Arch Microbiol (1990) 153:384–391

Archives of
Microbiology

© Springer-Verlag 1990

Effects of ethanol on *Saccharomyces cerevisiae* as monitored by in vivo ^{31}P and ^{13}C nuclear magnetic resonance

Maria C. Loureiro-Dias[1] and Helena Santos[2,3]

[1] Laboratory of Microbiology, Gulbenkian Institute of Science, Ap. 14, P-2781 Oeiras Codex, Portugal
[2] Centro de Tecnologia Química e Biológica, CTQB, Apartado 127, P-2780 Oeiras, Portugal
[3] Departamento de Química, Faculdade de Ciências e Tecnologia, U.N.L., Quinta da Torre, P-2825 Monte da Caparica, Portugal

Received July 12, 1989/Accepted November 28, 1989

Abstract. Cell suspensions of a respiratory deficient mutant of *Saccharomyces cerevisiae* were monitored by in vivo ^{31}P and ^{13}C Nuclear Magnetic Resonance in order to evaluate the effect of ethanol in intracellular pH and metabolism. In the absence of an added energy source, ethanol caused acidification of the cytoplasm, as indicated by the shift to higher field of the resonance assigned to the cytoplasmic orthophosphate. Under the experimental conditions used this acidification was not a consequence of an increase in the passive influx of H^+. With cells energized with glucose, a lower value for the cytoplasmic pH was also observed, when ethanol was added. Furthermore, lower levels of phosphomonoesters were detected in the presence of ethanol, indicating that an early event in glycolysis is an important target of the ethanol action. Acetic acid was identified as responsible for the acidification of the cytoplasm, in experiments where [^{13}C]ethanol was added and formation of labeled acetic acid was detected. The intracellular and the extracellular concentrations of acetic acid were respectively, 30 mM and 2 mM when 0.5% (120 mM) [^{13}C]ethanol was added.

Key words: *Saccharomyces cerevisiae* – Ethanol – Acetic acid – Cytoplasmic pH – ^{31}P-NMR – ^{13}C-NMR

Saccharomyces cerevisiae is able to ferment high concentrations of sugar and the slowing down of fermentation during the last stages of this process has been ascribed to end-product inhibition. Ethanol is the main end-product accumulated during fermentation, and although *S. cerevisiae* is the organism which can withstand higher ethanol concentrations, this may be primarily responsible for the cessation of fermentation. A number of mechanisms have been identified as being involved in the inhibitory action of ethanol. The cytoplasmic membrane has been shown to be an important target: H^+ passive influx into the cells was exponentially enhanced by ethanol (Leão and van Uden 1984; Cartwright et al. 1986) as well as the leakage of metabolites (Salgueiro et al. 1988). The fatty acyl composition of the membrane lipids was shown to play an important role on ethanol resistance (Thomas and Rose 1979; Mishra and Prasad 1989). These findings were interpreted as a result of increased disorder and fluidity created by ethanol in the lipids of the plasma membrane (Jones and Greenfield 1987). Mediated transport through the plasma membrane is also inhibited in a non-competitive way and data for the transport of glucose, maltose, amino acids and ammonium have been reported (for a review see van Uden 1989).

The effect of ethanol on the metabolism of glucose was investigated in several works. Dombek and Ingram (1987) found that the activity and the stability of the glycolytic enzymes was not significantly affected during the decline of fermentation. Millar et al. (1982) observed no important inhibitory effect on enzymatic activities, unless very high ethanol concentrations were used (higher than could be attained by fermentation).

A controversial point in the discussion concerning the effect of ethanol is related to the internal pH. While Dombek and Ingram (1987) detected just a slight decrease in the values of the internal pH during the last stages of fermentation at high ethanol concentration, Cartwright et al. (1986), measuring the internal pH in the presence of ethanol, detected a significant acidification, more relevant in the absence of glucose. The same authors reported that the pH gradient (ΔpH) across the plasma membrane and the membrane potential ($\Delta\Psi$) were also affected, and interpreted their results in terms of an increase of the net influx of H^+ (by inhibition of the plasma ATPase and increase of the passive H^+ influx).

Offprint requests to: M. C. Loureiro-Dias

Abbreviations: P_i, inorganic phosphate; P_{ic}, inorganic phosphate in the cytoplasm; P_{iv}, inorganic phosphate in the vacuole; tP, terminal phosphate in polyphosphate

Compartmentation in yeast cells implies the existence of membranes other than the plasma membrane. During the last years, a number of biochemical processes have been identified at the level of the tonoplast. An ATPase was characterized and shown to behave as an electrogenic pump, translocating H^+ from the cytoplasm into the vacuole (Kakinuma et al. 1981; Okorokov and Lichko 1983). A number of active transport systems were identified at the tonoplast, able to lead to the accumulation of ions in the vacuole (Ohsumi and Anraku 1981; Okorokov 1985) at the expense of the electrochemical H^+ gradient at the tonoplast level.

Since ethanol increases passive movements of H^+ through the plasma membrane it may also conceivably disturb ΔpH across the tonoplast. To assess this parameter ^{31}P-NMR is a very suitable technique. In vivo ^{31}P-NMR can provide information about intracellular pH, phosphorylation potentials, rates of enzymatic reactions and transport of phosphorylated metabolites (for reviews see Gadian 1982; Balaban 1984; Campbell-Burk and Shulman 1987). In particular, measurement of the intracellular pH by in vivo ^{31}P-NMR is straightforward (Gillies et al. 1982), and with yeast cells it is possible to monitor vacuolar and cytoplasmic pH with a time resolution of about 1 min (Nicolay et al. 1982).

In the work reported here, an external pH slightly higher than the cytoplasmic pH was used, in order to minimize the movements of H^+ between the exterior and the cytoplasm, and to keep the resonance due to the external P_i well separated from the resonances of intracellular P_i. A strong acidification of the cytoplasmic compartment was observed upon addition of ethanol to a suspension of *S. cerevisiae* cells. Our results indicate that metabolic events, rather than alterations in passive or mediated transport, are responsible for the observed acidification of the cytoplasm.

Materials and methods

Organism and growth

A respiratory-deficient mutant of *Saccharomyces cerevisiae* (IGC 3507-III) was used in all the experiments. Cells were grown in a mineral medium (van Uden 1967) with vitamins and glucose (2%, w/v) at 25°C in an orbital incubator. The cells were harvested in the early exponential phase (O.D. $\cong$ 0.2), by centrifugation and washed twice with demineralized water (4°C). Cells were kept on ice until use (less than 2 h after harvesting).

NMR measurements

NMR spectra were recorded at 20°C using a Bruker CXP-300 spectrometer, operating at 121.47 MHz for ^{31}P-NMR and at 75.47 MHz for ^{13}C-NMR. For each experiment, about 150 mg dry weight of cells were suspended in Tris 50 mM buffer adjusted to pH 7.5 with citric acid, giving a final volume of 4 ml; 100 µl of D_2O were added to provide a lock signal. 10 mm NMR tubes were used and an efficient mixing of the cell suspension was achieved by using a simple air-lift system (Santos and Turner 1986). For a typical ^{31}P-NMR spectrum, 300 scans were accumulated using 45° pulses with a repetition time of 0.4–0.5 s. Chemical shifts were referenced with respect to 85% H_3PO_4 contained in a capillary tube. Resonance assignments were taken from the literature (Navon et al. 1979). In order to calibrate pH values, a calibration curve was obtained using a cell-free extract.

^{13}C-NMR spectra were obtained under similar physiological conditions, with proton broadband decoupling using 30° pulses and a repetition delay of 0.6 s. 8000 data points were acquired over 18 KHz spectral width. Chemical shifts were measured using methanol ($\delta = 49.3$) as an external reference. When indicated, 120 mM [1-^{13}C]ethanol was added to the assay mixture. [1-^{13}C]ethanol (isotopic enrichment 90%) was purchased from the Service and Molécules Marquées, CEN Saclay, France and [1-^{13}C]acetic acid (isotopic enrichment 99%) was obtained from Sigma.

Results

Effect of ethanol on deenergized yeast cells

In previously reported ^{31}P-NMR studies of *Saccharomyces cerevisiae* cells (Navon et al. 1979; den Hollander et al. 1981; Nicolay et al. 1982), only one resonance was observed for the intracellular P_i of deenergized cells, and separate resonances for vacuolar and cytoplasmic P_i were apparent only if the cells were supplied with an energy source (Nicolay et al. 1982). In order to be able to monitor passive H^+ movements across the tonoplast in the presence of ethanol, our first task was the establishment of the experimental conditions under which distinct resonances for the vacuolar P_i and the cytoplasmic P_i were observed in the ^{31}P-NMR spectra of deenergized cells. This aim was attained by using young cell cultures (O.D. < 0.3) grown on minimal medium, and by selecting a value of 7.5 for the external pH. Under this set of conditions, two additional goals were achieved: 1) the resonance due to the external P_i did not overlap the small resonance originated by the vacuolar P_i; 2) the component of the pH change in the cytoplasm, associated with the H^+ passive flux through the plasma membrane was eliminated, since the external pH was made higher than the cytoplasmic pH.

Figure 1 illustrates the changes in the ^{31}P-NMR spectra of a cell suspension of *S. cerevisiae*, upon addition of ethanol (no added energy source). The bottom spectrum is a typical example of the spectra obtained under the conditions described above. It is characterized by resonances due to orthophosphate in different compartments (cytoplasm, vacuole and extracellular space), and strong resonances due to polyphosphate. From the chemical shifts of the resonances P_{ic} and P_{iv}, pH values were determined for the cytoplasm and the vacuole.

After the acquisition of the initial spectrum, ethanol was added (5% (v/v) for the experiment illustrated in Fig. 1) and spectra acquired for a period of about 90 min. The resonances due to the cytoplasmic P_i and the external P_i shifted gradually to higher field, indicating acidification of both compartments. However, the vacuolar pH was not affected, as judged from the constant positions both of the resonances due to the vacuolar P_i and the terminal phosphates in polyphosphate. The intensity of the resonance assigned to the middle phosphates in polyphosphate strongly decreased, indicating the occur-

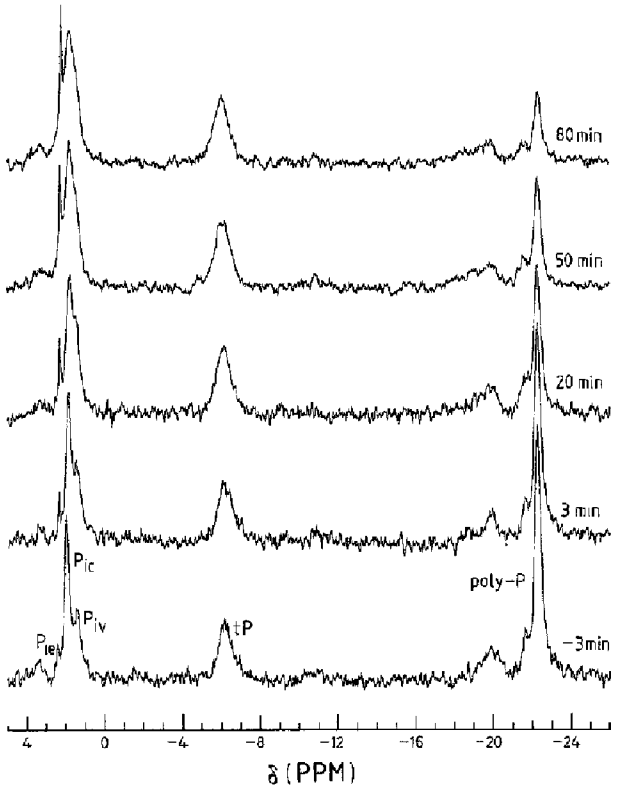

Fig. 1. In vivo ^{31}P-NMR spectra of a deenergized cell suspension of *Saccharomyces cerevisiae* (IGC 3507-III). Each spectrum represents the sum of 300 free induction decays acquired over 2.5 min. At time zero, ethanol was added to a final concentration of 5% (v/v) and consecutive spectra were acquired. A selection of spectra accumulated at the times indicated on the right hand side is shown. P_{ie}, external P_i; P_{ic}, cytoplasmic P_i; P_{iv}, vacuolar P_i; tP, terminal phosphates in polyphosphate; poly-P, middle phosphates in polyphosphate

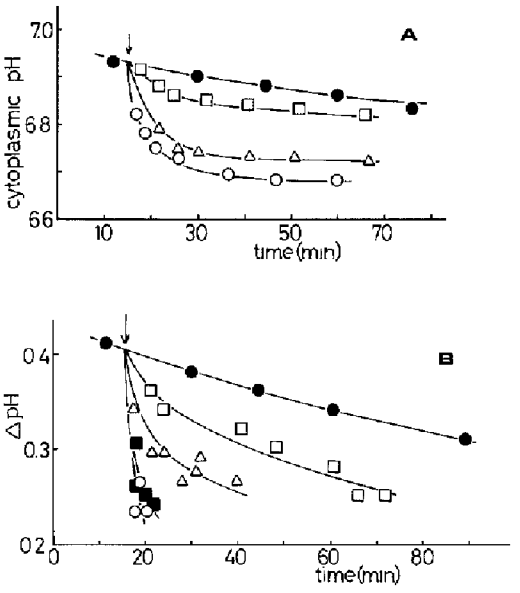

Fig. 2A, B. Time course of the cytoplasmic pH (**A**) and the ΔpH across the tonoplast (**B**) in deenergized cells of *Saccharomyces cerevisiae* (IGC 3507-III), upon addition of several concentrations of ethanol. The pH values were determined from the chemical shifts of the resonances P_{ic} and P_{iv} in the series of spectra shown partly in Fig. 1, by using a calibration curve. No ethanol (●), 2% (□); 5% (△); 7.5% (■) and 10% (○). Concentrations of ethanol are expressed in (v/v)

rence of hydrolysis of polyphosphates. In contrast with this behaviour, the resonance due to the terminal phosphates slightly increased in the course of the experiment; these results suggest breakdown of polyphosphate into smaller fragments, probably including pyrophosphate. Leakage of inorganic phosphate also occurred, since the intensity of the external P_i resonance increased with time (Fig. 1).

In other similar experiments the effects of adding different concentrations of ethanol were probed. It was found that, both the rates of the hydrolysis of polyphosphates and the leakage of P_i to the external medium, were constant and independent of the ethanol concentration, although a sudden release of P_i into the external medium was observed when ethanol was added. Furthermore, hydrolysis of polyphosphate was also observed when the cells were energized with glucose (see below).

Ethanol had an important effect on the cytoplasmic pH (Fig. 2). The time courses of the changes observed in the cytoplasmic pH and in the ΔpH across the tonoplast are presented, respectively in Fig. 2A and Fig. 2B. Although in the absence of ethanol a slight acidification of the cytoplasm was observed, addition of ethanol strongly accelerated this process, and for high concentrations of ethanol, ΔpH vanished very rapidly.

The internal acidification promoted by the addition of ethanol to deenergized yeast cells was tentatively explained by Cartwright et al. (1986) as a result of the increased H^+-permeability of the plasma membrane induced by ethanol. Under the experimental conditions used in the work reported here, this explanation is not valid since the extracellular pH was higher than the cytoplasmic pH and passive H^+ flux should occur in the opposite direction. Furthermore, the alternative explanation that H^+ would flow from the vacuole is not sustainable, since no change in the vacuolar pH was observed.

Effect of ethanol on energized yeast cells

In order to investigate the effects of ethanol on energized yeast cells, ^{31}P-NMR spectra of cell suspensions were acquired consecutively after the addition of a pulse of 0.4 mmol glucose (100 mM final concentration), in the absence and in the presence of ethanol (10% v/v). Figure 3 presents some of the spectra in the series run under these conditions. The bottom traces correspond to the spectra of the cellular suspensions, immediately after resuspension in the NMR tube and are identical to the initial spectrum in Fig. 1, discussed above. In the absence of ethanol (Fig. 3A), the general features of the spectra are similar to those published earlier (Navon et al. 1979; den Hollander et al. 1981; Nicolay et al. 1982), except for the absence of the resonance appearing in the phosphodiester region of the spectrum and assigned to phosphomannan (Gage et al. 1984). Upon addition of

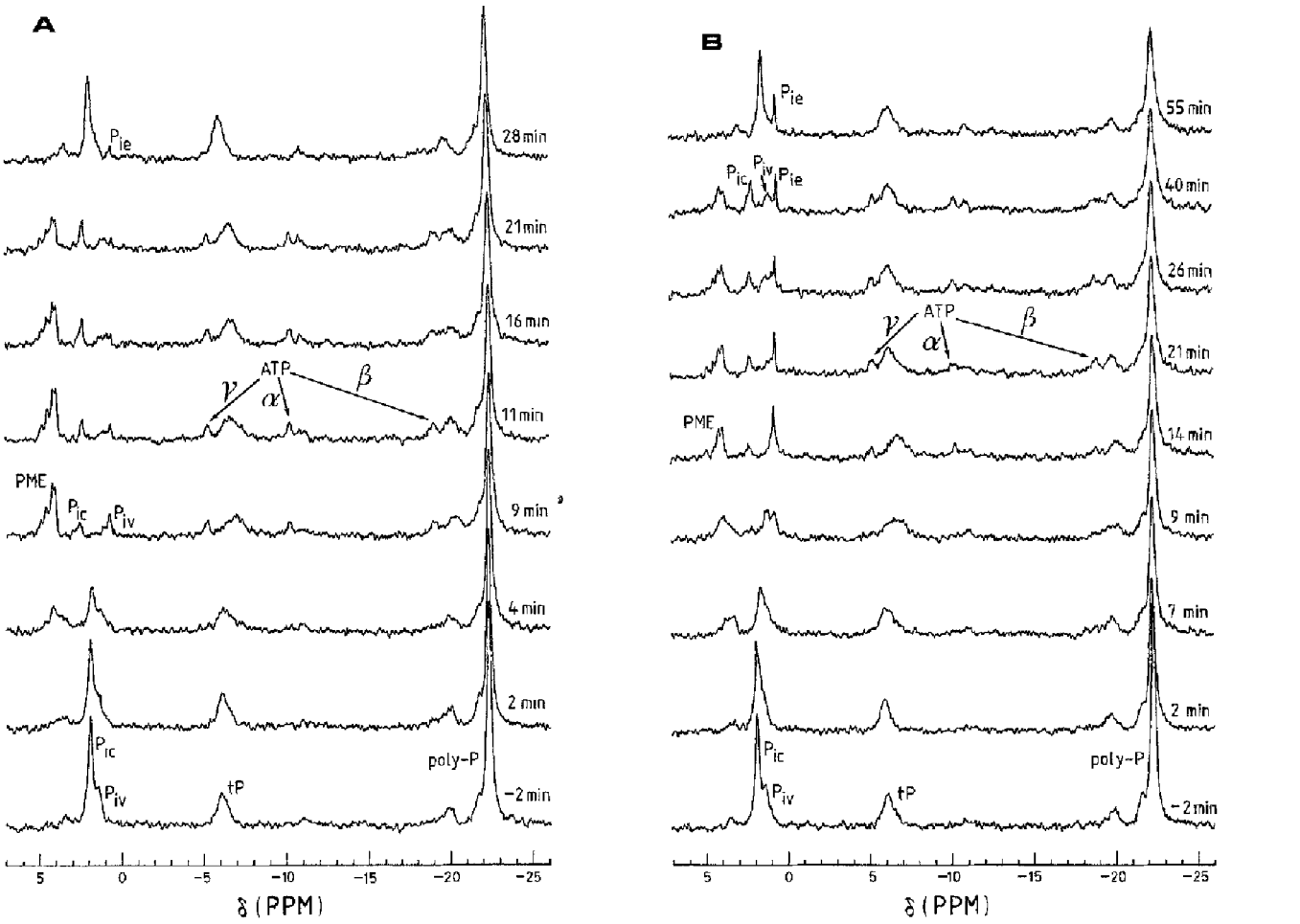

Fig. 3A, B. ^{31}P-NMR spectra of cell suspension of *Saccharomyces cerevisiae* (IGC 3507-III). Each spectrum represents the sum of 300 scans acquired over 2 min. At time zero, a pulse of 100 mM glucose (**A**) or 100 mM glucose + 10% (v/v) ethanol (**B**) were added. PME, phosphomonoesters; ATP, α, β and γ peaks of adenosine triphosphate; other symbols as in Fig. 1

glucose, significant changes occurred in the ^{31}P-NMR spectra. The intensities of the resonances P_{ic} and P_{iv} were significantly reduced and strong shifts of their positions were observed, indicating alterations in the cytoplasmic and vacuolar pH values. After a transitory acidification of the cytoplasm, its pH increased to a value of 7.3 in the steady state observed in the absence of ethanol. In contrast with this alkalinization of the cytoplasm, the vacuolar pH changed from 6.5 to 6.2. This acidification was also revealed by the shift to higher field of the resonance assigned to the terminal phosphates in polyphosphate (tP). It is interesting to remark that, at the onset of glucose depletion, a shift of tP in the opposite direction was observed, indicating alkalinization of the compartment containing the polyphosphates (Fig. 3). The observed parallel between the behaviour of the resonances P_{iv} and tP, supports the conclusion that a significant part of the NMR visible polyphosphate is localized in the vacuole (Nicolay et al. 1982), and not in the extracellular space. In fact, the tP resonance due to the polyphosphate supposedly localized outside the plasma membrane, would experience a monotonic shift to the acidic direction, reflecting the observed extracellular acidification. Attempts to use the chemical shift of tP to determine absolute pH values in the vacuole were, however, frustrated presumably by the strong dependence of the chemical shift of tP, on the ionic composition of the solution, as previously reported (MacDonald and Mazurek 1987).

In the course of the experiments, polyphosphate was hydrolyzed, as deduced from the decrease in the intensity of the resonance at −22.3 ppm. The rate of polyphosphate hydrolysis was independent, either of the energy status of the cells, or the presence of ethanol. The phosphate starvation conditions of the cells may play a definite role in this process.

Concomitantly with the alkalinization of the cytoplasm and the acidification of the vacuole, a strong increase in the levels of the phosphomonoesters and of ATP was observed. These effects, induced by the administration of glucose, were reversed when this substrate was depleted (Fig. 3). Fructose 1,6-bisphosphate and glucose 6-phosphate have been identified as the major components in the phosphomonoester resonances of the ^{31}P-NMR spectra of *S. cerevisiae* (den Hollander et al. 1981).

A detailed presentation of the comparison between the time courses of the cytoplasmic pH, in the absence

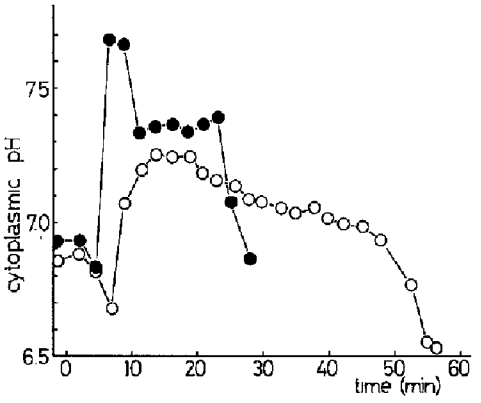

Fig. 4. Time course of the effect of ethanol on the cytoplasmic pH of *Saccharomyces cerevisiae* (IGC 3507-III), fermenting 100 mM glucose added at time zero, in the absence (●) and in the presence of 10% (v/v) ethanol (○)

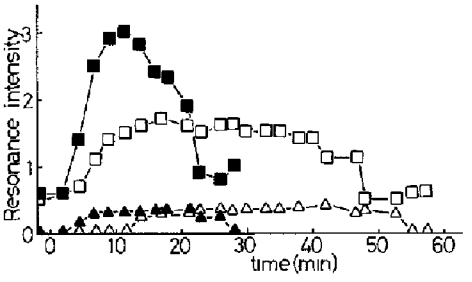

Fig. 5. Time course of the effect of ethanol on the level of phosphomonoesters and ATP in *Saccharomyces cerevisiae* (IGC 3507-III) fermenting 100 mM glucose added at time zero. The ordinates represent the intensities (arbitrary units) of the resonances due to phosphomonoesters (squares) and the γ-phosphate of ATP (triangles), measured from the series of spectra partly presented in Fig. 3. Closed symbols in the absence of ethanol; open symbols in the presence of 10% (v/v) ethanol

and in the presence of ethanol is given in Fig. 4; the levels of the phosphomonoesters and ATP are presented in Fig. 5. Measurements were made in the series of spectra partly shown in Fig. 3 (A and B).

The comparison between the two experiments (absence and presence of ethanol) reveals significant differences. In the absence of ethanol, 0.4 mmol of glucose was consumed about twice as fast as in the presence of ethanol. This conclusion can be drawn from the comparison between the times when an abrupt acidification of the cytoplasm together with the return of the phosphomonoesters and the ATP levels to the initial values were observed (Figs. 4 and 5), in the absence and in the presence of ethanol. The faster utilization of glucose in the absence of ethanol is to be expected, since it is well known that fermentation is slowed down by ethanol. The pH in the cytoplasm reached higher values in the absence of ethanol, and clear steady state conditions were observed for some minutes (Fig. 4). In contrast with this, in the presence of ethanol, the cytoplasmic pH gradually decreased during the time required for the consumption of glucose and the level of phosphomonoesters was significantly lower.

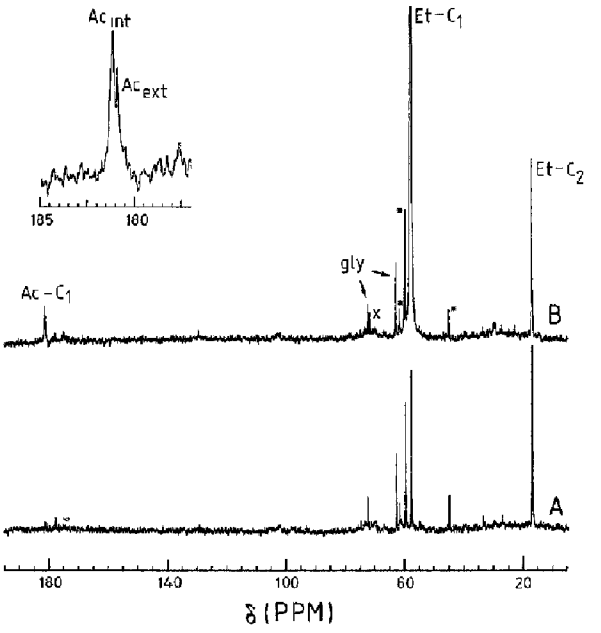

Fig. 6 A, B. ^{13}C-NMR spectra of cell suspensions of *Saccharomyces cerevisiae* (IGC 3507-III) after fermenting 0.4 mmol of glucose in the absence (**A**) and in the presence of 120 mM [1-^{13}C]ethanol (**B**). Ac_{int}, carboxylic group of the intracellular acetic acid; Ac_{ext}, carboxylic group of the extracellular acetic acid; gly, glycerol; X, unknown compound; Et, ethanol. Resonances labeled with * are due to the natural abundance ^{13}C present in the buffer. The insert shows an expansion of the carboxylate region of spectrum

Acetic acid formation as evidenced by ^{13}C-NMR

The results described above demonstrate that the acidification of the cytoplasm induced upon addition of ethanol, at the extracellular pH used, was not due to an increase of passive H^+ movements. Thus, formation of an acidic compound must be responsible for that acidification. In order to investigate the nature of such compound(s), ^{13}C-NMR was successfully used. In an initial experiment, [1-^{13}C]ethanol (120 mM) was added to a deenergized yeast cell suspension and the production of compounds was monitored by ^{13}C-NMR. Under these conditions, acetic acid was found to accumulate with time, as deduced from the appearance and growth of a resonance at 181.6 ppm, and assigned to the C_1 of acetic acid (results not shown).

The formation of acetic acid was also detected when the experiment was run with glucose-energized cells. Fig. 6 shows the ^{13}C-NMR spectra of yeast cell suspensions acquired after fermenting 0.4 mmol of glucose, in the absence (A) and in the presence of added [1-^{13}C] ethanol (B). In addition to the resonances due to the natural abundance ^{13}C present in the buffer, resonances arising from the usual fermentation products (ethanol and glycerol) were readily identified in spectrum A. In the presence of added [1-^{13}C]ethanol (spectrum B), the most striking difference was the appearance of two clear resonances at 181.1 and 181.3 ppm and assigned to the C_1 of the intracellular and the extracellular acetic acid, respectively (inset in Fig. 6). Two distinct resonances were

originated by the same carbon atom in acetic acid because of the pH difference between the external medium (acidified during the utilization of glucose) and the cytoplasm.

Quantification of the acetic acid pools was done by adding a known amount of [1-^{13}C]acetic acid. About 2 mM and 30 mM were determined respectively for the extracellular, and the intracellular acetic acid concentrations, using 1.5 $\mu l \cdot mg^{-1}$ dry weight for the internal volume of yeast cells (Pampulha and Loureiro-Dias 1989) in the calculation of the intracellular concentration.

A new resonance at 71.7 ppm also appeared when [1-^{13}C]ethanol was present (resonance X in spectrum B), but was not assigned. The resonance does not coincide with resonances of any of the following compounds: acetoin, butanediol, erythritol or acetaldehyde.

Discussion

The inhibitory effect of ethanol in fermentation by yeast is a complex process, as has been pointed out by several authors (Pamment 1989 and references therein). In the present work a new aspect is focused: the conversion of ethanol into acetic acid, a more toxic compound.

The physiological situation of absence of respiration under conditions of catabolic repression, usually found when *Saccharomyces cerevisiae* is fermenting high concentrations of glucose, which result in the production of high levels of ethanol, were simulated in our experiments by using a respiratory-deficient mutant. With this strain, the effect of the addition of ethanol was not masked by the energization resulting from the production of ATP due to ethanol respiration, as was observed with the wild strain (unpublished results). However, it was not expected that this mutant would metabolize ethanol to acetic acid since this process involves the reduction of NAD^+ (in the conversion of ethanol into acetaldehyde, catalysed by an alcohol dehydrogenase), and the reduction of $NADP^+$ (in the conversion of acetaldehyde into acetic acid catalysed by an acetaldehyde dehydrogenase). This latter enzyme has been described as a cytoplasmic, partially non-repressible enzyme in *S. cerevisiae* (Jacobson and Bernofsky 1974). As previously pointed out (van Dijken and Scheffers 1986), redox balance has a central role in metabolism and since the reoxidation of the pyridine nucleotides in a respiratory mechanism is not possible in this mutant, it will probably occur at the level of cytoplasmic cytochromes. In fact, consumption of O_2 by a cyanide resistant mechanism has been previously described in the same strain (Arrabaça and Loureiro-Dias 1982).

The data concerning the changes observed in the internal pH upon addition of ethanol can be interpreted as a result of the production of acetic acid in the cytoplasm. In deenergized cells (Figs. 1 and 2), acidification of both the cytoplasm, and the extracellular medium was observed without any appreciable change in the pH of the vacuole. Acetic acid formed in the cytoplasm dissociates, causing a decrease in the pH of this compartment. The small undissociated fraction will slowly leak out, decreasing pH outside. In energized cells (Figs. 3 and 4), the typical rise in the cytoplasmic pH was observed upon addition of glucose, but this value slowly decreased during glucose utilization in the presence of ethanol, reflecting the time course of acetic acid production.

The comparison and interpretation of results from different authors with respect to the effect of ethanol on yeast is often a difficult task. Differences among strains and among experimental conditions lead to ambiguous conclusions. Cartwright et al. (1986), measuring intracellular pH, found that ethanol promoted a significant pH drop, but their measurements were performed, as in our work, upon addition of ethanol to cells that had been grown under a lower ethanol concentration. Dombek and Ingram (1987) measured the effect of ethanol on the internal pH, but in their case, ethanol reached a high concentration by being slowly produced in the medium and these cells were well adapted to high levels of ethanol. Under these conditions, a much smaller drop in the internal pH was observed. In both cases, acetic acid was probably produced, causing a decrease of the cytoplasmic pH. However, adapted cells may be able to overcome this unfavourable situation by an activation of the plasma-membrane ATPase. The observation of such activation induced by an acidic pH was previously reported by Eraso and Gancedo (1987).

The acetic acid produced inside the cells at physiological pH, in the course of fermentation is essentially in the ionized form; only about 1% is non-ionized and able to cross the plasma membrane by simple diffusion, since the transport system for acetate is repressed in the presence of glucose (Cassio et al. 1987). Usually during fermentation processes the external pH is quite acidic, acetic acid being then essentially non-ionized. This means that the driving force outwards is low and the acid will be trapped inside the cells, where in synergism with ethanol it may play a definite role in the inhibition of fermentation (Pampulha and Loureiro 1989).

An interesting feature emerges from the data depicted in Fig. 5. The level of phosphomonoesters is clearly lower in the presence of ethanol. This suggests that the main inhibitory mechanism of ethanol should affect an early event in fermentation: transport and/or phosphorylation of the sugar. Inhibition of the glucose transport system by ethanol was reported by Leão and van Uden (1982); inhibition of phosphorylation as a consequence of ethanol accumulation has been proposed (Dombek and Ingram 1988; Alterthum et al. 1989). According to these authors, the inhibition of the phosphorylation of the sugar is not due to a direct inhibition of the hexokinase, but due to the accumulation of AMP which would compete with ATP for the active site of the enzyme. Dombek and Ingram (1988) further proposed that the increase of AMP during fermentation would be a consequence of a series of related events: ethanol would increase passive H^+-permeability, causing drop in the internal pH; the cells would overcome this acidification by activation of the plasma-membrane ATPase, which in turn would lead to a depletion of ATP and accumulation of AMP. Assuming this as a valid model, our results indicate that the whole sequence of phenomena is triggered by the

production and accumulation of acetic acid in the cytoplasm of the yeast cell, (although at low pH the increase in passive proton permeability may play an important role). Such activation of the ATPase is, however, controversial. Cartwright et al. (1987) reported the inhibition of the ATPase *in vitro* by ethanol and Pascual et al. (1988) found an inhibitory effect of ethanol on proton extrusion through the plasma membrane.

The overall effect of ethanol on yeast remains difficult to interpret, however, our results definitely show that acetic acid is formed inside the cells upon addition of ethanol, and this piece of information must be taken into account in this discussion. It is worthwhile emphasising that a strategy to produce mutants with a high resistance to ethanol should consider the hypothesis that a strain lacking acetaldehyde dehydrogenase may present properties of increased ethanol tolerance.

Acknowledgements. We thank Prof. N. van Uden and Prof. A. V. Xavier for constant support, valuable discussions and the critical reading of the manuscript. This research was supported by the Calouste Gulbenkian Foundation, Lisbon, Portugal and the Agency for International Development, Washington DC, USA, Grant No. DPE-5542-G-SS-8071-00 (M. C. L.-D.) and by Instituto Nacional de Investigação Científica and Junta Nacional de Investigação Científica e Tecnológica (H. S.).

References

Alterthum F, Dombek KM, Ingram LO (1989) Regulation of glycolytic flux and ethanol production in *Saccharomyces cerevisiae:* Effects of intracellular adenine nucleotide concentrations on the in vitro activities of hexokinase, phosphofructokinase, phosphoglycerate kinase and pyruvate kinase. Appl Environ Microbiol 55:1312–1314

Arrabaça JD, Loureiro-Dias MC (1982) Cyanide-resistant respiration in a respiration-deficient mutant of *Saccharomyces cerevisiae*. Z Allg Mikrobiol 22:437–442

Balaban RS (1984) The application of nuclear magnetic resonance to the study of cellular physiology. Am J Physiol 246:C10–C19

Campbell-Burk SL, Shulman RG (1987) High-resolution NMR studies of *Saccharomyces cerevisiae*. Ann Rev Microbiol 41: 595–616

Cartwright CP, Juroszek JR, Beavan MJ, Ruby FMS, Morais SMF, Rose AH (1986) Ethanol dissipates the proton-motive force across the plasma membrane of *Saccharomyces cerevisiae*. J Gen Microbiol 132:369–377

Cartwright CP, Veazey FJ, Rose AH (1987) Effect of ethanol on activity of the plasma-membrane ATPase in, and accumulation of glycine by, *Saccharomyces cerevisiae*. J Gen Microbiol 133:857–865

Cassio F, Leão C, van Uden N (1987) Transport of lactate and other short-chain monocarboxylates in the yeast *Saccharomyces cerevisiae*. Appl Environ Microbiol 53:509–513

den Hollander JA, Ugurbil K, Brown TR, Shulman RG (1981) Phosphorus-31 nuclear magnetic resonance studies of the effect of oxygen upon glycolysis in yeast. Biochemistry 20:5871–5880

Dombek KM, Ingram LO (1987) Ethanol production during batch fermentation with *Saccharomyces cerevisiae:* Changes in glycolytic enzymes and internal pH. Appl Environ Microbiol 53:1286–1291

Dombek KM, Ingram LO (1988) Intracellular accumulation of AMP as a cause for the decline in rate of ethanol production by *Saccharomyces cerevisiae* during batch fermentation. Appl Environ Microbiol 54:98–104

Dijken JP van, Scheffers WA (1986) Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol Rev 32:199–224

Eraso P, Gancedo C (1987) Activation of yeast plasma membrane ATPase by acid pH during growth. FEBS Lett 224:187–192

Gadian DG (1982) Nuclear Magnetic Resonance and its applications to living systems. Clarendon, Oxford

Gage RA, Wijngaarden W van, Theuvenet APR, Borst-Pauwels GWFH, Haasnoot CAG (1984) Localization and identification of the compound causing peak 'X' in the ^{31}P-NMR spectrum of *Saccharomyces cerevisiae*. Biochim Biophys Acta 804:341–347

Gillies RJ, Alger JR, den Hollander JA, Shulman RG (1982) Intracellular pH measured by NMR: Methods and results. In: Intracellular pH: Its measurement, regulation and utilization in cellular functions. Alan R Liss, Inc, New York, pp 79–104

Jacobson MK, Bernofsky C (1974) Mitochondrial acetaldehyde dehydrogenase from *Saccharomyces cerevisiae*. Biochim Biophys Acta 350:277–291

Jones RP, Greenfield PF (1987) Ethanol and the fluidity of the yeast plasma membrane. Yeast 3:223–232

Kakinuma Y, Ohsumi Y, Anraku Y (1981) Properties of H^+-translocating adenosine triphosphatase in vacuolar membranes of *Saccharomyces cerevisiae*. J Biol Chem 256:10859–10863

Leão C, van Uden N (1982) Effects of ethanol and other alkanols on the glucose transport system of *Saccharomyces cerevisiae*. Biotechnol Bioeng 24:2601–2604

Leão C, van Uden N (1984) Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 774:43–48

MacDonald JC, Mazurek M (1987) Phosphorus magnetic resonance spectra of open-chain linear polyphosphates. J Magn Reson 72:48–60

Millar DG, Griffiths-Smith K, Algar E, Scopes RK (1982) Activity and stability of glycolytic enzymes in the presence of ethanol. Biotechnol Lett 4:601–606

Mishra P, Prasad R (1989) Relationship between ethanol tolerance and fatty acyl composition of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 30:294–298

Navon G, Shulman RG, Yamane T, Eccleshall TR, Lam K-B, Baronofsky JJ, Marmur J (1979) Phosphorus-31 nuclear magnetic resonance studies of wild-type and glycolytic pathway mutants of *Saccharomyces cerevisiae*. Biochemistry 18:4487–4499

Nicolay K, Scheffers WA, Brunenberg PM, Kaptein R (1982) Phosphorus-31 nuclear magnetic resonance studies of intracellular pH, phosphate compartmentation and phosphate transport in yeasts. Arch Microbiol 133:83–89

Ohsumi Y, Anraku Y (1981) Active transport of basic amino acids driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. J Biol Chem 256:2079–2082

Okorokov LA, Lichko LP (1983) The identification of a proton pump on vacuoles of the yeast *Saccharomyces cerevisiae*. FEBS Lett 155:102–106

Okorokov LA (1985) Main mechanisms of ion transport and regulation of ion concentration in the yeast cytoplasm. In: Kulaev IS, Dawes EA, Tempest DW (eds) Environmental regulation of microbial metabolism. Academic Press, London, pp 339–349

Pamment NB (1989) Overall kinetics and mathematical modeling of ethanol inhibition in yeasts. In: van Uden N (ed) Alcohol toxicity in yeasts and bacteria. CRC Press., Inc, Boca Raton, Florida, pp 1–76

Pampulha ME, Loureiro V (1989) Interaction of the effects of acetic acid and ethanol on inhibition of fermentation in *Saccharomoyces cerevisiae*. Biotechnol Lett 11:269–274

Pampulha ME, Loureiro-Dias MC (1989) Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. Appl Microbiol Biotechnol 31:547–550

Pascual C, Alonso A, Garcia I, Romay C, Kotyk A (1988) Effect of ethanol on glucose transport, key glycolytic enzymes and proton extrusion in *Saccharomyces cerevisiae*. Biotechnol Bioeng 32:374–378

Salgueiro SP, Sá-Correia I, Novais JM (1988) Ethanol-induced leakage in *Saccharomyces cerevisiae*: kinetics and relationship to yeast ethanol tolerance and alcohol fermentation productivity. Appl Environ Microbiol 54:903–909

Santos H, Turner DL (1986) Characterization of the improved sensitivity obtained using a flow method for oxygenating and mixing cell suspensions in NMR. J Magn Reson 68:345–349

Thomas DS, Rose AH (1979) Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition. Arch Microbiol 122:49–55

Uden N van (1967) Transport-limited fermentation and growth of *Saccharomyces cerevisiae* and its competitive inhibition. Arch Microbiol 58:155–168

Uden N van (1989) Effects of alcohols on membrane transport in yeasts. In: van Uden N (ed) Alcohol toxicity in yeasts and bacteria. CRC Press, Inc, Boca Raton, Florida, pp 135–146