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Cholesteryl-(2'-hydroxy)-ethyl ether—A potential cholesterol substitute for studies in membranes

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Abstract. The yeast sterol auxotroph GL-7, which grows well on ergosterol and cholesterol, was used to study the ability of cholesteryl-(2'–hydroxy)-ethyl ether to substitute for cholesterol. In this compound the 3j3-hydroxyl group of cholesterol is replaced by ethylene glycol and the resulting ether still retains the amphiphilic character of cholesterol. Cholesteryl-(2^{L}hydroxy)-ethyl ether was found to support the growth of GL-7 as effectively as cholesterol. Crystal violet permeability and membrane order parameter determined using a spin label were similar for cells grown on these sterols. The ability of such ethylene glycol derivatives to substitute for cholesterol in both artificial and natural membranes should help in designing suitable spacers through which molecules can be linked to cholesterol without affecting the normal function of cholesterol in membranes. This in turn should prove useful in studies with surface -modified liposomes.

Keywords. Cholesteryl-(2'hydroxy)-ethyl ether; yeast sterol auxotroph; membranes; anchor molecule.

Introduction

Cholesterol is an integral component of various biological membranes. It also affects the fluidity of biological membranes and is found to be associated with various membrane-associated processes like permeability, activity of membrane-bound enzymes and receptors, endocytosis and immune response (Demel and De Kruyff, 1976; Yeagle, 1985). The interaction of cholesterol with phospholipids and proteins is well documented (Yeagle, 1985). A planar ring system (Demel *et al.,* 1972), an isoctyl chain (Suckling *et al.,* 1979; Bloch, 1983), a C_5-C_6 double bond (Ranadive and Lala, 1987) and a 3β-hydroxyl group at C_3 (Demel and De Kruyfff, 1976) have been considered essential for optimal interaction of cholesterol with other membrane components. We have for some time been involved in examining the role of hydroxyl group of cholesterol in membranes (Lala, 1981) and have recently reported that a free hydroxyl group in cholesterol is not necessary for the normal cholesterol-associated properties observed in model membranes (Demel *et al.,* 1984). This study involved the use of various alkyl ethers of cholesterol and revealed that increasing the hydrophobic bulk of the ether *i.e*., going from cholesteryl methyl ether to cholesteryl butyl ether, decreases its ability to condense membranes as judged by monolayer, differential scanning calorimetric and glucose permeability studies on liposomes. Nevertheless cholesteryl methyl ether substituted very well for cholesterol. Interestingly the introduction of a hydroxyl group in place of the terminal methyl

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Abbreviations used: CH-OEG, Cholesteryl-(2'–hydroxy)-ethyl ether; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; PC, egg phosphatidylcholine.

group of cholesteryl n-propyl ether gives rise to a compound, cholesteryl-(2′-hydroxy)-ethyl ether (CH-OEG), which also substitutes for cholesterol.

We have also reported that cholesteryl methyl ether is as effective as cholesterol in supporting the growth of the *Saccharomyces cerevisiae* double mutant GL-7 (Lala *et al.,* 1979). This yeast mutant is an effective sterol auxotroph (Gollub *et al.,* 1977) and has been successfully used to study the role of sterol in natural membranes (Buttke and Bloch, 1981; Nanda Kumari *et al.,* 1982; Bloch, 1983). We reported here our studies with the novel compound, CH-OEG using GL-7.

Materials and methods

Cholesterol was obtained from SRL, Bombay and crystallised twice from methanol before use. CH-OEG (figure 1) was prepared as reported earlier (Demel *et al.,* 1984). Both sterols were purified by high performance liquid chromatography (HPLC) and found to be homogeneous. HPLC analysis was carried out on a Dupont 8800 system or a Shimadzu LC-4A system using an RI or UV detector. Methanol or methanol: water (98:2) was used as mobile phase. Cholesterol appeared at 13·6 min and CH-OEG at 15 min when methanol was the mobile phase. Increasing water in the mobile phase or gradient elution did not lead to improved separation of the two sterols.

The *S. cerevisiae* mutant GL-7 was grown on these sterols. Growth of the cells and isolation of nonsaponifiable lipid extract were carried out as described earlier (Nanda Kumari *et al.,* 1982). HPLC analysis of the extract from cells grown on CH-OEG indicated no trace of cholesterol. Thin-layer chromatographic (TLC) analysis was also carried out on silica gel G coated plates using 15% ethyl acetate in benzene as developing solvent system. For NMR spectroscopic analysis, the nonsaponifiable lipid extract of cells grown on CH-OEG (2 L batches) was subjected to TLC and a broad band between R_f 's 0·3 and 0·7 was cut and extracted with chloroform: methanol (2:1). The solvent was then removed and the NMR spectrum of the residue in $CDCl_3$ was recorded on a Bruker 270 MHz spectrometer.

Crystal violet was obtained from Glaxo, Bombay. A fresh 18 h GL-7 culture was shaken well. Three ml aliquots were taken in glass centrifuge tubes. Crystal violet was added as a solution in ethanol (30 μl, 0·5 mg/ml) to each tube, and the tubes incubated at room temperature (30°C) for 10 min. The cells were centri fuged out and the absorbance at 590 nm of the supernatant was determined. The crystal violet index was obtained by dividing the mean absorbance at 590 nm of the supernatant of a culture grown on cholesterol by the mean absorbance at 590 nm of the supernatant of the test culture. A correction factor was applied for the difference in the number of cells.

7-Doxyl stearic acid was prepared by published procedure (Jost *et al.,* 1971) Electron spin resonance spectra were recorded on a Varian E-12 spectrometer. 7-Doxyl stearic acid was incorporated in GL-7 cells grown on cholesterol and CH-OEG and order parameter determined from the ESR spectra as reported earlier (Lees *et al.,* 1979).

Results and discussion

The yeast mutant GL-7 was grown on cholesterol and on CH-OEG (figure 1). Both

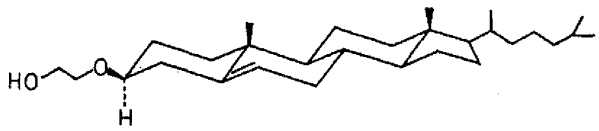

Figure 1. Structure of CH-OEG.

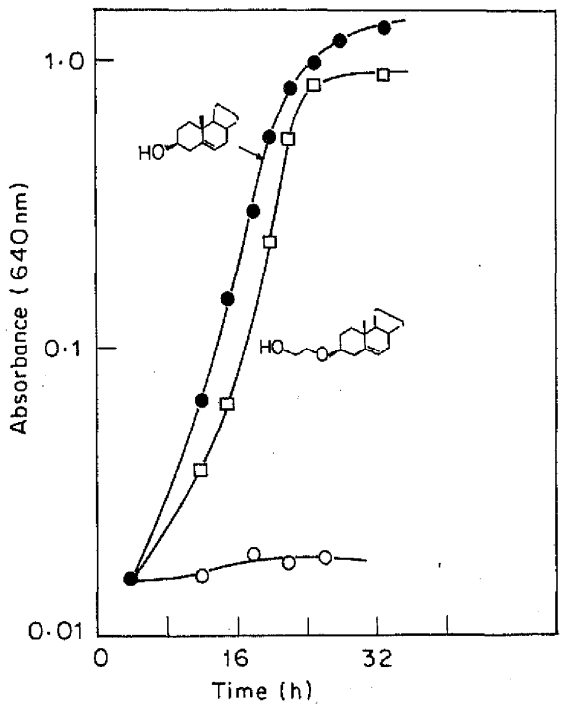

Figure 2. Growth curve of GL-7 cells grown on cholesterol(●), CH-OEG (□), and in the absence of sterol (O).

the sterols were found to be quite effective in supporting growth over several cycles (figure 2). It has been reported that cholesterol is not metabolically transformed while supporting growth of GL-7 (Lala *et al.,* 1979; Buttke and Bloch, 1981). To confirm that CH-OEG is not metabolized during growth, the nonsaponifiable lipid extract was analysed by reversed phase HPLC. The analysis confirmed the identity of the compound and showed no trace of cholesterol. To further confirm the identity of CH-OEG, NMR spectroscopy was used. This method has been used in the past to identify other sterols which support the growth of GL-7 (Nanda, Kumari *et al.,* 1982). Although it is much less sensitive than HPLC, NMR spectroscopy is an independent method for sterol identification. Cells were grown in bulk on CH-OEG and the sterol fraction of the nonsaponifiable extract of the cells was isolated. The NMR spectrum of the sterol fraction was obtained and found to match that of authentic CH-OEG clearly indicating that this compound had not undergone metabolic transformation while supporting the growth of GL-7.

It is well known that increasing the amount of cholesterol in membranes leads to change in membrane fluidity which can be monitored by a variety of methods

(Demel and De Kruyff, 1976; Yeagle, 1985). In order to see the effect of cholesterol and CH-OEG on membrane-associated properties in GL-7, permeability to crystal violet and membrane order parameter were determined for cells grown on these sterols. Crystal violet is a cationic dye and has been used to determine the permeability of yeast mutants (Bard *et al.,* 1978). The data given in table 1 clearly indicate similarity in crystal violet permeability for GL-7 cells grown on cholesterol and those grown on CH-OEG. Spin labelled probes like 7-doxyl stearic acid have been quite useful in assessing the degree of order in membranes, which increases with increasing concentration of cholesterol (Schreier *et al.,* 1978; Lees *et al.,* 1979). The membrane order parameter determined for cells grown on cholesterol and that for cells grown on CH-OEG were also found to be similar (table 1). In order to see the effect of the two sterols on artificial membranes, order parameter for egg phosphatidyl choline (PC): cholesterol and PC: CH-OEG vesicles was also determined. The order parameter values for the two vesicle preparations were also found to be similar (table 1).

Table 1. Crystal violet permeability and membrane order parameter [S_{7DS}]- for GL-7cells grown on cholesterol and on CH-OEG

Experiment	Cholesterol	CH-OEG
Crystal violet permeability for cells	1·00 ± 0·02	0·91 ± 0·01
[S_{7DS}] for cells	0·67 ± 0·02	0·68 ± 0·04
[S_{7DS}] for PC:sterol vesicles	0·64 ± 0·03	0·63 ± 0·02

Values are mean ± of seat least 3 determinations.
[S_{7DS}] for PC: vesicles (33 mol% sterol) included for comparison

The GL-7 growth data and the preliminary studies on membrane-associated properties of these cells indicate that CH-OEG is quite effective in simulating the role of cholesterol. This confirms similar conclusions made on the basis of artificial membrane studies (Demel *et al.,* 1984). The ability of CH-OEG to substitute for cholesterol indicates that it should be possible to synthesise cholesterol analogues with the hydroxyl group substituted by other groups without affecting the normal function of cholesterol in membranes. Such chemical modifications are important for the use of cholesterol as an anchor molecule for attaching membrane surface-active agents like sugars and antibodies. Earlier studies along these lines have involved the use of carbamate links for attaching sugars (Slama and Rando, 1980) and fluorescent probes (Alecio *et al.,* 1982) and ester links for attaching proteins (Kinsy *et al.,* 1983). The use of these functional groups, specially cholesteryl esters, is likely to perturb the membrane and thus affect the normal function of cholesterol in membranes. For effective liposome targeting and related studies it will be desirable to have cholesterol analogues with spacers which would permit convenient attachment of molecules of interest and still retain the normal membrane-associated properties of cholesterol. The use of cholesteryl ethers recently in the synthesis of cholesterol analogues carrying β-aminogalactose to study effects of surface modification on aggregation of phospholipid vesicles (Wu *et al.,* 1981) is encouraging. The use of ethylene glycol based ethers of cholesterol as membrane anchor sites thus has considerable potential for future studies. It should be interesting to see the effect of similar diethylene glycol

and triethylene glycol based ethers of cholesterol on membranes. These studies are currently in progress.

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