Ethanol Dissipates the Proton-motive Force across the Plasma Membrane of Saccharomyces cerevisiae

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Populations of Saccharomyces cerevisiae NCYC 431, harvested after 16 h incubation from self-induced anaerobic cultures, were more tolerant to the inhibitory effect of ethanol on fermentation rate and viability than organisms harvested from 8 h cultures. Ethanol increased the rate of passive influx of protons into de-energized organisms at a rate which was greater with organisms from 8 h compared with 16 h cultures. Rates of passive influx of protons into spheroplasts were significantly greater than into intact organisms, although culture age did not affect rates of ethanol-induced influx of protons into spheroplasts. Ethanol retarded both the initial net rate of proton efflux and the final extent of acidification produced by suspensions of energized organisms, both effects being more pronounced with organisms from 8 h as compared with 16 h cultures. The magnitude of the proton-motive force (Δp) was decreased by ethanol in both energized and de-energized organisms. Although culture age did not affect the extent of ethanol-induced decrease in Δp in de-energized organisms, in energized organisms harvested from 8 h cultures ethanol produced a significantly greater decrease in Δp as compared with organisms from 16 h cultures. If the ability of ethanol to decrease the Δp value is important in its inhibitory effect on growth, it is suggested that some phenomenon other than proton uncoupling is involved.

INTRODUCTION

Although Saccharomyces cerevisiae is on the whole more tolerant of ethanol than other yeasts, concentrations of this alkanol approaching 2 M are inhibitory to even the most tolerant strains (Rose, 1980). Membranes, and in particular the plasma membrane, are considered to be prime targets for ethanol inhibition in S. cerevisiae (Ingram & Buttke, 1984). Evidence that plasma-membrane lipids are targets for the interaction of ethanol with S. cerevisiae came from Thomas and her colleagues (Thomas et al., 1978; Thomas & Rose, 1979), who showed that the inhibitory effect of ethanol on viability of, and solute accumulation by, S. cerevisiae was less marked when membranes were enriched in linoleyl as compared with oleyl residues. Accumulation of amino acids (Thomas & Rose, 1979; Leão & van Uden, 1984a), ammonium ions (Leão & van Uden, 1983), maltose (Loureiro-Dias & Peinado, 1982) and glucose (Thomas & Rose, 1979; Leão & van Uden, 1982) is non-competitively inhibited by ethanol. With the exception of the glucose-transport system, which involves facilitated diffusion (Eddy, 1982), all of these transport

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Abbreviations: TPP⁺, tetraphenylphosphonium ion; Δp, proton-motive force.

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systems are active and therefore probably require a proton-motive force (Δp; Borst-Pauwels, 1981; Serrano, 1977; Seastone et al., 1976). One possibility therefore is that ethanol may affect the formation and maintenance of Δp across the plasma membrane. Evidence to support this notion has recently come from Leão & van Uden (1984b) who reported that ethanol and other alkanols accelerated passive re-entry of protons into S. cerevisiae in a manner resembling the action of a proton-uncoupling agent. The present paper describes dissipation by ethanol of the Δp across the plasma membrane of S. cerevisiae, the magnitude of the dissipation depending on the age of the batch culture from which organisms are harvested.

METHODS

Organisms. The two strains of S. cerevisiae used in this study, NCYC 431 (Beavan et al., 1982) and Y185 (Caldenberg et al., 1984), were maintained on slopes of malt extract/yeast extract/glucose/mycological peptone (MYGP) medium (Wickerham, 1951) solidified with 2% (w/v) agar.

Experimental cultures. The majority of experiments were done with organisms from self-induced anaerobic cultures of S. cerevisiae NCYC 431 grown as described by Beavan et al. (1982). Growth was followed by measuring optical density at 600 nm, measurements being related to dry weight of organisms by a standard curve. Organisms were harvested from cultures by centrifugation (3500 g; 5 min; 4 °C) after approximately 8 h (0.28 mg dry wt ml⁻¹) or 16 h incubation (2.4 mg dry wt ml⁻¹), and washed twice with water. S. cerevisiae Y185 was grown under strict anaerobic conditions in a medium supplemented, as indicated in the text, with a sterol (5 mg l⁻¹) and an unsaturated fatty acid (30 mg l⁻¹) (Alterthum & Rose, 1973). Growth was followed as described previously. Organisms were harvested and washed as described for strain NCYC 431.

Preparation of spheroplasts. Harvested organisms were washed first in water, and then were washed once in buffered sorbitol (20 mM-Tris containing 10 mM-MgCl₂ and 1.2 M-sorbitol, adjusted to pH 7.2) and resuspended in the same solution to 10 mg dry wt ml⁻¹. After supplementation with Zymolyase 60 000 (0.1 mg (6 mg dry wt organisms)⁻¹), the suspension was incubated at 30 °C with reciprocal shaking (120 r.p.m.). After incubation for 1 h, a check that formation of spheroplasts was complete was made by diluting 0.1 ml portions of the suspension into 2.9 ml of either 1.2 M-sorbitol or water and measuring optical density at 600 nm. Spheroplasts were harvested by centrifugation (1500 g for 3 min) and resuspended in 1.2 M-sorbitol to 2.5-5.0 mg dry wt organisms equiv. ml⁻¹.

Measurements of rates of proton flux. To measure rates of passive influx of protons, a suspension (50 ml; 2.5-5.0 mg dry wt ml⁻¹) of organisms in water was placed in a 100 ml round-bottomed flask fitted with two ports (1.5 cm diam.), in addition to a Suba-seal port and a short gas-entry port (0.5 cm diam.). The flask was maintained in a water bath at 30 °C and the suspension stirred with a magnetic flea. Changes in pH value in the suspension were measured by two combination pH electrodes (type CMWL; Russell pH Ltd, Auchtermuchty, Fife, UK) inserted through ports. One was connected to a digital read-out pH meter and the other through a second pH meter measuring the rise in pH value of the suspension for a period of approx. 5 min. Rates of passive influx, ethanol (95%, v/v) was added to the suspension, after the pH had been lowered to 4.0, to give final concentrations of 0.5, 1.0, 1.5 or 2.0 M, and changes in extracellular pH value followed over a 5 min period. Rates of passive influx of protons are quoted as nequiv. H⁺ (mg dry wt⁻¹ min⁻¹). Rates of proton influx into spheroplasts of S. cerevisiae NCYC 431, suspended in 1.2 M-sorbitol, were measured as described for intact organisms except that the suspension was supplemented with ethanol to a maximum concentration of 1.0 M; supplementation with higher concentrations caused spheroplast lysis. After a proton influx rate had been measured on a suspension, the number of spheroplasts present was determined using a haemocytometer. Rates of passive influx of protons into spheroplasts are quoted as nequiv. H⁺ (mg dry wt equiv.⁻¹ min⁻¹). With organisms from 8 h cultures, 1 mg dry wt was equivalent to 3.0 × 10⁸ organisms and with those from 16 h cultures 3.9 × 10⁹ organisms. Passive influx of protons into S. cerevisiae Y185 was followed in a manner similar to that described for S. cerevisiae NCYC 431 except that the headspace in the flask was flushed continuously throughout the experiment with oxygen-free nitrogen gas (Alterthum & Rose, 1973) fed through the gas port.

Glucose-stimulated proton efflux from energized S. cerevisiae NCYC 431 was followed as already described for proton influx, except that 2-deoxy-D-glucose was not included and, after adjusting the suspension to pH 4.0, glucose (20 mM) was added before proton efflux was measured over a 5 min period. The effect of ethanol on glucose-induced proton efflux was measured by supplementing the suspension with 95% (v/v) ethanol after adjusting its pH value to 4.0, to give final concentrations of 0.5, 1.0, 1.5 or 2.0 M, before addition of glucose (20 mM) and then following proton flux over a 5 min period.
Ethanol and proton-motive force in yeast

Measurement of intracellular pH values of organisms. Intracellular pH values of energized and de-energized organisms were calculated by determining the equilibrium distribution of a weak acid across the plasma membrane (Conway & Downey, 1950; Seaston et al., 1976). A suspension of washed organisms (2.5–5.0 mg dry wt ml⁻¹) was incubated as already described for measuring rates of proton flux and, after the pH value had been adjusted to 4.0, incubation was continued for a further 5 min. A portion (1·8 ml) of the suspension was then removed, placed in a Bijou bottle and stirred at 30 °C. [2⁻¹⁴C]Propionic acid [sodium salt; 0·1 mm; 0·5 μCi ml⁻¹ (18·5 kBq ml⁻¹); 0·2 ml] was added to the suspension and, after 3 min equilibration, triplicate portions (0·3 ml) were rapidly removed and filtered through membrane filters (0·45 μm pore size; 25 mm diam.; Millipore). Organisms on filters were washed with 4 × 1 ml 0·1 mm-propionic acid (4 °C), the filters with organisms transferred to scintillation vials containing 7·5 ml Cocktail T (BDH) and the radioactivity of the contents measured in a LKB Rackbeta liquid scintillation spectrometer (model 1217). When measuring the effect of ethanol on the intracellular pH values of organisms, ethanol (95%, v/v) was added to the suspension after the pH value had been lowered to 4.0, to give a final ethanol concentration of 0·5, 1·0, 1·5 or 2·0 m. In experiments with energized organisms, glucose (20 mM) was incorporated in the suspension 30 s after addition of ethanol. Intracellular pH values were calculated from the expression derived by Waddell & Butler (1959):

\[
pH = pK + \log \left[ \frac{R(10^{pH} - pK)}{10^{pH} + 1} \right]
\]

where \( R = TA; V_i; TA_e; V_i; pK_i; pK_e \), respectively, the internal and external pH values, \( TA_i \) and \( TA_e \), the internal and external total amounts of propionic acid, \( V_i \) and \( V_e \), the intracellular and extracellular water volumes, and \( pK_i \) and \( pK_e \), the dissociation constants for propionic acid in the internal and external environments. Intracellular volumes were calculated from the dry weight of organisms used assuming that, for organisms from 8 h cultures, the cell volume was 1·93 μl (mg dry wt)⁻¹ and for organisms from 16 h cultures 1·61 μl (mg dry wt)⁻¹ (Beavan et al., 1982). The internal and external dissociation constants for propionic acid were calculated from the Davies' simplified version of the Debye–Hückel equations (Davies, 1962), assuming that the ionic strength within organisms was in the region 0·15–0·25 (Conway & Downey, 1950). Values for \( pK_i \) and \( pK_e \) were calculated to be 4·68 and 4·75, respectively.

Measurement of the plasma-membrane potential (\( \Delta \psi \)) of organisms. This value was derived from the equilibrium distribution of tetra[¹³H]phenylphosphonium (TPP⁺) across the yeast plasma membrane (Hauer & Höfer, 1978). Suspensions of energized and de-energized organisms (2·5–5·0 mg dry wt ml⁻¹), prepared as already described, were incubated at pH 4·0 for 5 min. A portion (1·8 ml) was then supplemented with 0·2 ml TPP⁺ solution [200 μM; 0·5 μCi ml⁻¹ (1·8·5 kBq ml⁻¹)] and, after equilibration for 25 min, triplicate samples (0·3 ml) were taken and rapidly filtered through prewashed (10 ml; 10 mM-TPP⁺; 4 °C) membrane filters (0·45 μm pore size; 25 mm diam.; Millipore). Organisms on filters were washed four times with 2 ml portions of 10 mM-TPP⁺ at 4 °C after which filters and organisms were transferred to scintillation vials containing 7·5 ml Optiphase Safe (Fisons) and radioactivity was measured in a liquid scintillation spectrometer (LKB Rackbeta, model 1217). When measuring the effect of ethanol on \( \Delta \psi \), suspensions after adjustment to pH 4·0 were supplemented with 95% (v/v) ethanol to give the stated concentration. To determine the extent of TPP⁺ binding to the yeast wall and plasma membrane, 2,4-DNP (1 mM; Eilam, 1984) was added at the same time as radioactive TPP⁺ and the incubation and filtration procedures were done as already described. \( \Delta \psi \) in mV was calculated using the equation:

\[
\Delta \psi = -2·3 \frac{RT}{F} \log \left[ \frac{[\text{TPP}^+_{\text{inside}}]}{[\text{TPP}^+_{\text{outside}}]} \right]
\]

Recent evidence suggests that under certain conditions a positive \( \Delta \psi \) may exist across the yeast plasma membrane, indeed by accumulation of thiocyanate ions (SCN⁻; Höfer & Künemund, 1984). Suspensions (50 ml; 2·5–5·0 mg ml⁻¹) of energized or de-energized organisms from 8 h or 16 h cultures were incubated for 5 min at pH 4·0 with 2·0 mM-ethanol. A sample (9 ml) of the suspension was then removed and added to KS¹⁴CN [1 ml; 50 μM; 2·5 μCi ml⁻¹ (92·5 kBq ml⁻¹)]. At 10 min intervals up to 50 min, 0·5 ml samples were removed, filtered through washed (5 mm-KSCN; 10 ml) membrane filters (25 mm diam.; 0·45 μm pore size; Millipore), washed with KSCN (4 × 2 ml; 5 μM) and organisms measured as described for TPP⁺ accumulation. To indicate the extent of non-specific SCN⁻ binding, de-energized organisms were incubated in the absence of ethanol and filtered as already described. Since, under these conditions, S. cerevisiaeNCYC 431 has a negative \( \Delta \psi \), no SCN⁻ accumulation should occur.

Viability measurements. Viability of yeast populations was measured by staining with methylene blue (Fink & Kühles, 1933). A portion (0·5 ml) of culture was mixed with an equal volume of methylene blue solution (0·01%, w/v, sodium citrate). After 5 min incubation at room temperature, the numbers of live and dead cells were established in a population of at least 500 organisms.

Measurement of fermentation rate. Harvested organisms were washed in citrate buffer (100 mM; pH 4·5) and suspended in the same buffer to 1·0 mg dry wt ml⁻¹. The fermentative activity of organisms was measured by...
Conventional Warburg manometry (Umbreit et al., 1964). Each flask contained 1.5 ml citrate buffer (100 mM; pH 4.5) containing 300 mM-D-glucose and, where indicated, 1.0 mM-ethanol. A suspension containing 1.0 mg dry wt organisms was placed in the side arm. The well contained 20% (w/v) KOH solution or water.

RESULTS

Effect of culture age on the response of organisms to ethanol

Ethanol (1.0 M) completely inhibited fermentation of glucose by organisms from 8 h cultures. The same concentration caused only a 12% decrease in the fermentative activity of organisms from 16 h cultures. Ethanol, up to 3.0 M, also caused a greater loss of viability in populations of organisms from 8 h cultures as compared with those from 16 h cultures (Table 1).

Effect of culture age and ethanol on proton fluxes

Ethanol, up to 2.0 M, accelerated passive influx of protons into de-energized organisms, as reported by Leão & van Uden (1984b). However, ethanol-induced acceleration of proton influx was greater with organisms from 8 h cultures compared with 16 h cultures (Fig. 1). With organisms from 8 h cultures, the ethanol-induced increase in rate of proton influx was 11.6 nequiv. H⁺ (mg dry wt organisms⁻¹ min⁻¹ (ethanol concn; M)⁻¹, while with organisms from 16 h cultures the increase in rate was 6.8 nequiv. H⁺ (mg dry wt organisms⁻¹ min⁻¹ (ethanol concn; M)⁻¹. Proton influx was considerably accelerated when spheroplasts were used instead of intact organisms, but the influx rate was only slightly greater with spheroplasts from organisms from 8 h compared with 16 h cultures (Fig. 2). The ethanol-induced increase in the rate of passive influx of protons into spheroplasts from organisms from 8 h cultures was 412.0 nequiv. H⁺ (mg dry wt organisms equiv.)⁻¹ min⁻¹ (ethanol concn; M)⁻¹, while that with spheroplasts from organisms from 16 h cultures was 365.8 nequiv. H⁺ (mg dry wt equiv.)⁻¹ min⁻¹ (ethanol concn; M)⁻¹.

The extent of extracellular acidification of suspensions of energized organisms was decreased in the presence of ethanol, less so with organisms from 16 h compared with 8 h cultures (Fig. 3). Ethanol-induced decrease in the initial net rate of proton efflux, calculated from the linear portions of the acidification curves (measured over the period approximately 0.5–2.0 min), was greater with organisms from 8 h compared with 16 h cultures. Comparable measurements were not possible with spheroplasts since their incubation in glucose-containing buffer resulted in lysis.

Effect of plasma-membrane lipid composition on ethanol-induced passive influx of protons into de-energized organisms

Ethanol, up to 1.5 M, caused a virtually identical acceleration of proton influx into de-energized S. cerevisiae Y185 with plasma membranes enriched in ergosterol and either oleyl or linoleyl residues. This strain of S. cerevisiae was used because it is known to incorporate into membranes high proportions of exogenously supplied sterol and unsaturated fatty acids when grown under anaerobic conditions (Calderbank et al., 1984). Higher concentrations of ethanol

Table 1. Effect of ethanol on the viability of suspensions of S. cerevisiae NCYC 431 harvested from 8 h and 16 h cultures

<table>
<thead>
<tr>
<th>Age of culture (h)</th>
<th>Viability (% of zero-time value, ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>8</td>
<td>98.0 ± 2.0</td>
</tr>
<tr>
<td>16</td>
<td>99.4 ± 1.3</td>
</tr>
</tbody>
</table>
Fig. 1. Effect of ethanol on influx of protons into de-energized \textit{S. cerevisiae} NCYC 431 harvested from 8 h cultures (O) and 16 h cultures (●). Vertical bars indicate SE.

Fig. 2. Effect of ethanol on influx of protons into spheroplasts from de-energized \textit{S. cerevisiae} NCYC 431 harvested from 8 h cultures (O) and 16 h cultures (●). Vertical bars indicate SE.

Fig. 3. Time-course of acidification of suspensions of energized \textit{S. cerevisiae} NCYC 431 harvested from 8 h cultures (a) and 16 h cultures (b). The data are representative of those obtained in five separate experiments.

caused a decrease in viability in suspensions, particularly of organisms enriched in linoleyl residues. Values for rates of proton influx were similar to those obtained with \textit{S. cerevisiae} NCYC 431 from 8 h self-induced anaerobic cultures (Fig. 1). Nor were differences detected in rates of passive influx of protons into \textit{S. cerevisiae} Y185 with plasma membranes enriched in oleyl residues and either ergosterol or cholesterol.
Table 2. Effect of ethanol on intracellular and extracellular pH values of suspensions of de-energized S. cerevisiae NCYC 431

<table>
<thead>
<tr>
<th>Ethanol concn (M)</th>
<th>Extracellular pH value</th>
<th>Intracellular pH value</th>
<th>ΔpH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>4.68 ± 0.12</td>
<td>5.85 ± 0.11</td>
<td>1.17</td>
</tr>
<tr>
<td>0.5</td>
<td>4.73 ± 0.09</td>
<td>5.75 ± 0.06</td>
<td>1.02</td>
</tr>
<tr>
<td>1.0</td>
<td>4.81 ± 0.15</td>
<td>5.71 ± 0.06</td>
<td>0.90</td>
</tr>
<tr>
<td>1.5</td>
<td>4.82 ± 0.22</td>
<td>5.63 ± 0.08</td>
<td>0.81</td>
</tr>
<tr>
<td>2.0</td>
<td>4.85 ± 0.19</td>
<td>5.56 ± 0.12</td>
<td>0.71</td>
</tr>
</tbody>
</table>

pH Values are quoted ± SE.

Table 3. Effect of ethanol on intracellular and extracellular pH values of suspensions of energized S. cerevisiae NCYC 431

<table>
<thead>
<tr>
<th>Ethanol concn (M)</th>
<th>Extracellular pH value</th>
<th>Intracellular pH value</th>
<th>ΔpH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3.55 ± 0.16</td>
<td>6.36 ± 0.14</td>
<td>2.81</td>
</tr>
<tr>
<td>0.5</td>
<td>3.66 ± 0.23</td>
<td>6.31 ± 0.14</td>
<td>2.65</td>
</tr>
<tr>
<td>1.0</td>
<td>3.93 ± 0.18</td>
<td>6.22 ± 0.44</td>
<td>2.29</td>
</tr>
<tr>
<td>1.5</td>
<td>4.14 ± 0.11</td>
<td>6.10 ± 0.08</td>
<td>1.96</td>
</tr>
<tr>
<td>2.0</td>
<td>4.20 ± 0.34</td>
<td>5.97 ± 0.12</td>
<td>1.77</td>
</tr>
</tbody>
</table>

pH Values are quoted ± SE.

Table 4. Effect of ethanol on Δψ values of suspensions of de-energized S. cerevisiae NCYC 431

Values quoted are means of five separate determinations, ± SE. ND, Δψ value not detectable.

<table>
<thead>
<tr>
<th>Ethanol concn (M)</th>
<th>Δψ Value (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 h cultures</td>
</tr>
<tr>
<td>0.0</td>
<td>-44 ± 8</td>
</tr>
<tr>
<td>0.5</td>
<td>-19 ± 12</td>
</tr>
<tr>
<td>1.0</td>
<td>ND</td>
</tr>
<tr>
<td>1.5</td>
<td>ND</td>
</tr>
<tr>
<td>2.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

Effect of culture age and ethanol on values for ΔpH, Δψ and Δρ

Supplementing suspensions of organisms with ethanol, up to 2·0 M, caused a decrease in the intracellular pH value which, coupled with the simultaneous increase in extracellular pH value, caused a decrease in ΔpH. With de-energized organisms, the decrease in ΔpH was approximately the same in organisms from 8 h and 16 h cultures (Table 2) but, with energized organisms, was proportionately greater with organisms from 8 h compared with 16 h cultures (Table 3). Ethanol supplementation also decreased the magnitude of Δψ in de-energized organisms, 1·0 M-ethanol being sufficient to abolish Δψ in organisms from either 8 h or 16 h cultures (Table 4). However, even in the presence of 2·0 M-ethanol, SCN⁻ ions were not accumulated. The effect of ethanol on values for ΔpH and Δψ lowered the values for Δρ [ = -2·3 (RT/F)·ΔpH + Δψ; Table 5]. The ethanol-induced decrease in Δρ was about the same for de-energized organisms from 8 h and 16 h cultures, but for energized organisms was greater for those from 8 h compared with 16 h cultures.
Table 5. Effect of culture age and ethanol on the $\Delta p$ values in de-energized and energized S. cerevisiae NCYC 431

<table>
<thead>
<tr>
<th>Ethanol concn (M)</th>
<th>8 h cultures</th>
<th>16 h cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>De-energized</td>
<td>Energized</td>
</tr>
<tr>
<td>0.0</td>
<td>-113</td>
<td>-165</td>
</tr>
<tr>
<td>0.5</td>
<td>-79</td>
<td>-155</td>
</tr>
<tr>
<td>1.0</td>
<td>-53</td>
<td>-134</td>
</tr>
<tr>
<td>1.5</td>
<td>-48</td>
<td>-115</td>
</tr>
<tr>
<td>2.0</td>
<td>-42</td>
<td>-104</td>
</tr>
</tbody>
</table>

DISCUSSION

An increase in the rate of passive influx of protons following addition of ethanol to de-energized organisms confirms the report by Leão & van Uden (1984b) that this alkanol dissipates proton gradients. However, it is worth noting that rates of passive influx of protons, in the presence or absence of ethanol, were greater than those reported by Leão and van Uden (1984b). The considerable increase in the rate of proton influx when spheroplasts rather than intact organisms were examined demonstrated the large proton-buffering capacity of the yeast wall. Since the rate of influx was very similar with spheroplasts from organisms from 8 h and 16 h cultures, it would appear that the different rates observed with intact organisms can be attributed to the greater capacity of the wall of organisms from 16 h cultures to buffer protons.

Leão & van Uden (1984b), when describing ethanol-induced passive influx of protons into S. cerevisiae, also reported on the effect of other alkanols. They showed that the exponential enhancement constants for different alkanols increased with the lipid solubility of the alkanol, and this led them to suggest that hydrophobic membrane regions, possibly lipid regions, are target sites for the inhibitory effect of ethanol. Our finding that enrichment of the plasma membrane in S. cerevisiae Y185 with linoleyl as compared with oleyl residues, or with cholesterol rather than ergosterol, did not affect ethanol-induced passive influx of protons into organisms does not eliminate plasma-membrane lipids as targets for the cause of this influx. It is conceivable that lipid properties other than unsaturation or the magnitude of the sterol-phospholipid interaction, which would differ in ergosterol- as compared with cholesterol-enriched membranes, are involved in the interaction of ethanol with yeast plasma membranes.

The rapid extracellular acidification produced by energized S. cerevisiae NCYC 431 is indicative of a net proton efflux, presumably caused by the activity of a Mg$^{2+}$-dependent proton-translocating ATPase in the plasma membrane (Willsky, 1979; Malpartida & Serrano, 1981). The acidification curves obtained were similar in shape to those obtained with other strains of S. cerevisiae in unbuffered suspensions (Sigler et al., 1981; Leão & van Uden, 1984b). Both the increase in final extracellular pH value and the decrease in initial net proton efflux rate observed following addition of ethanol to suspensions were greater with organisms from 8 h as compared with 16 h cultures. Leão & van Uden (1984b) suggested that the increase in the final extracellular pH value was attributable to the ethanol-induced enhancement of the rate of passive influx of protons. It is possible, however, that other factors are involved in both the ethanol-mediated increase in the final extracellular pH value and the ability of the alcohol to decrease the initial net proton efflux rate. One obvious possibility is that ethanol inhibits the activity of the plasma-membrane ATPase thereby retarding proton extrusion, and this effect augmented by the ethanol-induced increase in passive influx of protons could contribute to the observed decrease in acidification. It was unfortunate that, because of the instability of spheroplasts in glucose-containing buffer, the importance of the cell wall under energized conditions could not be assessed.

With de-energized organisms, ethanol caused a progressive decrease in both components which contribute to the magnitude of $\Delta p$, i.e. $\Delta pH$ and $\Delta \psi$. This strongly suggests that the proton-uncoupling action of ethanol is alone sufficient to dissipate $\Delta p$. However, there was no
significant difference between de-energized organisms from 8 h and 16 h cultures with respect to the effect of ethanol on $\Delta p$, supporting the idea that the extent of proton uncoupling in organisms from 8 h and 16 h cultures is very similar. We conclude that, if maintenance of a $\Delta p$ value is important for $S. cerevisiae$ to tolerate ethanol, then the uncoupling effect of ethanol on the proton gradient is not the determining parameter.

Assuming that the extent of proton uncoupling induced by ethanol is the same for energized and de-energized organisms (Leão & van Uden, 1984b), then the notion that factors other than simple uncoupling are involved in the effect of ethanol on $\Delta p$ is supported by data obtained with energized organisms. Our inability to measure a negative $\Delta\psi$ value under these conditions, i.e. with an extracellular pH value of 4.0 or below, is in agreement with previous reports (Hauer et al., 1981). Höfer & Küнемund (1984) showed that a positive $\Delta\psi$ value could exist in $Rhodotorula glutinis$. Our inability to demonstrate accumulation of SCN$^-$ ions under conditions where a negative $\Delta\psi$ value could not be measured suggests that either the plasma membrane of $S. cerevisiae$ is impermeable to these ions or a positive $\Delta\psi$ cannot exist in this organism. It is of interest that the intracellular pH value in energized organisms was significantly higher than the value for de-energized organisms, which suggests that the activity of the plasma-membrane ATPase may be involved in regulation of intracellular pH (Sanders et al., 1981). Since the inhibitory effect of ethanol on $\Delta p$ differed in energized organisms from 8 h compared with 16 h cultures, it is possible that ethanol has a greater inhibitory effect on the plasma-membrane ATPase of organisms from 8 h rather than 16 h cultures. Finally, there is the need to comment on the physiological significance to $S. cerevisiae$ of an inability to maintain a constant $\Delta p$ when energized and in the presence of ethanol. There is considerable evidence that a $\Delta p$ across the plasma membrane is required for energization of solute transport into $S. cerevisiae$ (Eddy, 1982).

If 1.5 M ethanol is introduced into cultures of $S. cerevisiae$ NCYC 431 after 8 h incubation under self-induced anaerobic conditions, growth ceases immediately (Beavan et al., 1982). We have demonstrated that this ethanol concentration is sufficient also to cause an appreciable decrease in $\Delta p$ in energized organisms from 8 h cultures. It is conceivable, therefore, that this cessation of growth is a result of an inhibition of solute accumulation.

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