Original Papers



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

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Abstract. ³¹P 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 ³¹P 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 ³¹P 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

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

physiological pH values, ³¹P 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 ³¹P 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 ³¹P 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 ³¹P 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 ³¹P 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 MgCl₂, 10 mM KCl and 2.4 mM CaCl₂. Finally, the cells were washed once and resuspended in the same medium, except that 7 mM

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potassium phosphate, pH 6.0, and 10% ²H₂O 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.

$^{31}P NMR$

³¹P 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 $22 \pm 1^{\circ}$ 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.

 $50\,\mu$ l 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 μ l 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 ³¹P 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. 1A) two inorganic phosphate pools at different pH environments were observed. Aeration (Fig. 1B), 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 ³¹P NMR: P_{i,e}, P_{i,v} and P_{i,ex}. The resonance labeled P_{i,e} was assigned to cytoplasmic inorganic phosphate on the basis of the following observations:



Fig. 1A, B. ³¹P 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 $P_{i,e}$, $P_{i,v}$ plus $P_{i,exx}$, t^P and P^P and X peaks in Fig. 1A. 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; $P_{i,ex}$, cytoplasmic P_i ; $P_{i,v}$, intracellular P_i , most probably from the vacuole; $P_{i,exx}$, 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 $P_{i,e}$ 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 $P_{i,e}$, 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. 1A and 1B, 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 ³¹P 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^{q} , terminal phosphate of adenosine triphosphate

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

The addition of 20 mM NH₄Cl 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 ³¹P NMR spectra of *C. utilis*, also those of the yeasts Saccharomyces cerevisiae and Zygosaccharomyces bai-

lii revealed two significant internal P_i pools. ³¹P 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 ³¹P 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,e} 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. O₂ indicates the start of oxygen bubbling while at the time indicated by N₂ the gas phase was changed to nitrogen. 100 mM glucose (*glc*) was added to the yeast suspension and ³¹P NMR spectra were accumulated in consecutive blocks of 250 scans

upon addition of the substrate (Fig. 2B). Furthermore, it has been established that, within experimental error, all the polyphosphate observed by ³¹P NMR was intracellular: both EDTA-shock and MnCl₂ 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_{iv} 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.



Fig. 4. Orthophosphate region of ³¹P 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

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 Mg²⁺-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 ³¹P 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 ³¹P NMR at 8°C, a number of interesting observations were made. Figure 4 shows the monophosphate region of ³¹P 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 $(pH_{in,e})$ and vacuolar pH $(pH_{in,e})$. 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 buildup 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,e} 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 Pi,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. ³¹P 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, ³¹P 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 Mg²⁺-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 energydependent 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 ³¹P 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 ³¹P 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 ³¹P NMR resonances (Jacobson et al. 1982). Hence, it is likely that all polyphosphate in yeast is observed by ³¹P 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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References

- Barton JK, Den Hollander JA, Lee TM, MacLaughlin A, Shulman RG (1980) Measurement of the internal pH of yeast spores by ³¹P nuclear magnetic resonance. Proc Natl Acad Sci USA 77:2470-2473
- Busby SJW, Gadian DG, Radda GK, Richards RE, Seeley PJ (1978) Phosphorus nuclear-magnetic-resonance studies of compartmentation in muscle. Biochem J 170:103-114

- 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
- Dürr M, Urech K, Boller Th, Wiemken A, Schwencke J, Nagy M (1979) Sequestration of arginine by polyphosphate in vacuoles of yeast (Saccharomyces cerevisiae). Arch Microbiol 121:169-175
- Gadian DG (1982) Nuclear magnetic resonance and its applications to living systems. Oxford University Press, Oxford
- Gillies RJ, Ugurbil K, Den Hollander JA, Shulman RG (1981) ³¹P NMR studies of intracellular pH and phosphate metabolism during cell division cycle of *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 78:2125-2129
- Goffeau A, Slayman CW (1981) The proton-translocating ATPase of the fungal plasma membrane. Biochim Biophys Acta 639:197-223
- Hüber-Wälchli V, Wiemken A (1979) Differential extraction of soluble pools from the cytosol and the vacuoles of yeast (*Candida utilis*) using DEAE-dextran. Arch Microbiol 120:141-149
- Jacobson L, Cohen JS (1981) Improved technique for investigation of cell metabolism by ³¹P NMR spectroscopy. Bioscience Reports 1:141– 150
- Jacobson L, Halmann M, Yariv J (1982) The molecular composition of the volutin granule of yeast. Biochem J 201:473-479
- 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
- Lichko LP, Okorokov LA, Kulaev IS (1980) Role of vacuolar ion pool in Saccharomyces carlsbergensis: potassium efflux from vacuoles is coupled with manganese or magnesium influx. J Bacteriol 144:666– 671
- Marin B, Blasco F (1982) Further evidence for the proton pumping work of tonoplast ATPase from *Hevea latex* vacuole. Biochem Biophys Res Commun 105:354-361
- Mason PW, Carbone DP, Cushman RA, Waggoner AS (1981) The importance of inorganic phosphate in regulation of energy metabolism of *Streptococcus lactis*. J Biol Chem 256:1861-1866
- 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
- Nickerson WJ, Carroll WR (1945) On the metabolism of Zygosaccharomyces. Arch Biochem 7:257-271

- Nicolay K, Lolkema JS, Hellingwerf KJ, Kaptein R, Konings WN (1981) Quantitative agreement between the values for the light-induced ΔpH in *Rhodopseudomonas sphaeroides* measured with automated flow-dialysis and ³¹P NMR. FEBS Lett 123:319-323
- Nicolay K, Hellingwerf KJ, Van Gemerden H, Kaptein R, Konings WN (1982) ³¹P NMR studies of photophosphorylation in intact cells of *Chromatium vinosum*. FEBS Lett 138:249-254
- Ogawa S, Boens CC, Lee T-M (1981) A ³¹P nuclear magnetic resonance study of the pH gradient and the inorganic phosphate distribution across the membrane in intact rat liver mitochondria. Arch Biochem Biophys 210:740-747
- 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, Kulaev IS (1980) Vacuoles: main compartments of potassium, magnesium, and phosphate ions in Saccharomyces carlsbergensis cells. J Bacteriol 144:661-665
- Pollard HB, Shindo H, Creutz CE, Pazoles CJ, Cohen JS (1979) Internal pH and state of ATP in adrenergic chromaffin granules determined by ³¹P nuclear magnetic resonance spectroscopy. J Biol Chem 254:1170-1177
- Roberts JKM, Jardetzky O (1981) Monitoring of cellular metabolism by NMR. Biochim Biophys Acta 639:53-76
- Roberts JKM, Wade-Jardetzky N, Jardetzky O (1981) Intracellular pH measurements by ³¹P nuclear magnetic resonance. Influence of factors other than pH on ³¹P chemical shifts. Biochemistry 20:5389-5394
- Tielens AGM, Nicolay K, Van den Bergh SG (1982) ³¹P-NMR studies of pH homeostasis in intact adult *Fasciola hepatica*. Molec Biochem Parasitology 6:175-180
- Ugurbil K, Shulman RG, Brown TR (1979) High-resolution ³¹P and ³¹C nuclear magnetic resonance studies of *Escherichia coli* cells in vivo. In: Shulman RG (ed) Biological applications of magnetic resonance. Academic Press, New York, pp 537–589
- Urech K, Dürr M, Boller Th, Wiemken A, Schwencke J (1978) Localization of polyphosphate in vacuoles of Saccharomyces cerevisiae. Arch Microbiol 116:275-278
- Wohlrab H, Flowers N (1982) pH-Gradient dependent phosphate transport catalyzed by the purified mitochondrial phosphate transport protein. J Biol Chem 257:28-31

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