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# Phosphorus-31 Nuclear Magnetic Resonance Studies of Intracellular pH, Phosphate Compartmentation and Phosphate Transport in Yeasts

K. Nicolay[1], W. A. Scheffers[2], P. M. Bruinenberg[2], and R. Kaptein[1]

[1] Department of Physical Chemistry, University of Groningen, Nijenborgh 16, NL-9747 AG Groningen, The Netherlands
[2] Department of Microbiology, Delft University of Technology, Julianalaan 67a, NL-2628 BC Delft, The Netherlands

**Abstract.** $^{31}$P NMR spectra were obtained from suspensions of *Candida utilis*, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* grown aerobically on glucose. Direct introduction of substrate into the cell suspension, without interruption of the measurements, revealed rapid changes in pH upon addition of the energy source. All $^{31}$P NMR spectra of the yeasts studied indicated the presence of two major intracellular inorganic phosphate pools at different pH environments. The pool at the higher pH was assigned to cytoplasmic phosphate from its response to glucose addition and iodoacetate inhibition of glycolysis. After addition of substrate the pH in the compartment containing the second phosphate pool decreased. A parallel response was observed for a significant fraction of the terminal and penultimate phosphates of the polyphosphate observed by $^{31}$P NMR. This suggested that the inorganic phosphate fraction at the lower pH and the polyphosphates originated from the same intracellular compartment, most probably the vacuole. In this vacuolar compartment, pH is sensitive to metabolic conditions. In the presence of energy source a pH gradient as large as 0.8 to 1.5 units could be generated across the vacuolar membrane. Under certain conditions net transport of inorganic phosphate across the vacuolar membrane was observed during glycolysis: to the cytoplasm when the cytoplasmic phosphate concentration had become very low due to sugar phosphorylation, and into the vacuole when the former concentration had become high again after glucose exhaustion.

**Key words:** *Candida utilis* – *Saccharomyces cerevisiae* – *Zygosaccharomyces bailii* – Compartmentation – Vacuoles – Internal pH – Phosphate – Glycolysis – Nuclear magnetic resonance

$^{31}$P NMR has become a well-established method in the study of the bioenergetics and metabolism of living systems (Ugurbil et al. 1979; Roberts and Jardetzky 1981; Gadian 1982; Nicolay et al. 1982). Since the chemical shifts of $P_i$ and other phosphorylated metabolites are sensitive to pH near physiological pH values, $^{31}$P NMR has been extensively used to evaluate the intracellular pH (Den Hollander et al. 1981; Nicolay et al. 1981; Tielens et al. 1982). Although the NMR method can provide information about intracellular compartmentation, the number of systems in which this phenomenon has been demonstrated by $^{31}$P NMR is limited (Cohen et al. 1978; Busby et al. 1978; Roberts and Jardetzky 1981). Navon et al. (1979) and Gillies et al. (1981) have reported the presence of two $^{31}$P NMR resonances from intracellular inorganic phosphate in the yeast *Saccharomyces cerevisiae*. One of these could easily be assigned to cytoplasmic $P_i$ while the other was tentatively proposed to originate from the vacuoles that store polyphosphate. Here we report on a detailed $^{31}$P NMR investigation of the phosphate pools in the yeasts *Candida utilis*, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. In the present study we have focussed on the response of cytoplasmic and vacuolar pH in intact yeast cells upon the addition of substrate under both aerobic and anaerobic conditions. Furthermore, the in vivo transport of inorganic phosphate across the vacuolar membrane during aerobic glycolysis in *Candida utilis* will be discussed. So far, most $^{31}$P NMR studies in yeasts have concentrated on *S. cerevisiae* (Navon et al. 1979; Den Hollander et al. 1981; Gillies et al. 1981; Jacobson and Cohen 1981). Here we compare this organism with *C. utilis* and *Z. bailii*. We included the latter yeast since it is known to be an organism with a strong tendency for acid production (Nickerson and Carroll 1945).

## Materials and Methods

### *Growth and Harvesting of Cells*

*Candida utilis* CBS 621, *Saccharomyces cerevisiae* CBS 8066 and *Zygosaccharomyces bailii* CBS 749 were obtained from Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands. The yeasts were grown in 100 ml Erlenmeyer flasks, containing 50 ml yeast extract with 2% glucose, incubated at 30°C under aerobic conditions in a rotary shaker. In some experiments a growth medium was used containing higher phosphate concentrations, as indicated in the text. Cells were harvested at the end of the logarithmic phase of growth. Before centrifugation, the cultures were cooled to about 5°C in an ice bath under vigorous aeration. The cells were collected by low-speed centrifugation at 5°C and washed twice in the ice-cold resuspension medium which contained 2.5 mM $MgCl_2$, 10 mM KCl and 2.4 mM $CaCl_2$. Finally, the cells were washed once and resuspended in the same medium, except that 7 mM

*Offprint requests to:* K. Nicolay

*Non-Standard Abbreviations:* NMR, nuclear magnetic resonance; ppm, parts per million; PP, polyphosphate; $P_{i,c}$, cytoplasmic inorganic phosphate; $P_{i,v}$, vacuolar inorganic phosphate; $pH_{in,c}$, cytoplasmic pH; $pH_{in,v}$, vacuolar pH; FCCP, carbonyl p-trifluoromethoxyphenylhydrazone

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potassium phosphate, pH 6.0, and 10% $^2H_2O$ were added. The cell pellet volume was usually 30–40% of the total sample volume. The cells were stored on ice until studied by NMR.

### *$^{31}P$ NMR*

$^{31}P$ NMR spectra were obtained at 145.8 MHz using a Bruker HX-360 spectrometer operating in the Fourier-transform mode. Accumulation was carried out employing 60° pulses and a 0.34 s repetition time. Routinely, time profiles were obtained by sequentially storing on disk free induction decays, each consisting of 100 to 250 scans. Glycerophosphorylcholine at 0.49 ppm relative to 85% orthophosphoric acid was used as an internal chemical shift marker.

Unless otherwise indicated, NMR experiments were carried out at 22 ± 1 °C. Samples consisted of 3 ml of the yeast cell suspension in 10 mm tubes.

Aerobic conditions in the NMR tube were obtained by bubbling pure $O_2$ gas through the suspensions; anaerobiosis was created by using $N_2$ gas. In all experiments, gas was bubbled through a capillary just above the bottom of the NMR tube at a rate of 30–40 ml/min.

50 µl Antifoam was added to the cell suspension prior to each experiment.

Substrates were injected directly into the NMR tube inside the magnet. This was achieved by introducing 200 µl glucose or ethanol solution into a bypass of the silicone tubing used for the gas supply. Subsequently, the gas flow was directed through the bypass, thus pushing the substrate solution into the yeast suspension.

### *Perchloric Acid Extracts*

Cell extracts were prepared by perchloric acid digestion as described by Navon et al. (1979).

### *Intracellular pH*

The intracellular pH was determined from the chemical shifts of the internal $P_i$ resonances by using a calibration procedure described by Den Hollander et al. (1981).

### *Materials*

FCCP was purchased from Sigma while silicone antifoam was obtained from B.D.H.

## Results

### *Compartmentation of Intracellular Phosphate and pH*

Figure 1 shows 145.8 MHz $^{31}P$ NMR spectra of a suspension of *Candida utilis* cells under anaerobic and aerobic conditions. The spectra were taken in the absence of energy source. In the anaerobic suspension (Fig. 1 A) two inorganic phosphate pools at different pH environments were observed. Aeration (Fig. 1 B), however, split the $P_i$-peak at higher field (at 1.0 ppm) into two distinct resonances. Hence, in a suspension of *C. utilis* three $P_i$ pools were discriminated by $^{31}P$ NMR: $P_{i,c}$, $P_{i,v}$ and $P_{i,ex}$. The resonance labeled $P_{i,c}$ was assigned to cytoplasmic inorganic phosphate on the basis of the following observations:

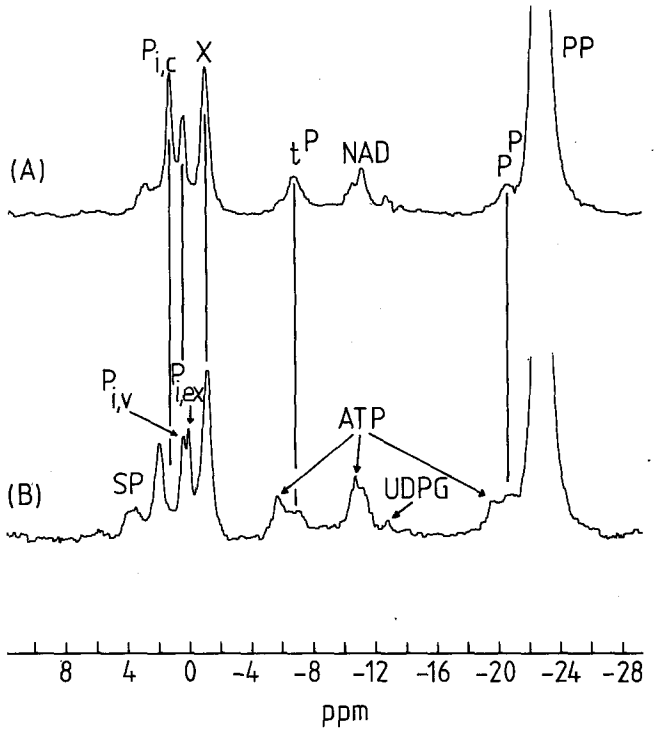


**Fig. 1 A, B.** $^{31}P$ NMR spectra of an aerobic and an anaerobic suspension of *Candida utilis* cells in the absence of substrate. Cells were resuspended at a density of 40% wet weight. The spectra represent the time-average of 500 scans. **A** Anaerobic suspension; **B** Aerobic suspension. Vertical lines indicate the chemical shift positions of the $P_{i,c}$, $P_{i,v}$ plus $P_{i,ex}$, $t^P$ and $P^P$ and *X* peaks in Fig. 1 A. Assignments were made on the basis of chemical shifts, by pH titration of perchloric acid extracts, and by addition of specific compounds to the extracts. *SP*, sugarphosphates; $P_{i,c}$, cytoplasmic $P_i$; $P_{i,v}$, intracellular $P_i$, most probably from the vacuole; $P_{i,ex}$, extracellular $P_i$; *X*, unassigned; *ATP*, adenosine triphosphate; *NAD*, nicotinamide adenine dinucleotides (reduced plus oxidized); *UDPG*, uridine diphosphoglucose; $t^P$, $P^P$ and *PP*, terminal penultimate and middle peaks of polyphosphates, respectively. In $t^P$, inorganic pyrophosphate is included

a) upon glucose introduction $P_{i,c}$ became very low initially when sugarphosphate levels were high while it regained its original intensity after sugar consumption (see below);

b) when glycolysis was inhibited by iodoacetate, glucose addition led to accumulation of sugar phosphates (mainly fructose-1,6-bisphosphate) and complete depletion of $P_{i,c}$, leaving the other inorganic phosphate resonances unaffected (data not shown);

c) the pH as determined from the chemical shift of $P_{i,c}$ agreed, within the accuracy of the method, with the pH as calculated from the chemical shift of the sugar phosphates. Since glycolysis is carried out in the cytoplasm, $P_{i,c}$ must originate from this intracellular compartment. The rise in cytoplasmic pH from 6.40 to 6.96 upon aeration (Fig. 1 A and 1 B, respectively) can be explained by higher ATP concentrations under aerobic conditions. Hydrolysis of ATP via the plasma membrane ATPase (Goffeau and Slayman 1981) leads to proton ejection causing alkalinization of the cytoplasm and acidification of the external medium, in agreement with the results presented in Fig. 1.

The other intracellular inorganic phosphate resonance labeled $P_{i,v}$ showed only a slight upfield shift upon aeration, indicating that the pH in the compartment containing this

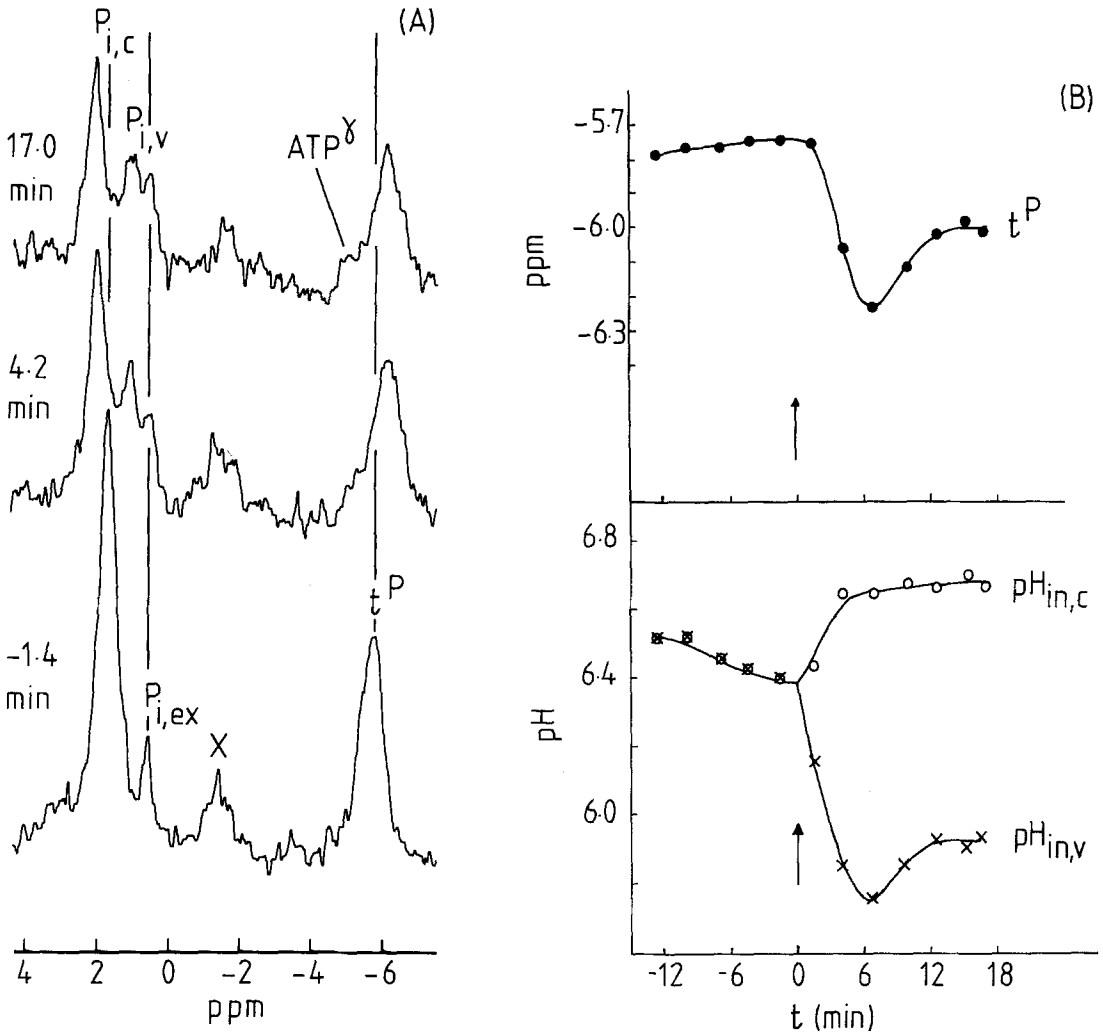


**Fig. 2A, B.** Response of *Saccharomyces cerevisiae* to addition of ethanol. **A** Orthophosphate region of $^{31}P$ NMR spectra of an aerobic suspension before and after addition of 200 mM ethanol at $t = 0$. Each spectrum represents the sum of two consecutive blocks of 250 scans. A cell density of 30% wet weight was used. Vertical lines indicate the chemical shift positions of the peaks $P_{i,c}$ plus $P_{i,v}$, $P_{i,ex}$ and $t^P$ resonances immediately before introduction of ethanol. Symbols as in the legend to Fig. 1. **B** Time evolution of the cytoplasmic pH ($pH_{in,c}$) and the pH in the second internal compartment ($pH_{in,v}$) determined from the chemical shifts of the peaks $P_{i,c}$ and $P_{i,v}$, respectively, as described in Materials and Methods. Before ethanol addition peaks $P_{i,c}$ and $P_{i,v}$ overlap completely. Hence, $pH_{in,v}$ cannot be determined accurately. Furthermore, the time course of the chemical shift of the terminal phosphate of PP($t^P$) is indicated. $ATP^\gamma$, terminal phosphate of adenosine triphosphate

phosphate pool underwent a minor acidification (from pH 5.48 to 5.43).

The addition of 20 mM $NH_4Cl$ to the cell suspension led to the superposition of the $P_{i,c}$ and $P_{i,v}$ resonances within 3 min (not shown). Since ammonium salts are known to eliminate pH gradients in many cellular systems (Pollard et al. 1979) this observation leads us to conclude that the interaction with $H^+$ (i.e. pH) is the main factor leading to different chemical shifts for the two intracellular inorganic phosphate fractions (see Discussion). This conclusion is further supported by our observation that superposition of the $P_{i,c}$ and $P_{i,v}$ resonances occurred upon addition of the protonophore FCCP.

A close examination of the chemical shift changes induced by the action of $NH_4Cl$ revealed that by far the largest shift was observed for the $P_{i,v}$ resonance (not shown). This might indicate that the cytoplasmic pH is more closely regulated than the pH in the second compartment (see also below).

Besides the $^{31}P$ NMR spectra of *C. utilis*, also those of the yeasts *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* revealed two significant internal $P_i$ pools. $^{31}P$ NMR spectra of an aerobic suspension of *S. cerevisiae* cells before and after ethanol addition are shown in Fig. 2A. It can be seen that after the introduction of ethanol at t = 0 cytoplasmic pH ($pH_{in,c}$) increased, probably due to proton translocation coupled to hydrolysis of ATP via the plasma membrane ATPase (see above). Another factor contributing to the observed alkalinization of the cytoplasm was the transport of protons from the cytoplasm to the compartment containing $P_{i,v}$ since the latter was acidified considerably upon ethanol addition (Fig. 2B). During the period of constant $pH_{in,c}$ and $pH_{in,v}$ the pH gradient across the membrane separating both internal aqueous phases was about 0.8 units.

Upon addition of ethanol to a suspension of *S. cerevisiae* cells the terminal peaks of polyphosphate experienced a shift to high field (Figs. 2A and 2B) indicating that a major fraction of the polyphosphate observed by $^{31}P$ NMR in *S. cerevisiae* was contained in a compartment undergoing a decrease in pH. This observation excluded the cytoplasm as the principal source of polyphosphate since $pH_{in,c}$ increased

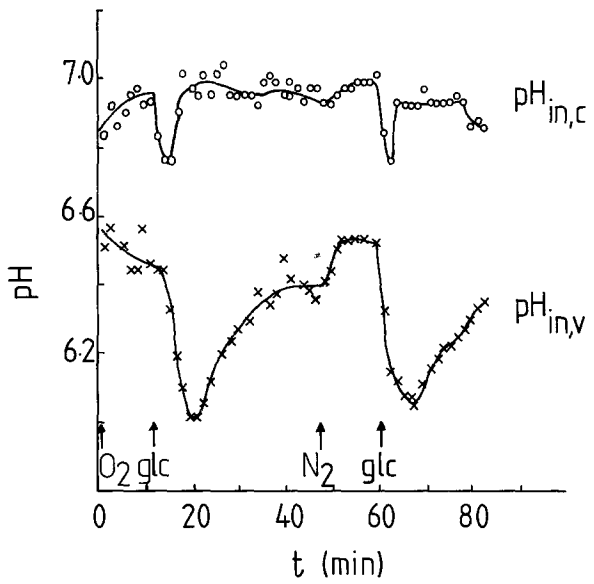


**Fig. 3.** Time course of cytoplasmic and vacuolar pH in a cell suspension of *Zygosaccharomyces bailii* during aerobic and anaerobic utilization of glucose. Cells were grown, harvested, and resuspended at a cell density of 40% wet weight. $O_2$ indicates the start of oxygen bubbling while at the time indicated by $N_2$ the gas phase was changed to nitrogen. 100 mM glucose (*glc*) was added to the yeast suspension and $^{31}P$ NMR spectra were accumulated in consecutive blocks of 250 scans

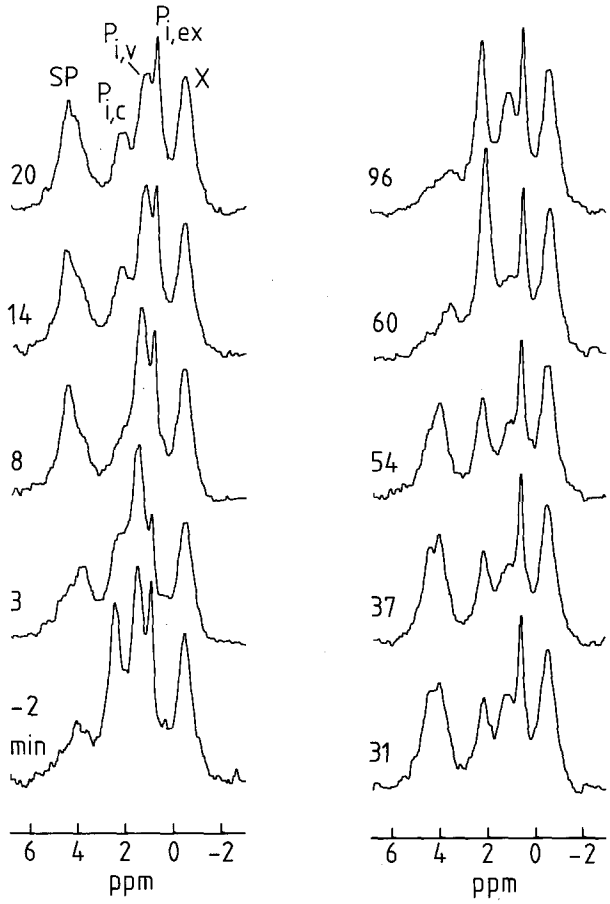


**Fig. 4.** Orthophosphate region of $^{31}P$ NMR spectra obtained during aerobic glucose utilization in a suspension of *Candida utilis* cells. At $t = 0$, 100 mM glucose was added. The cell density was 40% wet weight. Each spectrum represents the sum of 500 scans. The experiment was carried out at 8° C. Other conditions and symbols as in the legend to Fig. 1

upon addition of the substrate (Fig. 2B). Furthermore, it has been established that, within experimental error, all the polyphosphate observed by $^{31}P$ NMR was intracellular: both EDTA-shock and $MnCl_2$ addition had no effect upon the polyphosphate resonances (not shown). When a significant fraction of the polyphosphate would have been bound to the exterior of the cells in the periplasmic space, the treatments mentioned above would have led to an increase and a decrease in PP signal intensity, respectively. Urech et al. (1978) also concluded from other studies that no polyphosphate is located in the cell wall of *S. cerevisiae*. In this connection it is noteworthy that the chemical shift of the terminal phosphate of PP and $pH_{in,v}$ showed a very similar time course (Fig. 2B). Okorokov et al. (1980) have demonstrated that in yeasts $P_i$ can be concentrated in the vacuole relative to the cytosol. Moreover, under the growth conditions employed, the major PP-containing organelle in yeast is the vacuole (Urech et al. 1978), which also is the largest organelle (Ohsumi and Anraku 1981). Since changes in the chemical shift of resonance $P_{i,v}$ are strongly coupled to those in the terminal phosphate of PP, we conclude that the resonance labeled $P_{i,v}$ originates from vacuolar inorganic phosphate (see also Discussion).

In *Zygosaccharomyces bailii* cells, slightly different time courses of cytoplasmic and vacuolar pH upon glucose administration were observed (Fig. 3). Both under aerobic and anaerobic conditions a fast, transient decrease in cytoplasmic pH was seen at the onset of glucose metabolism. As the ATP level rose (not shown), cytoplasmic pH increased to 6.96 and 6.93 under aerobic and anaerobic conditions, respectively. Also the proton concentration in the vacuole became higher immediately after glucose addition. This period of acidification lasted for about 7 min, whereafter a slow increase in pH was observed. It is interesting to note that after the addition of glucose a time lag between the maximal responses of the cytoplasmic and vacuolar pH was registered in *Z. bailii*. The pH profile of the vacuole in this experiment cannot readily be explained. However, the cytoplasmic ATP concentration might play a crucial role, since the period of slow increase in $pH_{in,v}$ was parallelled by a slow decrease in ATP levels (not shown), which might result in lowered hydrolysis of ATP via the vacuolar membrane-bound $Mg^{2+}$-ATPase (Kakinuma et al. 1981) and, as a consequence, in lower $H^+$-translocation into the vacuole.

Figure 3 demonstrates the utmost importance of injecting the substrate directly into the NMR tube without interrupting the measurements. The metabolic response of the cells is so fast that the transient cytoplasmic acidification would have been largely missed, if the addition had been carried out outside the magnet. The experimental set-up employed allows $^{31}P$ NMR spectra to be taken immediately before and after the introduction of substrate into the cell suspension.

### *Intracellular Phosphate Transport*

Lowering the temperature at which yeast cells are incubated slows down the rates of metabolic processes, thereby allowing substrate consumption to be monitored with higher time resolution (Navon et al. 1979). When aerobic glycolysis in *C. utilis* was followed by $^{31}P$ NMR at 8° C, a number of interesting observations were made. Figure 4 shows the monophosphate region of $^{31}P$ NMR spectra obtained during

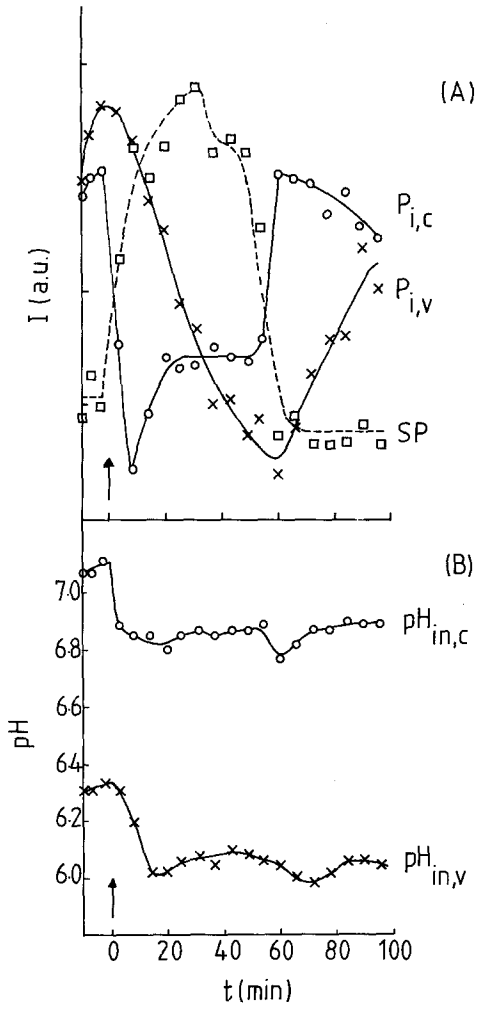


**Fig. 5A, B.** Time course of phosphate and sugarphosphate levels and cytoplasmic and vacuolar pH in an aerobic suspension of *Candida utilis* cells during glucose utilization at 8° C. Data were taken from the series shown partly in Fig. 4. 100 mM glucose was added at $t = 0$. **A** Time courses of the relative intensities of the cytoplasmic $P_i$, vacuolar $P_i$ and sugarphosphate resonances. **B** Time courses of the cytoplasmic ($pH_{in,c}$) and vacuolar pH ($pH_{in,v}$). Other conditions and symbols as described in the legend to Fig. 1

the course of this experiment. Figure 5A is a compilation of the time courses observed for the cytoplasmic $P_i$, vacuolar $P_i$ and total sugarphosphate resonance intensities while the cytoplasmic and vacuolar pH are plotted in Fig. 5B. The cytoplasmic $P_i$ concentration became very low during the first 10 min after glucose introduction. This was due to the build-up of high levels of sugarphosphate. However, in the period between 10 and 20 min $P_{i,c}$ showed a moderate increase, then remained constant between 20 and 50 min while sugarphosphate was also virtually constant. During this period vacuolar inorganic phosphate decreased until after about 60 min the sugar phosphates returned to their initial level. Then $P_{i,c}$ instantly rose to its original level. However, from 60 to 100 min it fell off again slowly while the vacuolar $P_i$ concentration increased significantly. Figures 4 and 5A clearly demonstrate antagonistic behaviour of the $P_{i,c}$ peak intensity on one hand and the $P_{i,v}$ and sugarphosphate peak intensities on the other hand. Exchange between $P_{i,c}$ and sugarphosphate could be ascribed to glycolytic activity. It should be stressed that during the experiment the intensity of the peaks originating from PP remained unchanged (not shown), while also external $P_i$ was essentially constant (Fig. 4). Hence, changes in cytoplasmic and vacuolar phosphate most probably originated from the transport of $P_i$ across the vacuolar membrane. In most experiments carried out at room temperature the vacuolar $P_i$ concentration did not experience the drastic changes shown in Figs. 4 and 5A. On the contrary, in these cases vacuolar $P_i$ remained essentially constant upon glucose introduction while the PP resonance intensities showed complex time courses with net polyphosphate hydrolysis occurring in the initial phase of glycolysis. Presumably, at 8° C polyphosphatases are inactive in hydrolyzing PP while at room temperature their action tends to keep vacuolar $P_i$ concentrations constant.

Under the experimental conditions both cytoplasmic and vacuolar pH were lower during glycolysis than in the resting cell suspension. It is interesting to note that after about 60 min a strong decrease in sugarphosphates was accompanied by a transient cytoplasmic acidification.

Figure 4 demonstrates that the sugarphosphate pool changed its composition during steady-state glycolysis. Between 10 and 20 min the sugarphosphate peak was most intense on the low field side while between 35 and 50 min the intensity was predominantly on the high field side. Through analysis of perchloric acid extracts of *C. utilis* it has been established that glucose-6-phosphate was the most abundant sugarphosphate in the early phase of glycolysis. Fructose-1,6-bisphosphate largely formed the sugarphosphate pool in the period between 35 and 50 min.

## Discussion

Many classical methods used in studying intracellular compartmentation require subcellular fractionation. Due to the vulnerability of many internal organelles it is often extremely difficult to isolate functionally intact preparations. $^{31}P$ NMR is not hampered by this drawback, since intact cells are used and monitored without perturbation. However, due to a combination of its limited sensitivity, the internal volume fraction of different compartments and the concentrations of free P-compounds therein, $^{31}P$ NMR registrates only two distinct intracellular aqueous phases in a yeast cell. These two internal compartments have been assigned to represent the cytoplasm and the vacuole. The resonance in the lower pH region cannot originate from mitochondrial $P_i$, because the respiring mitochondrion is expected to have a higher pH than the cytoplasm (Cohen et al. 1978).

The cytoplasmic and vacuolar pH have been calculated from the chemical shift of $P_i$ in both compartments, assuming the same calibration curve for both pools. The calibration curve has been measured in a medium made up to approximate the concentrations of the major intracellular cationic components in yeast (see Materials and Methods). However, it is well known that cations are unequally distributed over different organelles (Lichko et al. 1980; Okorokov et al. 1980). Consequently, different calibration curves, each obtained in the proper media, should be used to determine the pH in different compartments. This approach cannot be realized in practice, since the free concentrations of all ionic species interacting with $P_i$ in the cytosol and the vacuole are not known. The difference in apparent pH between the two compartments detectable by NMR is, however, much larger

than could have been caused by known interactions with $P_i$ (Roberts et al. 1981).

There are no gross differences in the cytoplasmic pH maintained by the yeasts *Candida utilis*, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. Although *Z. bailii* is known to have a strong tendency for acid production (Nickerson and Carroll 1945), the organism regulates its cytoplasmic pH to values near neutrality (Fig. 3). The acidification of the external medium which is observed upon substrate addition is somewhat larger in suspensions of *Z. bailii* than in suspensions of *C. utilis* and *S. cerevisiae* cells.

The transient cytoplasmic acidification observed in *Z. bailii* upon the introduction of glucose (Fig. 3) has also been reported for *S. cerevisiae* after glucose feeding (Den Hollander et al. 1981).

In all yeasts studied a large pH gradient (ranging from 0.8 to 1.5 pH units) across the vacuolar membrane is observed in the presence of energy source. Most probably, the main contribution to this gradient is generated by the vacuolar membrane-bound $Mg^{2+}$-dependent ATPase, the catalytic site of which is exposed to the cytoplasm (Kakinuma et al. 1981). The hydrolysis of cytoplasmic ATP by this enzyme is coupled to the translocation of protons into the vacuole. In right-side out vacuolar membrane vesicles an electrochemical potential gradient of protons across the tonoplast of 180 mV has been measured, with a contribution of the pH gradient of 1.7 pH units (interior acid) (Kakinuma et al. 1981). It has been shown that the electrochemical proton gradient is a driving force for the uptake of basic amino acids into the vacuole (Ohsumi and Anraku 1981). This organelle contains the bulk of the basic amino acids in yeast cells, especially arginine and ornithine (Dürr et al. 1979). The arginine is firmly retained in the vacuole, which has been partially explained by sequestration by polyphosphate (Dürr et al. 1979). Apart from being important in generating the driving force for energy-dependent transport processes across the tonoplast, the vacuolar ATPase might also play an important role in the regulation of cytoplasmic pH. Especially in plant vacuoles, the role of the tonoplast ATPase in cytosolic pH homeostasis has been demonstrated (Marin and Blasco 1982). In view of the results presented in Fig. 3, it is tempting to assume a role for the vacuolar membrane ATPase in an energy storage process. $H^+$ ions, accumulated in the vacuole during the early phase of glucose metabolism, and returning to the cytoplasm via the tonoplast ATPase in a later phase might, if leading to ATP synthesis, be a source of cytoplasmic energy.

The values for the vacuolar pH reported in the present study range from 5.4 to 6.6. Navon et al. (1979) reported a value of 6.5 for the vacuolar pH under energized conditions for intact cells of *S. cerevisiae*. In vacuolar membrane vesicles derived from *S. cerevisiae* a value of 5.2 was reported upon ATP addition (Kakinuma et al. 1981). Among the yeasts studied, there is a large difference in the pH gradient across the tonoplast before the addition of substrate. In *C. utilis* (Figs. 1 and 5B) and *Z. bailii* (Fig. 3) this pH gradient is 0.8 to 0.9 and 0.5 pH units, respectively; in *S. cerevisiae* (Fig. 2B), however, it is negligible. Although these observations are not completely understood, they might be explained by differences in the availability of endogenous reserve material.

By differential extraction of ions, it has been established that in yeast large concentration gradients of $P_i$ may occur across the tonoplast (Okorokov et al. 1980) showing predominant accumulation in the vacuole. From the present report it is clear that the relative concentrations of vacuolar and cytoplasmic $P_i$ may differ among yeasts but in particular are strongly dependent upon metabolic conditions (compare for example Figs. 2A and 4). Assuming the vacuolar volume to be 25% of the protoplast volume (Hüber-Wälchli and Wiemken 1979; Okorokov et al. 1980) the ratio of the concentrations of vacuolar to cytoplasmic $P_i$ ranges from 0.4 to 25. It is interesting to note that in *C. utilis* this ratio, as determined by $^{31}P$ NMR, is essentially independent of the phosphate concentration in the growth medium within the range from 1 to 50 mM (results not shown). The same holds for the levels and the average chain length of the polyphosphate in these cells.

The average chain length of PP can be estimated from the relative intensities of the middle and penultimate phosphate peaks in $^{31}P$ NMR spectra of yeasts. For *C. utilis* cells this number ranges from 20–40 which is consistent with previous reports upon vacuolar PP in yeasts (Urech et al. 1978). PP with mol.wt. up to 250,000 yields relatively sharp $^{31}P$ NMR resonances (Jacobson et al. 1982). Hence, it is likely that all polyphosphate in yeast is observed by $^{31}P$ NMR unless it is immobilized by complexation into rigid, high molecular weight structures.

Since the line widths of vacuolar and cytoplasmic $P_i$ are comparable (40–60 Hz), it can be concluded that there are no gross differences in the osmotic state between the cytoplasmic and vacuolar contents in yeasts as was also concluded for yeast ascospores (Barton et al. 1980).

The intracellular $P_i$ concentration is a major factor in the control of glycolysis both in bacteria (Mason et al. 1981) and yeasts (Den Hollander et al. 1981). One of the regulatory mechanisms for maintaining optimal cytoplasmic $P_i$ concentrations in yeasts might be the exchange of $P_i$ across the tonoplast (Figs. 4 and 5A). It is unclear from the present study whether the electrochemical potential gradient of protons across this membrane might represent a driving force for this transport. In rat liver mitochondria $P_i$ is distributed across the mitochondrial membrane according to the pH gradient only (Ogawa et al. 1981; Wohlrab and Flowers 1982). This is clearly not the case in yeast vacuoles (see for example Figs. 5A and 5B) since a drastic change in the pH gradient across the tonoplast does not trigger an analogous change in the $P_i$ concentration gradient and vice versa, as should be found if electroneutral exchange of $H_2PO_4^-$ versus $OH^-$ would occur. Although the experiment shown in Figs. 4 and 5 was clearly carried out under non-physiological conditions, it strongly suggests that the decrease in temperature allows the regulation of the exchange of $P_i$ between its cytosolic and vacuolar pools to be studied in vivo.

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