ELSEVIER

Biochimica et Biophysica Acta 1191 (1994) 309–316

BBA Biochimica et Biophysica Acta

Trehalose inhibits ethanol effects on intact yeast cells and liposomes

Jose J.C. Mansure [a,*], Anita D. Panek [a], Lois M. Crowe [b], John H. Crowe [b]

[a] *Departamento de Bioquimica, Instituto de Quimica, Universidade Federal do Rio de Janeiro, Cidade Universitaria 21941 Rio de Janeiro, RJ, Brazil*
[b] *Section of Molecular and Cellular Biology, Storer Hall, University of California, Davis, CA 95616, USA*

(Received 20 September 1993; revised manuscript received 24 January 1994)

Abstract

The effect of ethanol on stability of intact yeast cells has been investigated. Several strains with differences in trehalose metabolism were examined for their ability to survive in the presence of 10% (v/v) ethanol. A positive correlation was observed between cell viability and trehalose concentration. When leakage of electrolytes from the cells was recorded by observing changes in conductivity of the medium, we found that ethanol increases leakage, but the presence of trehalose reverses that effect. Similar studies were done with liposomes of similar composition to those seen in intact cells in log and stationary phases. In the presence of ethanol, carboxyfluorescein trapped in the liposomes leaked to the medium. When trehalose was added inside, outside or on both sides of the membrane, the ethanol-induced leakage was strongly inhibited. More leakage was observed in liposomes in gel phase state than in liquid-crystalline phase, suggesting that the thermotropic behavior of the lipids in the plasm membrane, together with trehalose, plays a role in enhancing ethanol tolerance.

Key words: Ethanol tolerance; Trehalose; Liposome; Plasma membrane; Phase transition; (*S. cerevisiae*)

1. Introduction

Ethanol production by microorganisms has been exploited by man for millennia, but much is still not understood about this process. For example, during fermentation, when ethanol concentration is high, yeasts experience inhibition of cell growth, loss of cell viability, loss of nutrient uptake, decreased proton fluxes and impaired fermentation performance [1–5].

Several authors have proposed that the plasma membrane can be one of the major target sites of ethanol toxicity [5–8]. Since ethanol and plasma membrane lipids are both amphiphatic molecules, it is likely that they interact directly with each other during the course of fermentation, resulting in physiological changes to the membrane [3].

A way in which ethanol could affect the permeability properties of a membrane has been hypothesized [5]. If we consider the hydrophobic interior of the membrane as a primary permeability barrier of the cell, preventing free exchange of polar molecules, intercalation of ethanol into this hydrophobic interior would tend to increase the polarity of this region, increasing permeability to water soluble molecules. An increase in permeability to protons and ions would, among other things, result in an uncoupling that should contribute to the inhibition of a secondary active transport across the yeast plasma membrane, decreasing substrate accumulation.

Ethanol can also induce changes in lipid composition of yeast and bacterial membranes as an adaptative response. In *Escherichia coli*, the presence of ethanol changes the ratio of saturated to unsaturated fatty acids, with an increase in the proportion of vaccenic acid and a reduction of palmitic acid. The reduced

* Corresponding author (at Section of Molecular and Cellular Biology, Storer Hall, University of California, Davis, CA 95616, USA). Fax: +1 (916) 7521449. E-mail: JJMANSURE@UCDAVIS.

Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Tes, (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; CF, carboxyfluorescein.

SSDI 0005-2736(94)00019-L

acid and a reduction of palmitic acid. The reduced level of saturated fatty acids has been suggested to be due to a decrease in the amount of saturated fatty acids available for phospholipid synthesis [9]. In yeast cells, the lipid composition of plasma membrane has been reported to affect ethanol tolerance [6,7]. Supplementation of cultures with ethanol at several concentrations led to an increase in the proportion of monounsaturated fatty acid residues in cellular phospholipids, which was accompanied by a decrease in the proportion of saturated residues [2,10].

Some information about basic mechanisms can be derived from simpler model studies. Using a multilamellar liposome model, some workers have described unusual thermotropic behavior for pure saturated phosphatidylcholines (PC) of various chain lengths [11–15] in the presence of ethanol. At low ethanol concentrations, the phase transition temperature decreases as ethanol intercalates into the membrane and spreads the phospholipid headgroups apart, while at higher concentrations the ethanol spreads the lipids so much that the chains interdigitate. Additionally, ethanol has been shown to induce lateral phase separation in mixed bilayers of PC and phosphatidylethanolamine (PE) [15]. Clearly, such events could lead to loss of membrane function.

Trehalose, a non-reducing disaccharide (α-D-glucopyranosyl-α-D-glucopyranoside) has been considered for a long time as a storage carbohydrate in yeast [16,17]. However, an additional function has recently been proposed. This sugar is widely found in many organisms capable of surviving under several adverse environmental conditions such as freezing, heat shock and dehydration [18–26]. In *Saccharomyces cerevisiae*, accumulation of trehalose was observed when cells were shifted to temperatures above 28°C or exposed to either ethanol or hydrogen peroxide, suggesting that it might correspond to a general response to a physiological stress [27–29]. These observations have shifted the focus from trehalose as storage carbohydrate to a new role as a membrane and protein protectant.

Other studies from this laboratory have shown that trehalose is highly effective in preserving structure and function in dry biological membranes. Further biophysical studies show that the sugar acts by forming hydrogen bonds with the phospholipid head group, thereby maintaining the bilayer structure in a 'fluid' condition, preventing fusion during drying and phase transitions (which cause leakage) during rehydration [30–34].

In this report, we have investigated some mechanisms by which ethanol affects yeasts and liposomes. This study was undertaken to test the hypothesis that ethanol may be toxic to yeast because of its effects on lipid bilayers and that trehalose can partially or wholly reverse these effects, leading to decreased leakage and increased survival.

2. Materials and methods

Yeast strains. The control strain used in this study was D273-10B. The mutant strains had the following characteristics: Klg (*cif*) and Q6R2 (*fdp*) show no trehalose accumulation due to the lack of detectable UDPG trehalose-6-phosphate synthase activity (EC 2.4.1.15) [35,36]. Strains $308T_1$, $308T_2$ and $308T_5$ are mutants lacking the trehalose transporter [37].

Growth conditions. Growth media consisted of 1.3% yeast extract and 2% glucose (YED) supplemented with 0.2% $(NH_4)_2SO_4$, 0.2% KH_2PO_4 and the pH adjusted to 5.2. Cultures were incubated at 28°C on a rotary shaker at 160 rpm. Growth of cultures was followed by turbity measurements at 570 nm.

Heat shock treatment (temperature shift). Cells grown at 28°C up to mid-log phase were submitted to a shift in temperature to 40°C for 60 min.

Trehalose extraction and determination. Trehalose was extracted from 12 mg (dry weight) of cells with 0.5 M trichloroacetic acid and determined by the antrone method according to Oliveira et al. [38].

Viability determination. Cell viability was determined after appropriate dilution and plating on solidified yeast extract-peptone-glucose medium (YPD). The colonies were counted after 48 h at 28°C. All plates were run in duplicate.

Measurement of conductivity. Cells were harvested 30 mg (dry weight) and washed twice with cold distilled water. The cells suspension were dispensed in electrode systems with water; water and 2 M ethanol; water, 2 M ethanol and 100 mM trehalose. Changes in conductance were measured after 10 min with a conductivity meter (model YSI 35, Yellow Spring Instruments, OH) using alternating current at 60 Hz.

Preparation of liposomes. All phospholipids were purchased in chloroform from Avanti Polar Lipids and stored at −20°C. Liposomes were made by drying 20 mg of phospholipids under dry nitrogen and then placing them in a high vaccum for at least 2 h. The lipid was rehydrated with 10 mM Tes buffer (pH 7.5) and 250 mM CF (carboxyfluorescein). When necessary, 100 mM trehalose was also trapped in the liposomes. Unilamellar liposomes were prepared by a simple extrusion method according to MacDonald et al. [39] through 0.1 μm polycarbonate membrane filters, obtained from Poretics (Livermore, CA).

Fluorescence measurement. Fluorescence was measured in a Perkin-Elmer LS-5 fluorescence spectrophotometer. The excitation wavelength was 460 nm and emission wavelength 550 nm. Maximal carboxyfluorescein was determined by lysing the vesicles with 0.1% Triton X-100.

Special chemicals. Carboxyfluorescein was obtained from Eastman Kodak (Rochester, NY). Trehalose was purchased from Pfanstiehl Laboratories (Waukegan,

IL). All other chemicals were purchased from Sigma (St. Louis, MO).

3. Results and discussion

Trehalose and ethanol toxicity

Trehalose has emerged as a protectant against ethanol toxicity, although the mechanism(s) of the protection remain unknown. Cells of one wild-type and several mutant strains were grown at 28°C and harvested at first log phase (before glucose exhaustion) when endogeneous trehalose is low [16–17]. Cells were rinsed, and one aliquot was subjected to a mild heat stress (40°C/1 h). Another aliquot was kept at 28°C. The temperature shift promotes a rapid trehalose accumulation in *Saccharomyces cerevisiae*, which has been correlated with 3 fold-increase in the level of trehalose-6-phosphate synthase activity [27,40–44]. Both aliquots were then incubated for 24 h in 10% ethanol at 28°C. Trehalose content and survival were determined for all strains both before and after ethanol incubation and with and without heat stress. Results are shown in Table 1.

As seen in Table 1, the wild-type D273-10B showed low trehalose and low survival when the temperature was not shifted. Following a temperature stress, trehalose increased significantly with a further increase following ethanol stress. The high trehalose content

Table 1
Survival of several strains as a function of trehalose content, before and after 10% (v/v) ethanol incubation for 24 h

Strains		Trehalose (mg/g dry wt. cells)		Survival (%)
		before EtOH	after EtOH	
D27310-B	no heat	3	3	6
	heat	46	65	60
KLG 102	no heat	3	3	3
	heat	3	3	12
Q6R2	no heat	3	3	3
	heat	3	3	3
308 T1	no heat	3	21	15
	heat	30	20	19
308 T2	no heat	3	19	42
	heat	29	26	45
308 T3	no heat	3	32	22
	heat	48	36	31

Cells grown at 28°C up to mid-log phase were submitted to a shift in temperature to 40°C for 60 min for heat-shocked samples. Trehalose was extrated from 12 mg (dry weight) of cells with 0.5 M trichloroacetic acid and determined by the antrone method according to Oliveira et al. [38]. Cell viability was determined after appropriate dilution and plating on YPD medium. The colonies were counted after 48 h at 28°C. Survival was obtained as percentage of control.

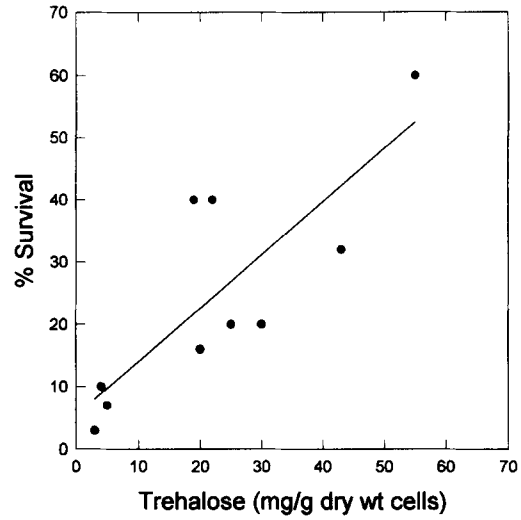

Fig. 1. Yeast cells survival as a function of trehalose content. A correlation coefficient, $r^2 = 0.65704204$, was obtained by a linear regression program.

was accompanied by high survival following ethanol stress. Two strains (Klg-102 and Q6R2) which have no trehalose-6-phosphate synthase activity showed low trehalose and low survival under all conditions. Three other strains ($308T_1$, $308T_2$, and $308T_5$), which are mutants lacking a trehalose transporter, showed low trehalose before both a heat and an ethanol stress. However, trehalose increased following these stresses in parallel with an increase in survival (compare survival with wild-type). These results can be better visualized by plotting the survival data from all strains against trehalose content (Fig. 1), which shows a positive correlation between cell viability and trehalose concentration. This finding indicates that depending on the strain, yeast cells can respond to heat and ethanol stress with increased trehalose content and increased survival. Similar effects have been reported in brewing strains that showed an increase in ethanol tolerance accompanied by an increase in the level of intracellular trehalose, when these cells were submitted to heat or ethanol stress [28,45].

Effect of trehalose and ethanol on leakage from cells

Previous studies on ethanol toxicity for yeast have shown that it decreases the activity of glycolytic enzymes in whole cells and cell extracts, while apparently not inhibiting glucose uptake at least up to 2 M ethanol [46]. One of the most important effects of ethanol is to alter transmembrane proton flow, leading to changes in the transmembrane gradient [47], which can clearly affect solute accumulation, growth, and survival. Interestingly, the study by Cartwright et al. [47] found that older cultures were less affected by ethanol; these are the cells that would be expected to have higher trehalose contents [16–17].

Leakage from cells of D273-10B (*wt*) and Q6R2

Table 2
Conductivity from cells of D273-10B and Q6R2 with different levels of intracellular trehalose

Strain D277310-B	Conductivity (μmho/g cell)	Trehalose (mg/g cell)	Strain Q6R2	Conductivity (μmho/g cell)	Trehalose (mg/g cell)
A. Growth on glucose			B. Growth on glucose		
Log phase, no heat			Log phase, no heat		
Control	2.0	6	Control	2.0	4
2 M EtOH	2.4	6	2 M EtOH	3.0	4
2 M EtOH + 100 mM trehalose	2.1	6	2 M EtOH + 100 mM trehalose	2.3	4
Log phase, heat			Log phase, heat		
Control	0.6	58	Control	0.7	5
2 M EtOH	0.8	58	2 M EtOH	0.8	5
2 M EtOH + 100 mM trehalose	0.7	58	2 M EtOH + 100 mM trehalose	0.7	5
			C. Growth on maltose		
Stationary phase			Log phase		
Control	0.7	48	Control	1.8	40
2 M EtOH	0.8	48	2 M EtOH	2.4	40
2 M EtOH + 100 mM trehalose	0.7	48	2 M EtOH + 100 mM trehalose	2.3	40

(*fdp*) were compared after exposure for ten minutes to either water, 2 M ethanol, or 2 M ethanol with 100 mM trehalose, and the increase in conductivity (a measurement of ions in the solution) of the solution was determined. Results are shown in Table 2.

Both strains showed similarity in the effects of ethanol on leakage, as determined by conductivity of the medium; in general, the presence of trehalose decreased ethanol-induced leakage. Leakage from strain D273-10B collected during log phase is intrinsically higher than in stationary phase. However, log phase cells submitted to heat stress (temperature shift), which promotes trehalose accumulation [27,40–44], showed levels of leakage similar to stationary phase cells. In the case of strain Q6R2 grown on glucose and collected at log phase (conditions under which the cells have low levels of internal trehalose), ethanol again increased the conductivity in the solution, and this effect was reversed when trehalose was added. When the same cells were grown on maltose as carbon source and collected at log phase the levels of conductivity in the presence of ethanol were lower compared to growth on glucose. Q6R2 produces trehalose when it is grown on maltose [48].

Despite the general pattern that trehalose decreases ethanol-induced leakage from the cells, the presence of the sugar trehalose does not seem to be the only factor promoting ethanol tolerance. Cells of Q6R2 grown on glucose and submitted to a temperature shift did not produce trehalose, but also showed low levels of leakage compared with the control. Because this effect was induced by a temperature shift, it seems reasonable to assume that alterations in lipid compositon could be involved in the increased tolerance, a possibility that we have pursued with studies on model membranes.

Effect of trehalose and ethanol on leakage from liposomes

Control liposomes composed of egg PC and PS (9:1) do not leak trapped carboxyfluorescein during storage for 30 min at room temperature, but upon exposure to 2 M ethanol they leak about 30% of their contents over the same time (Fig. 2). Addition of trehalose significantly decreased the ethanol-caused leakage, regardless of whether the sugar was present only on the outside of the liposomes or on both sides of the bilayer (Fig. 2).

In yeast membranes, phosphatidylcholine (PC) is the most abundant phospholipid and quantitatively the most predominant fatty acids substituents of yeast phospholipids are 16:0 (palmitic acid); 16:1 (palmitoleyl acid); 18:1 (oleic acid) and a smaller proportion of 18:0 (stearic acid) [49–50].

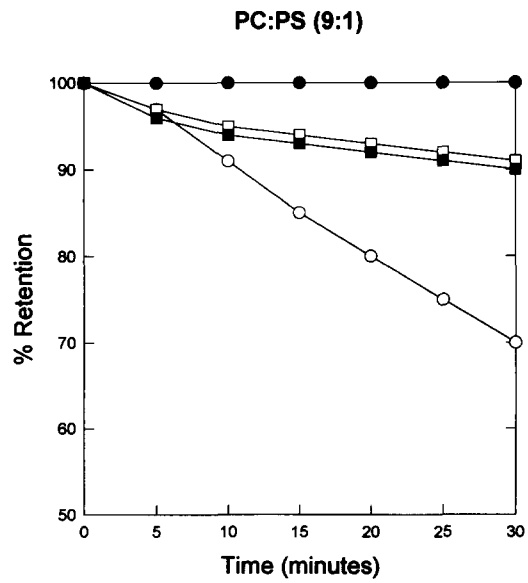

Fig. 2. Leakage of carboxyfluorescein from PC/PS (9:1), control (●). Measurements were also made in the presence of 2 M ethanol: without trehalose (○); with 100 mM trehalose outside (■) or with 100 mM trehalose on both sides of the membrane (□).

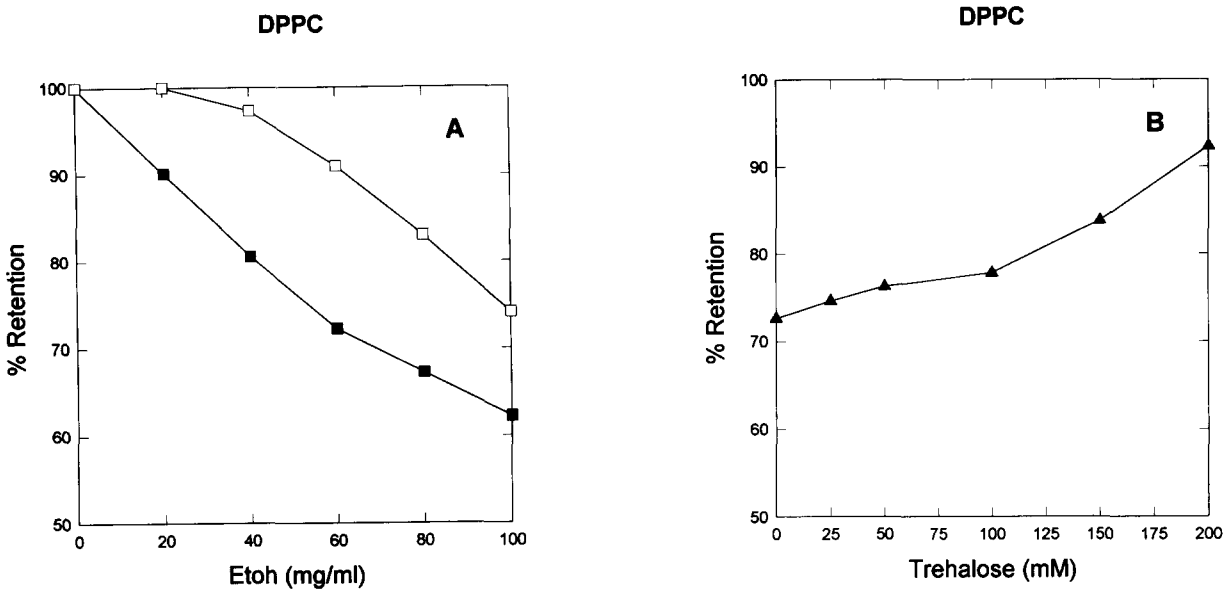

Fig. 3. Leakage of carboxyfluorescein from DPPC. (A) Increasing ethanol concentration, with (□) and without (■) addition of 100 mM trehalose. (B) Constant ethanol concentration at 2 M, with increasing trehalose concentration (▲).

Some organisms change their plasma lipid composition in response to high concentrations of ethanol in their growth medium. These changes include an increase in the proportion of mono-unsaturated fatty acids in cellular phospholipids, especially in C_{18} residues which is accompanied by a decrease in the proportion of saturated residues as C_{16} [2,5,9,51,52] while a small effect has been observed in stearic acid (18:0) [28,53]. Therefore, studies were made of effects of ethanol on leakage in liposomes constituted with the major phospholipids identified in the yeast membrane. Furthermore, we varied the ethanol concentration, maintaining trehalose concentration constant, and then at a constant ethanol concentration we varied the trehalose concentration.

An increase in the ethanol concentration was accompanied by an increase in the permeability of all liposomes tested, regardless of the composition (Figs. 3–7). In all liposomes tested, trehalose mitigated the ethanol-caused leakage, and this protective effect was dependent on trehalose concentration (Figs. 3–7).

These results also show that liposomes prepared with unsaturated phospholipids showed less leakage than those containing saturated fatty acids (Figs. 3–7).

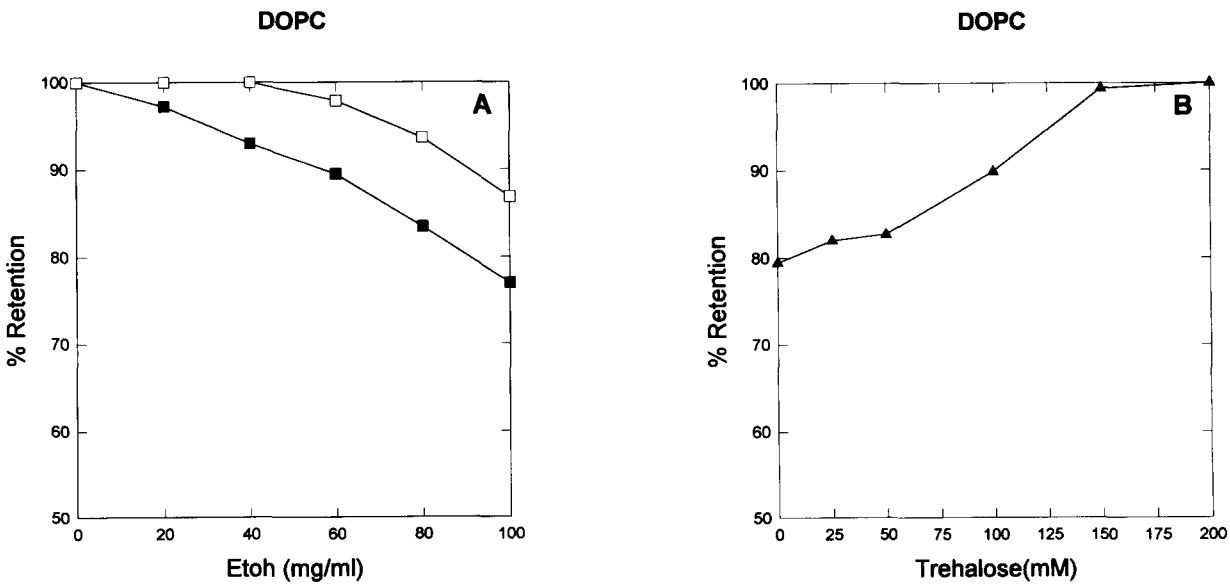

Fig. 4. Leakage of carboxyfluorescein from DOPC. (A) Increasing ethanol concentration, with (□) and without (■) addition of 100 mM trehalose. (B) Constant ethanol concentration at 2 M, with increasing trehalose concentration (▲).

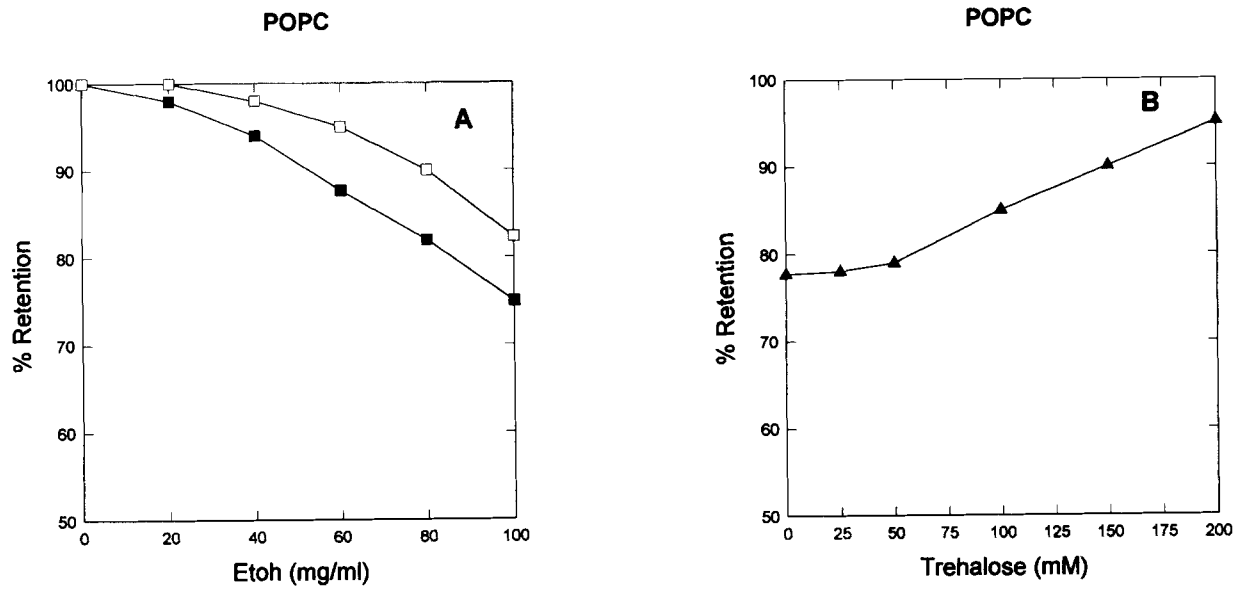

Fig. 5. Leakage of carboxyfluorescein from POPC. (A) Increasing ethanol concentration, with (□) and without (■) addition of 100 mM trehalose. (B) Constant ethanol concentration at 2 M, with increasing trehalose concentration (▲).

Indeed, a mixture of two unsaturated phospholipids POPC and DOPC (1:1) showed a lower level of leakage than a mixture including a third, saturated lipid POPC/DOPC/DPPC (1:1:1) (Figs. 6–7). One possible explanation for this effect is that at room temperature liposomes composed of saturated fatty acids (DPPC) are in gel phase state while liposomes containing unsaturated fatty acids are in liquid-crystalline phase. Thus there may be some lipid phase separation, which could lead to increased permeability. However, since the controls made from this mixture showed low leakage rates, phase separation in itself would not seem to explain this effect. At present, we have no satisfactory explanation for this effect, but suggest that the more rigid acyl chains of DPPC may not accommodate the presence of ethanol in the hydrophobic interior of the bilayer without defects, while more fluid acyl chains could conceivably do so. In this regard, it is satisfying to note again the response of some organisms to the presence of ethanol by altering their lipid composition with a increase in mono-unsaturated fatty acids and a decline in saturated fatty acids.

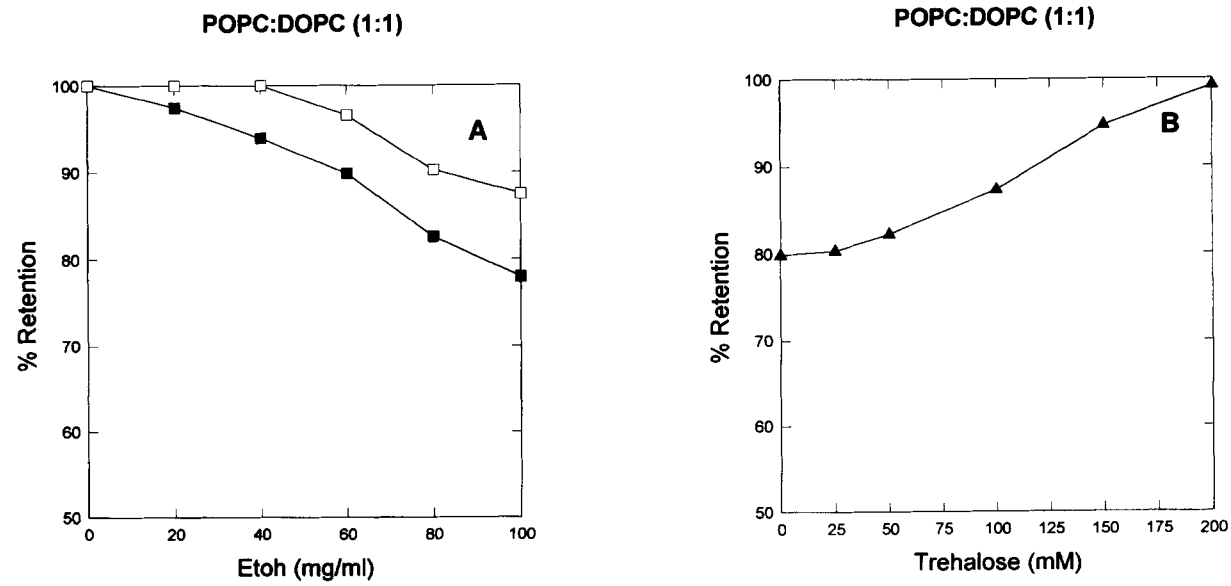

Fig. 6. Leakage of carboxyfluorescein from a mixture of POPC/DOPC (1:1). (A) Increasing ethanol concentration, with (□) and without (■) addition of 100 mM trehalose. (B) Constant ethanol concentration at 2 M, with increasing trehalose concentration (▲).

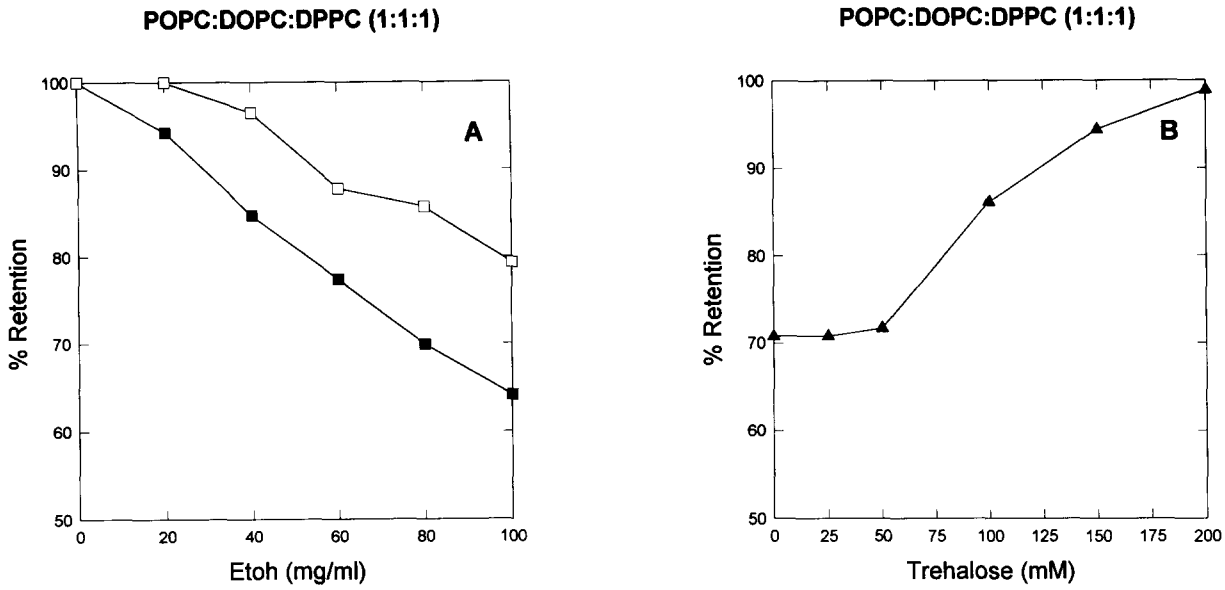

Fig. 7. Leakage of carboxyfluorescein from a mixture of POPC/DOPC/DPPC (1:1:1). (A) Increasing ethanol concentration, with (□) and without (■) addition of 100 mM trehalose. (B) Constant ethanol concentration at 2 M, with increasing trehalose concentration (▲).

Acknowledgements

The authors would like to thank Dr. David W. Deamer for helpful discussion and Dr Ann Oliver for her critical reading of the manuscript. The authors would also like to thank Samuel B. Leslie for help in solving some computation problems. This work was supported by grant from CNPq (Brazil) to J.J.C.M. and A.D.P. and by grant IBN-9308581 from National Science Foundation (USA) to J.H.C. and L.M.C.

References

[1] D'Amore, T., Panchal, C.J., Russel, I. and Stewart, G.G. (1990) CRC Crit. Rev. Biotechnol. 9, 287–304.
[2] Beaven, M.J., Charpentier, C. and Rose, A.H. (1982) J. Gen. Microbiol. 128, 1447–1455.
[3] Casey, G.P. and Ingledew, W.M. (1986) CRC Crit. Rev. Microbiol. 13, 219–280.
[4] Rose, A.H. (1980) Soc. Appl. Bacteriol. 9, 103–113.
[5] Ingram, L.O. and Buttke, T.M. (1984) Adv. Microbiol. Physiol. 25, 253–300.
[6] Thomas, D.S and Rose, A.H. (1979) Arch. Microbiol. 122, 49–55.
[7] Thomas, D.S., Hossack, J.A. and Rose, A.H. (1978) Arch. Microbiol. 117, 239–245.
[8] Van Uden, N. (1985) Annu. Rep. Ferment. Processes 8, 11–58.
[9] Buttke, T.M. and Ingram, L.O. (1980) Arch. Biochem. Biophys. 203, 565–571.
[10] Ghareib, M., Youssef, K.A. and Khalil, A.A. (1988) Folia Microbiol. 33, 447–452.
[11] Nambi, P., Rowe, E.S. and McIntosh, T.J. (1988) Biochemistry 27, 9175–9182.
[12] Rowe, E.S. (1983) Biochemistry 22, 3299–3305.
[13] Rowe, E.S. (1985) Biochim. Biophys. Acta 813, 321–330.
[14] Simon, S.A. and Mantsch, H.H. (1984) Biochim. Biophys. Acta 773, 169–172.
[15] Rowe, E.S. (1987) Biochemistry 26, 46–51.
[16] Lillie, S.H. and Pringle, J.R. (1980) J. Bacteriol. 143, 1384–1394.
[17] Thevelein, J.M. (1984) Microbiol. Rev. 48, 42–59.
[18] Panek, A.C., Bernardes, E. and Panek, A.D. (1986) in Membranes, Metabolism and Dry Organisms (Leopold, A.C., ed.), pp. 123–142, Cornell University Press, New York.
[19] Hottiger, T., Boller, T. and Wiemken, A. (1987) FEBS Lett. 220, 113–115.
[20] Hottiger, T., Boller, T. and Wiemken, A. (1989) FEBS Lett. 255, 431–434.
[21] Van Laere, A. (1989) FEMS Microbiol. Rev. 63, 201–210.
[22] Wiemken, A. (1990) Antonie van Leeuwenhoek 58, 209–217.
[23] Panek, A.C., Mansure, J.J., Paschoalin, V.M.F. and Panek, A.D. (1991) Biochimie 72, 77–79.
[24] Coutinho, C.C., Bernades, E., Felix, D. and Panek, A.D. (1988) J. Bacteriol. 7, 23–32.
[25] Gadd, G.M., Chalmers, K. and Reed, R.H. (1987) FEMS Microbiol. Lett. 48, 249–254.
[26] Panek, A.D. (1993) Cryobiology 30, 238–239.
[27] Attfield, P.V. (1987) FEBS Lett. 225, 259–263.
[28] Odumeru, J.A., D'Amore, T., Russel, I. and Stewart, G.G. (1993) J. Ind. Microbiol. 11, 113–119.
[29] Crowe, J.H, Crowe, L.M. and Jackson, S.A. (1983) Arch. Biochem. Biophys. 220, 477–484.
[30] Crowe, J.H., Crowe, L.M. and Chapman, D. (1984) Arch. Biochem. Biophys. 232, 400–407.
[31] Crowe, J.H., Crowe, L.M. and Chapman, D. (1984) Science 233, 701–703.
[32] Crowe, L.M., Crowe, J.H., Rudolph, A., Womersley, C. and Appel, L. (1985) Arch. Biochem. Biophys. 242, 240–247.
[33] Crowe, J.H., Spargo, B. and Crowe, L.M. (1987) Proc. Natl. Acad. Sci. USA 84, 1537–1540.
[34] Crowe, J.H., Hoekstra, F.A. and Crowe, L.M. (1992) Annu. Rev. Physiology 54, 579–599.
[35] Panek, A.C., Francois, J. and Panek, A.D. (1988) Curr. Genet. 13, 15–20.

[36] Paschoalin, V.M.F., Silva, J.T. and Panek, A.D. (1989) Curr. Genet. 16, 81–87.
[37] Eleutherio, E.C.A., Araujo, P.S. and Panek, A.D. (1993) Biochim. Biophys. Acta 1156, 263–266.
[38] Oliveira, D.E., Rodrigues, E.G.C., Matton, J.R. and Panek, A.D. (1981) Curr. Genet. 3, 235–242.
[39] MacDonald, R.C., MacDonald, R.I., Menco, B.Ph.M., Takeshita, K., Subbarao, N.K. and Hu, L. (1981). Biochim. Biophys. Acta 1061, 297–303.
[40] Hottiger, T., Schmutz, P. and Wiemken, A. (1987) J. Bacteriol. 169, 5518–5522.
[41] Hottiger, T., De Virgilio, C., Bell, W., Boller,T. and Wiemken, A. (1992) Eur. J. Biochem. 210, 125–132.
[42] Panek, A.D., Ferreira, R. and Panek, A.C. (1989) Biochimie 71, 313–318.
[43] Neves, M.J. and Francois, J. (1992) Biochem. J. 288, 859–864.
[44] Panek, A.D. and Panek, A.C. (1990) J. Biotechnol. 14, 229–238.
[45] D'Amore, T., Crumplen, R. and Stewart, G.G. (1991) J. Ind. Microbiol. 7, 191–196.
[46] Pascual, C., Alonso, A., Garcia, I., Romey, C. and Kotyk, A. (1988) Biotechnol. Bioeng. 32, 374–378.
[47] Cartwright, C.P., Juroszek, J.K., Beavan, M.J., Ruby, F.M.S., De Morais, S.M.F. and Rose, A.H. (1986) J. Gen. Microbiol. 132, 369–377.
[48] Operti, M.S., Oliveira, D.E., Freitas-Valle, A.B., Oestreicher, E.G., Mattoon, J.R. and Panek, A.D. (1982) Curr. Genet. 5, 69–76.
[49] Ratledge, C. and Evans, C.T (1989) in The Yeast (Rose, A.H. and Harrison, J.S., eds.), Vol. 3, pp. 385–455, Academic Press, London.
[50] Cottrell, M., Viljoen, B.C., Kock, J.L.F. and Lategan, P.M (1986) J. Gen. Microbiol. 132, 2401–2403.
[51] Mishra, P. and Kaur, S. (1991) Appl. Microbiol. Biotechnol. 34, 697–702.
[52] Ingram, L.O. (1976) J. Bacteriol. 125, 670–678.
[53] Grego, J. and Sajbidor, J (1992) FEMS Microbiol. Lett. 93, 13–16.