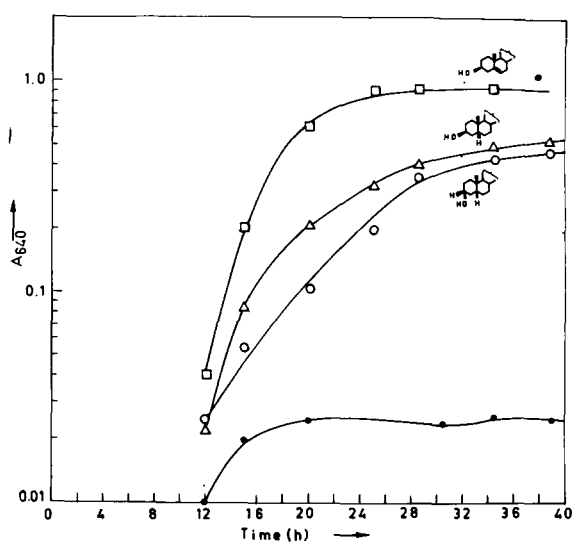



Fig. 2. Growth curves of GL 7 grown on cholesterol (□), cholestan-3β-ol (△), cholestan-3α-ol (○), and control, no steroid, (●).

To get a better insight of the structural requirements around the C-3 hydroxyl group and the effect of the 5,6-double bond, we decided to make compounds with double bonds in Ring A rather than in Ring B. Firstly because they would, by virtue of their proximity to C 3, affect this position more and secondly, because double bonds in Ring B, other than the 5,6-double bond, are more liable to be metabolised, i.e., cholest-7-en-3β-ol to 7-dehydrocholesterol [9]. The two positions at which double bonds may be introduced in Ring A to give stable compounds, are C1-C2 and C4-C5. The other two position C2-C3 and C3-C4, would give unstable enols. Accordingly, 5α-cholest-1-en-3β-ol and cholest-4-en-3β-ol were synthesized and tested for growth. Cholest-4-en-3β-ol barely supported growth (Fig. 4), whereas, cholest-1-en-3β-ol did so only slightly less efficiently when compared to cholesterol. In spite of the fact that molecular models indicate a slight flattening of Ring A more

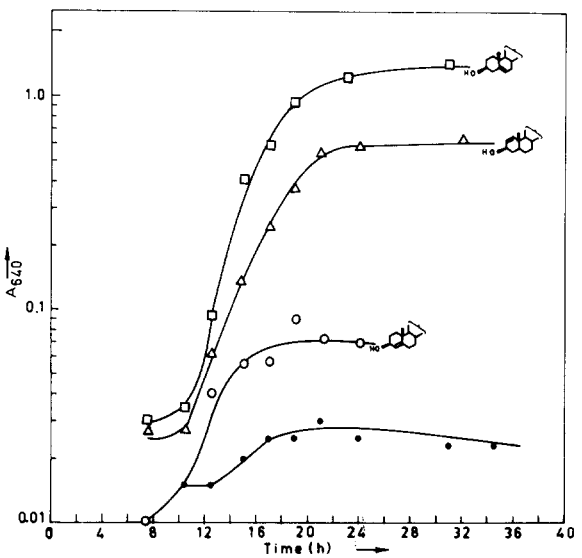



Fig. 4. Growth curves of GL 7 grown on cholesterol (□), cholest-1-en-3β-ol (△), cholest-4-en-3β-ol (○) and control, no steroid, (●).

in cholest-1-en-3β-ol than in cholest-4-en-3β-ol, there is no reason why one should have supported growth so much better than the other.

We must add here that the sterols tested are unlikely to undergo metabolic transformations, i.e. 3-hydroxymethyl compounds or cholest-4-en-3β-ol and cholest-1-en-3β-ol. Nevertheless one must check for the same before reaching any conclusion. This is easily done usually by extracting the non-saponifiable lipid fraction from harvested cells and analysing by GC-MS. Thus cholesterol grown cells gave MS corresponding to cholesterol alone. The tertiary alcohols and allylic alcohols are thermally unstable and dehydrated to give other compounds thus making it impossible for us to use GC-MS. To get over this problem and get conclusive evidence, we decided to grow cells on a large scale, isolate the sterol fraction by preparative thin-layer chromatography of nonsaponifiable lipid extract and analyse the sterol fraction by NMR spectroscopy. Towards this end we have isolated sterol

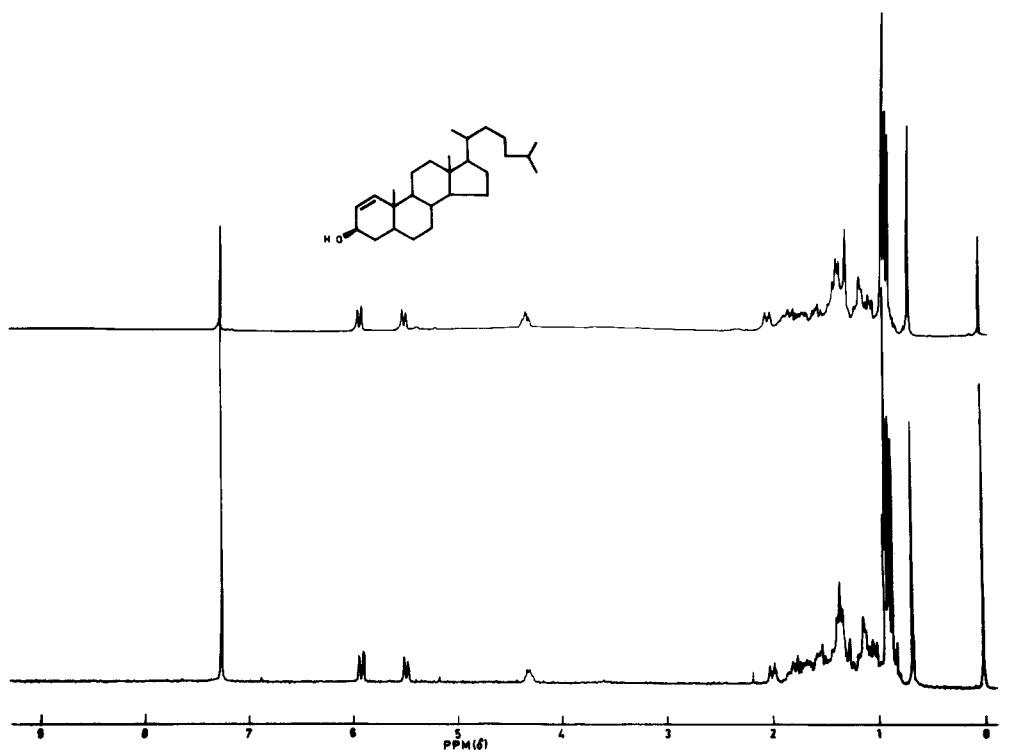



Fig. 5. NMR spectrum of cholest-1-en-3β-ol (lower) and of sterol fraction (upper) isolated from nonsaponifiable lipid entract of GL 7 cells grown on cholest-1-en-3β-ol.

fraction from cell grown on cholest-1-en-3β-ol and found the NMR of sterol fraction to be identical to that of the authentic compound (Fig. 5). Thus the NMR of sterol fraction clearly shows C 1H and C 2H at 5.92 and 5.48 ppm, respectively. ($^3J = 10$ Hz, *cis* coupling) and C 3H as a broad multiplet at 4.30 ppm (halfband width 16 Hz, axial proton) exactly as found in NMR of pure cholest-1-en-3β-ol. All the methyl groups protons were also in the same position as for pure cholest-1-en-3β-ol. Similarly, a sterol fraction isolated from cells grown on cholestan-3α-ol and 3α-hydroxy-3β-methyl-5α-cholestane gave an NMR spectrum identical to that of parent sterol. Metabolic transformation of these sterols if any would be less than 5% otherwise they would have been detected by NMR spectroscopy.

Thus the stereochemistry at C-3, and the 5,6-double bond can be considered to be important for cholesterol to exhibit optimal growth. It is tempting to speculate here on the structural differences between cholesterol and its analogs based on distance measurements using Drieding models, e.g. distances between C-18 and C-19 methyl carbon atoms or some other reference point to the oxygen atoms at C-3. The differences are so small that we feel such empirical correlations would not really help better our understanding of role and disposition of sterols in membranes and may hold only till someone comes up with data on some more compounds. In the absence of any single model which can explain all the compounds tried here based on structural differences alone, one can partially explain the results obtained here on the basis of following assumption. If two sterols support the growth of an organism to a similar extent, they need not be oriented in a identical way in membranes. Depending on their structure they may take up different orientation and thereby the structural requirement for favourable hydrophobic interaction of these sterols with membrane components may differ. Accordingly it is suggested that cholestan-3β-ol and cholestan-3α-ol, which support the growth to a similar extent, may be differently oriented. Thus while introduction of double bond at C5-C6 in cholestan-3β-ol enhances its growth promoting potential, the introduction of same in cholestan-3α-ol, inhibits growth. Similarly though introduction of methyl group at C-3 in cholestan-3β-ol inhibits growth, the same variation in cholestan-3α-ol i.e. 3α-hydroxy-3β-methyl cholestane, has no effect on its growth. The opposite effect observed on similar structural variations in cholestan-3β-ol and cholestan-3α-ol suggest that they have opposite structure requirements for interaction with membrane components. It is thus possible that cholesterol itself might have more than one orientation in membranes.

We would only like to conclude, that a lot more work needs to be done to understand how such minor variations around Ring A of cholesterol preclude the manifestation of its growth promoting or membrane associated properties.

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