Biochimica et Biophysica Acta, 774 (1984) 43–48
Elsevier

BBA 72152

EFFECTS OF ETHANOL AND OTHER ALKANOLS ON PASSIVE PROTON INFLUX IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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(Received December 9th, 1983)

Key words: Alkanol effect; Proton diffusion; Acidification; Transport uncoupling; (Kinetics)

Ethanol, isopropanol, propanol and butanol enhanced the passive influx of protons into deenergized cells of *Saccharomyces cerevisiae*. The influx followed first-order kinetics with a rate constant that increased exponentially with the alkanol concentration. The exponential enhancement constants increased with the lipid solubility of the alkanols, which indicated hydrophobic membrane regions as the target sites. While the enhancement constants were independent of pH over the range tested (3.3–5.0), the rate constants decreased linearly with increasing extracellular proton concentration, indicating the presence of an additional surface barrier against proton penetration, the effectiveness of which increased with protonation. The alkanols affected the acidification curves of energized yeast suspensions in such a way that the final pH values were linear functions of the alkanol concentrations. These results were consistent with a balance between active and passive proton movements at the final pH, the exponential enhancement constants calculated from the slopes being nearly identical with those obtained with deenergized cells. It was concluded that passive proton influx contributes to the kinetics of acidification in *S. cerevisiae* and that uncoupling contributes to the overall kinetics of alkanol-inhibited secondary active transport across the yeast plasma membrane.

Introduction

Ethanol inhibited the uptake by *Saccharomyces cerevisiae* of glucose and other nutrients. The inhibitory effect could be modulated by changing the lipid composition of the plasma membrane, which indicates interference of ethanol with membrane transport [1,2]. In the transport systems of *S. cerevisiae* that have so far been tested, the glucose system [3], the maltose system [4], the ammonium system [5] and the general amino acid permease [6], ethanol and other alkanols inhibited transport in a noncompetitive exponential way. The exponential inhibition constants were positively correlated with the lipid/buffer partition coefficients of the alkanols, which indicated hydrophobic membrane sites as the primary targets of alkanol inhibition of transport.

In the case of the glucose transport system, which in *S. cerevisiae* is electroneutral (for a discussion see Ref. [7]), the inhibitory effects of alkanols may be explained exclusively by interference with the carrier, either directly or by changing its lipid environment in the membrane. In the case of ammonium transport, which in all likelihood is an electrogenic uniport [8], possible effects of the alkanols on the membrane potential should also be taken into account, while in the case of maltose transport and the general amino acid permease, which in *S. cerevisiae* are electrogenic proton symports [9,10], possible effects of alkanols on the membrane potential as well as on the transmembrane proton gradient should be considered, in addition to alkanol interference with

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the carriers themselves. The membrane potential and the proton gradient may in principle be affected by alkanols either through inhibition of active proton extrusion or through enhancement of passive proton influx (uncoupling) or by a combination of the two mechanisms.

When cells of *S. cerevisiae* are suspended in water with glucose, protons are extruded and the pH drops to a final value following a characteristic acidification curve. Sigler et al. [11,12] identified a number of mechanisms that contributes to the kinetics of the acidification process including CO_2 production, extrusion of organic acids, buffering by surface polyelectrolytes and, above all, active proton efflux. Here we present evidence that passive proton influx contributes to the kinetics of the acidification process and that alkanols exponentially enhance the passive influx of protons in *S. cerevisiae* and thus, to some degree, must act as uncouplers of secondary electrogenic transport in addition to their more direct effects on the respective carriers.

Materials and Methods

S. cerevisiae IGC 3507, a respiration-deficient mutant, was grown with mechanical shaking at 25 °C in Erlenmayer flasks containing a liquid mineral medium with vitamins and 2% (w/v) glucose [13]. Cells were harvested in mid-exponential phase, centrifuged, washed with distilled water and resuspended in distilled water with a final concentration of 60 mg dry wt./ml.

Proton movements were measured at 26 °C with a standard pH meter PHM62 (Radiometer, Copenhagen) connected to a flat-bed recorder LKB 6500. The pH electrode was immersed in a water-jacketed cell of 10-ml capacity with magnetic stirring. To the cells were added 4.5 ml water and 0.5 ml yeast suspension and either glucose (final concentration 2%) or 2-deoxyglucose (final concentration 5 mM) for energizing or deenergizing the yeast cells, respectively. Alkanols were added to obtain the desired final concentrations.

Results and Discussion

Passive proton influx in deenergized yeast

In suspensions in water of yeast cells deenergized by the use of 2-deoxyglucose, the pH increased with time and alkanols increased the rate of the increase. After adjustment of the initial pH to the desired value within the range used in the experiments (3.3–5.0), the pH change was linear over a period of time and then ceased to be so, probably due to an increase in the intracellular proton concentration. The latter effect became more pronounced the higher the initial pH, i.e., the lower the initial proton gradient across the plasma membrane.

The time-dependence of the pH variation over the linear part was consistent with unidirectional diffusion kinetics:

$$-\frac{d[H]^+}{dt} = c[H]^+ \quad (1)$$

where $[H]^+$ is the extracellular proton concentration and c the apparent rate constant of proton influx applicable to the process under our experimental conditions. Integrating Eqn. 1 gives:

$$[H^+]_t = [H^+]_0 e^{-ct} \quad (2)$$

which leads to the following linear relation:

$$pH_t = pH_0 + c(\log_{10} e)t \quad (3)$$

where pH_t and pH_0 are the extracellular pH values after time t and at time zero, over the linear part of the pH trace.

Fig. 1 shows families of pH traces, linear with time, obtained at various initial pH values without added alkanols and in the presence of various concentrations of butanol. Similar results were obtained with ethanol, isopropanol and propanol.

Using Eqn. 3, estimates of rate constant, c, were obtained from the experimental pH traces. The estimates were corrected for pH buffering in the extracellular phase by applying a correction factor. The correction factor used was the amount of hydroxyl ions needed to increase the relevant pH value in the yeast suspension by 0.2 units divided by the amount needed in the same volume of water. The correction factors increased from unity between pH 3.3 and 3.8 to 12.5 at pH 5.0. The presence of alkanols at the concentrations used in the experiments did not affect the correction factors. Fig. 1 (inset) shows semilog plots of the

corrected estimates against the corresponding butanol concentrations. The semilog plots were essentially linear. The other alkanols gave similar linear semilog plots with a different slope for each alkanol (Fig. 2). We may thus write:

$$\ln c_x = \ln c_0 + kx \tag{4}$$

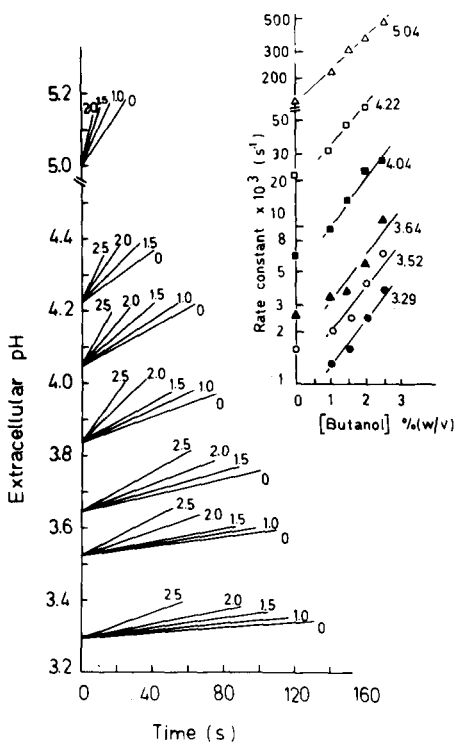
and

$$c_x = c_0 e^{kx} \tag{5}$$

where c_x and c_0 are the values of the rate constants at concentration x and 0 of the respective alkanol, and k is an exponential constant that expresses the enhancement of proton influx by the alkanol. The following values for k were obtained: ethanol 0.55 $l \cdot mol^{-1}$ (S.D. 0.085), isopropanol 0.82 $l \cdot mol^{-1}$ (S.D. 0.10), propanol 1.38 $l \cdot mol^{-1}$ (S.D. 0.06) and butanol 5.4 $l \cdot mol^{-1}$ (S.D. 0.57).

Since the rate of proton influx is expressed in Eqn. 1 as the rate of concentration decrease of extracellular protons, the rate constant c has the dimension of reciprocal time. It should be kept in mind though that it contains as a hidden dimension the cell density, which in our case was 6 g dry wt. $\cdot l^{-1}$. The enhancement constants, however, are independent of biomass concentration and dependent on the nature of the alkanol.

The exponential enhancement constants were independent of pH, as became evident from the parallelism of the semilog plots, as the ones depicted in the inset of Fig. 1 and Fig. 2. As is shown



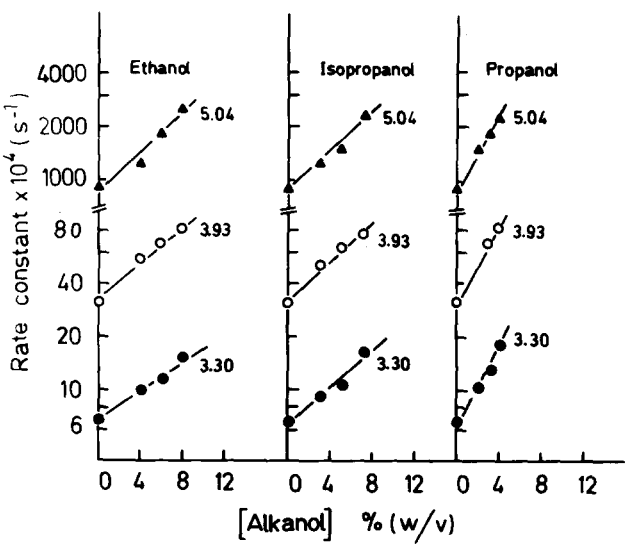


Fig. 1. Effects of butanol on passive proton influx in *S. cerevisiae*. The pH traces were obtained in aqueous suspensions of a respiration-deficient mutant deenergized with 2-deoxyglucose. Numbers indicate butanol concentrations (w/v). The inset shows the dependence of the diffusion rate constants on the butanol concentration. Numbers indicate the initial pH values of the diffusion experiments.

Fig. 2. Effects of ethanol, isopropanol and propanol on the rate constants of passive proton influx in aqueous suspensions of a respiration-deficient mutant of *S. cerevisiae* deenergized by preincubation with 2-deoxyglucose. Numbers indicate the initial pH values of the diffusion experiments.

in Fig. 3, the enhancement constants increased linearly with the lipid/buffer partition coefficients of the alkanols. The conclusion was that the alkanols interacted with hydrophobic regions of the membrane, increasing its permeability for protons and that this interaction was not influenced by the extracellular pH.

However, while the exponential relations between the rate constants, enhanced by alkanols, were the same at all pH values tested (Figs. 1 and 2), their absolute values decreased exponentially with pH, i.e., linearly with increasing extracellular proton concentration (Fig. 4). Thus, in addition to the pH-insensitive, alkanol-sensitive barrier constituted by hydrophobic regions in the membrane, also a pH-sensitive, alkanol-insensitive surface barrier against proton permeation existed and the effectiveness of this barrier apparently increased with protonation. As a consequence, the passive influx of protons was subject to opposite pH influences: increase with pH due to increase in the value of the rate constant and decrease with increasing pH due to decrease in the extracellular proton concentration. Superimposed on these pH effects on passive proton influx, the influx was enhanced, exponentially and independently of pH, by alkanols.

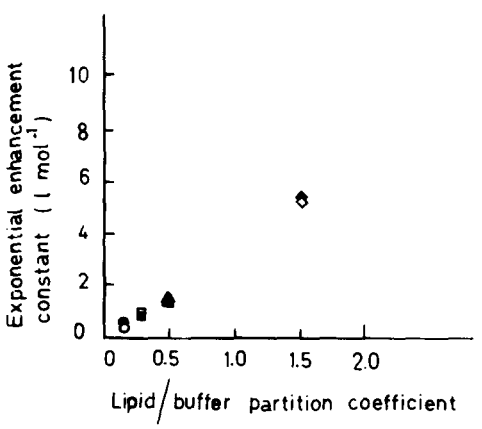


Fig. 3. Relation between the lipid/buffer partition coefficients (values from Ref. [14]) of ethanol (○, ●), isopropanol (□, ■), propanol (△, ▲) and butanol (◇, ◆) and their exponential enhancement constants of passive proton influx in aqueous suspensions of a respiration-deficient mutant of *S. cerevisiae* deenergized with 2-deoxyglucose (solid symbols) and energized with glucose (open symbols).

Passive proton influx in energized yeast

Addition of glucose to the yeast suspension in water induced proton extrusion and a concomitant decrease of the extracellular pH. The acidification curve had the shape described by Sigler et al. [11,12] and sloped off to a final external pH value. In the presence of alkanols, the curves had a similar shape but the final pH values that could be attained by the suspensions increased depending on the nature and the concentration of the alkanol. Fig. 5 depicts the results obtained with butanol. Similar results were obtained with the other alkanols.

We interpreted these results as follows. At the final pH values, the opposite proton movements (active extrusion and passive influx) were equal. Since the alkanols enhanced proton influx, the final pH values increased in their presence. We may write:

$$k_p = c_0 e^{kx} [H^+]_f \qquad (6)$$

where k_p is the rate of active proton extrusion,

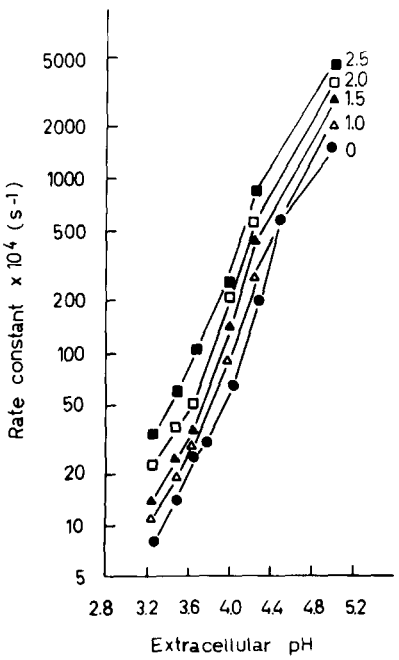


Fig. 4. Effects of pH on the rate constants of passive proton influx in aqueous suspensions of a respiration-deficient mutant of *S. cerevisiae* deenergized with 2-deoxyglucose with and without butanol. Numbers indicate butanol concentrations (w/v).

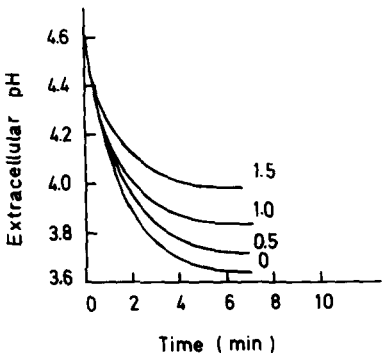


Fig. 5. Effects of butanol on the acidification curves in aqueous suspensions of a respiration-deficient mutant of *S. cerevisiae* energized with glucose. Numbers indicate butanol concentrations (w/v).

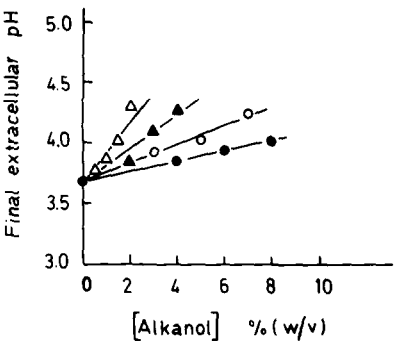


Fig. 6. Relations between alkanol concentrations and the final pH values of acidification curves of aqueous suspensions of a respiration-deficient mutant of *S. cerevisiae* energized with glucose. △, Butanol; ▲, propanol; ○, isopropanol; ●, ethanol.

$[H^+]_f$ the proton concentration at the final pH, c_0 the rate constant of proton diffusion at the final pH, x the alkanol concentration and k the respective enhancement constant. Taking logs and rearranging, we obtain the following relation between the alkanol concentration and the final pH:

$$pH_f = \log_{10} c_0 - \log_{10} k_p + kx \log_{10} e \qquad (7)$$

Under the conditions whereby the effects of the alkanols on active proton extrusion over the range of final pH values are not too great, so that $\log_{10} k_p$ is nearly a constant and the variation of c_0 over this range is small so that $\log_{10} c_0$ is also nearly a constant, Eqn. 7 should be linear, or nearly so, if Eqn. 6 holds. As is shown in Fig. 6, the relations were indeed linear and the estimates of enhancement constant, k, calculated from the slopes had nearly identitical values and relations with the lipid/buffer partition coefficients of the alkanols (Fig. 3) as the estimates obtained from the experiments with deenergized cells. These observations led us to conclude that passive proton influx takes place in energized cells with similar rates as in deenergized cells.

Table I compares the rates of passive proton influx at pH 4.5 observed in our strain with the maximum rates of transport in the same strain of methylammonium [5], D-xylose [3], maltose [4] and glycine [6]. While in the absence of ethanol the rate of passive proton influx was only about one-half of the maximum rate of glycine transport and much lower than the maximum transport rates of the other substrates, at 8% (w/v) ethanol passive

TABLE I

RATES OF PASSIVE PROTON DIFFUSION ACROSS THE PLASMA MEMBRANE OF A RESPIRATION-DEFICIENT MUTANT OF *S. CEREVISIAE* IN AQUEOUS SUSPENSION WITH AND WITHOUT ETHANOL COMPARED WITH MAXIMUM TRANSPORT RATES OF NUTRIENTS OR ANALOGUES

Rates are expressed as nmol/min per mg dry wt.

Ethanol concentration (%, w/v)	Passive proton influx at pH 4.5 (This paper)	Maximum rates of transport at pH 4.5 calculated from extrapolated Lineweaver-Burk plots			
		Methylammonium [5]	D-Xylose [3]	Maltose [a] [4]	Glycine [6]
0	17.8	76.5	131.6	166.7	41.7
8	46.2	20.6	51.8	77.8	7.9

[a] Rates at pH 5.25.

proton influx exceeded the maximum rate of glycine transport by a factor of nearly 6 and was more than double of that of methylammonium transport. However, at 8% (w/v) ethanol the rate of proton diffusion was still less than two-thirds of the maximum rate of maltose transport. Since the capacity of K^+ efflux is at least equal to the capacity of maltose transport in the strain studied [4], no enhancement of K^+ efflux by alkanols would be required should K^+ efflux compensate the enhanced H^+ influx. Using a K^+ electrode, this compensation was found to take place in deenergized cells (not shown). In energized cells, the charge compensation of passive proton influx is carried out, one expects, by active proton extrusion. These findings do of course not exclude that alkanols may enhance the capacity of K^+ efflux in yeast.

Alkanols as uncouplers

It was reported earlier that alkanols increase the passive leakage of K^+ and Na^+ through lipid membranes (references in Ref. 14), while ethanol acted as an uncoupler of lactose transport in *Escherichia coli* [15], presumably by enhancing passive proton influx. Our results with deenergized yeast cells showed that alkanols exponentially increase the permeability for protons of the yeast plasma membrane and that the kinetics are consistent with simple diffusion. The results with energized cells constituted evidence that a similar enhancement of passive proton influx by alkanols concurs with active proton extrusion, thus contributing to the kinetics of extracellular acidification. The results imply that uncoupling should contribute to the overall kinetics of alkanol-inhibited secondary active transport across the yeast plasma membrane and that alkanols should decrease the potential ratios of substrate accumulation. We have found that alkanols do indeed decrease the ratio of methylammonium accumulation in energized *S. cerevisiae* (unpublished data).

Our results raise a number of questions which require further study. What is the effect of alkanols on active proton extrusion? What is the molecular nature of the channels for passive proton diffusion? And finally, how does the interaction of alkanols with the plasma membrane result in exponential enhancement kinetics of proton diffusion, while extracellular pH decrease diminishes the permeability of the plasma membrane for protons?

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