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Measurement of the internal pH of yeast spores by ^{31}P nuclear magnetic resonance

(dormancy/germination/compartmentation)

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ABSTRACT **The asci and single spores of the yeast *Pichia pastoris* were examined by using ^{31}P NMR. The pH within the spores was determined from the pH-dependent chemical shifts of the internal orthophosphate (P_i) peaks. In spectra of asci, two internal P_i peaks corresponding to two compartments were observed. Only small variations in the internal pH values were found upon incubation of the asci in buffers ranging in pH from 3 to 10. For this range in external pH, the internal pH values calculated from the P_i chemical shifts were 5.5–6.3 and 5.1–5.9. The two internal P_i peaks, which have line widths of ≈60 Hz, have been assigned to spore and epiplasmic compartments, respectively. Spectra of single spores revealed only one P_i peak, and, upon germination of either the single spores or asci, only this P_i peak disappeared. Peaks corresponding to ATP were not found in the dormant spores and asci but did appear upon germination. These NMR studies show the yeast spore to have a mobile and relatively acidic interior.**

High-resolution ^{31}P nuclear magnetic resonance (NMR) has been used to measure internal pH in intact biological systems (1–6). Its advantages over previous methods (7, 8) are that it is noninvasive, that cellular compartments can be distinguished, and that changes in pH with time can be followed rapidly. The basis of this method is that the ^{31}P NMR chemical shifts of intracellular phosphate metabolites, in particular orthophosphate (P_i), are a function of pH. Application of this technique has revealed appreciable pH differences across plasma membranes in microorganisms. For example, in energized *Escherichia coli* (5) the intracellular pH determined in this fashion was found to be constant at 7.4 when the external pH varied from 6.0 to 8.0.

In bacterial (9, 10) and fungal (11) spores metabolic rates are slow for reasons that are not understood. It is known that they possess high concentrations of molecules that are potentially energy sources, such as 3-phosphoglycerate (12, 13) and trehalose (14–16), and that they possess a full complement of glycolytic enzymes (17). Although explanations of slow metabolic rates in terms of divalent metal ion concentrations or of low water content have been offered (18, 19), they have not been completely substantiated. Because glycolytic rates are pH dependent (5), it appeared to us important to determine the pH inside the spore. In a preliminary ^{31}P NMR study of spores of *Bacillus subtilis* we found a ^{31}P NMR signal whose position was interpreted as indicating an internal pH of 6.3. Because this peak was broad, presumably because of a high Mn^{2+} content (20), and not always reproducible, we examined instead, with more success, the asci and single spores of the yeast *Pichia pastoris*§ by ^{31}P NMR¶.

Fig. 1 shows a 145.7-MHz ^{31}P NMR spectrum of a suspension of yeast asci, each of which contains four haploid spores, in Tris·HCl buffer, pH 7.9, without added phosphate. The most prominent peaks of the spectrum, at −0.97 and −1.25 ppm, come from P_i. The pH values corresponding to these two chemical shifts are 5.7 and 6.1, both of which are considerably below the external pH. These pH values are assigned to the peaks by using a calibration curve measured in 210 mM salt (5). Because the pH of the asci is definitely lower than the external pH, and furthermore recalling that any external phosphate is too dilute to be observed, we conclude that these two pH values correspond to two different internal compartments of the ascus. Other NMR peaks in Fig. 1 are assigned to NAD^+ and polyphosphate.

The chemical shifts of the internal P_i peaks have been obtained for two different batches of asci and for one batch of single spores after incubation with external media at different pH values. The calculated pH values are plotted in Fig. 2.

It is important to notice that the single spores give rise to only one P_i peak and that this peak's chemical shift coincides with the lower-field peak of the ascus. It is therefore likely that the two P_i peaks of the ascus represent compartmentation of inorganic phosphate within the ascospores and epiplasmic space. The separated single spores, stripped of the mother cell wall and membrane, obviously lack this epiplasmic space and in the spectrum only one P_i peak is observed. The corresponding ΔpH between compartments is 0.4 pH units and the epiplasm is more acidic. The pH of both compartments has been determined from these measurements while the external pH was changed from 3 to 10 (Fig. 2). In both compartments the internal pH values were much more constant, varying by ≈0.8 pH unit over this large range of external values. The spore pH varied from 5.5 to 6.3 when the external pH was changed from 3 to 10, while the epiplasm pH varied from 5.1 to 5.9.

Similar internal pH values of resting yeast over the same range of external pH values have been reported (3). We have

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§ C. P. Kurtzman (U.S. Department of Agriculture, Peoria, IL) kindly pointed out (personal communication) that the yeast strain Y55, although closely related to *Saccharomyces cerevisiae*, is a member of the species *P. pastoris*.

¶ On the basis of our preliminary results, the pH in *B. megaterium* spores has been measured by conventional methods, by which it was also found to be acidic, as discussed by Setlow and Setlow in the companion paper (21).

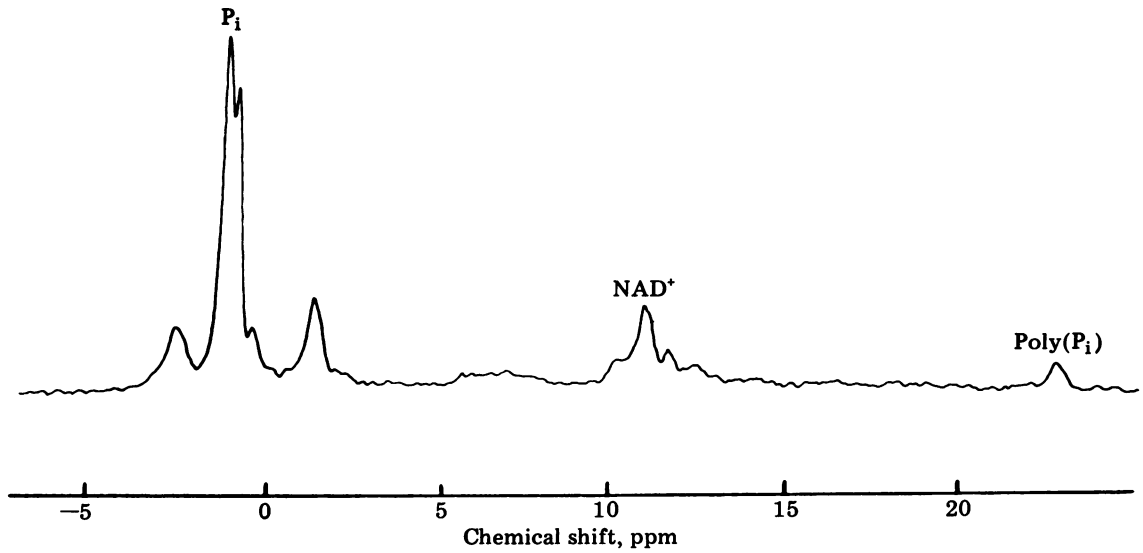

FIG. 1. The 145.7-MHz ^{31}P NMR spectra of a suspension of yeast asci (4×10^9 cells per ml) in 50 mM Tris·HCl, pH 7.9. Asci were incubated in buffer for 18 hr at 4°C and resuspended in fresh buffer before spectra were obtained. Each spectrum represents a sum of 1000 scans with a repetition time of 0.3 sec (line broadening = 8 Hz). Chemical shifts relative to H_3PO_4 were calibrated by using glycerophosphocholine. By comparison with acid extracts of yeast cells, the labeled peaks correspond to inorganic phosphate (P_i), nicotinamide dinucleotide (NAD^+), and the internal phosphates of polyphosphate [poly(P_i)]. Unlabeled peaks in the spectrum have not been assigned. Large quantities of asci were prepared by the following method (22, 23): The diploid strain *P. pastoris* Y55 (originally obtained from T. R. Eccleshall) was grown from an inoculum to stationary phase in standard YEPD (1% yeast extract/2% Bactopeptone/2% glucose) medium at 30°C and harvested after 41 hr. Thereafter cells were sonicated and subjected to density centrifugation through 20% mannitol in order to remove small buds (24). The cells were then transferred to well-aerated sporulation medium (2% potassium acetate, pH 7.0) at a density of 2×10^8 cells per ml. Incubation at 30°C for 1–4 days yielded ≥95% ascospores. Asci were then harvested, washed three times with sterile water, and stored at 4°C as a thick suspension until spectra were obtained. The percent ascospore formation was determined with a phase-contrast microscope by hemocytometer counts of cells containing refractile spores; subsequent germination (≥92%) was monitored by the loss in spore refractility.

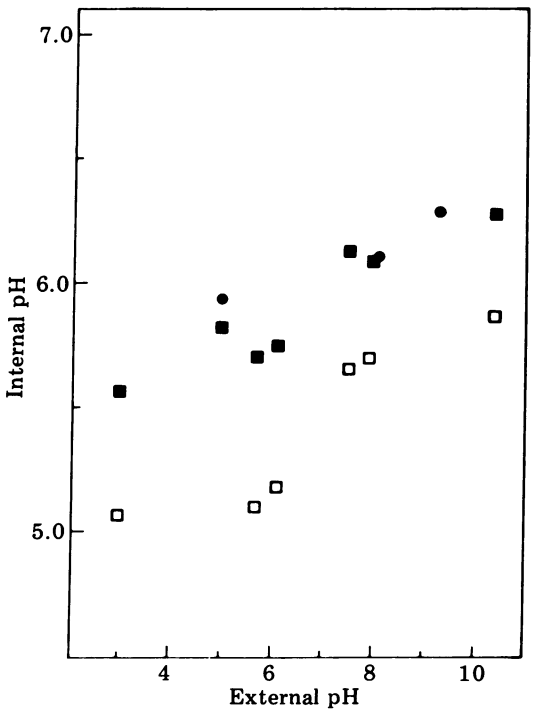

FIG. 2. Internal pH, measured from the chemical shifts of P_i, of yeast asci (■, □) and single spores (●) after incubation for 18 hr in buffers at various pH values. □s represent the pH derived for the secondary components of the P_i peaks. Single spores were obtained by using the following modified procedure (23, 25): Thick suspensions of yeast asci were incubated for 2 hr at 37°C with 4% (vol/vol) Glusulase (Endo Laboratories, New York, NY)/20 mM mercaptoethanol/20 mM citrate phosphate, pH 7.0, and thereafter sonicated and washed three times with sterile water containing 1% Tween 80. Centrifugation at low speed ($200 \times g$) prevented clumping of spore suspensions.

repeated these experiments, with similar results, using this strain of yeast.

It should be noted that because the chemical shift of P_i is most sensitive to pH near the pK (of 6.7), fully resolved pairs of P_i peaks are observed only at the highest external (and internal) pH values. At the lower pH values the peaks are not well resolved and the more acidic (higher-field) peak appears as a shoulder. In addition to these two peaks, in some instances an additional shoulder at lower field than the spore P_i peak has been observed in spectra of both asci and single spores. The pH corresponding to this P_i peak is ≈6.5. These points are not plotted in Fig. 2.

The germination of the yeast asci has been monitored by ^{31}P NMR and lends support to the compartmental assignments. Fig. 3 shows the spectrum of asci before and during germination. At the external pH of 5.8, before the addition of glucose, the P_i peak assigned to the epiplasm appears as a poorly resolved shoulder on the high-field side of the main spore P_i resonance (Fig. 3, spectrum A). The chemical shifts of −1.31 and −1.05 indicate that the spore and epiplasmic pH values are 6.13 and 5.84, respectively. After glucose addition (Fig. 3, spectra B and C), the low-field P_i peak (pH 6.13) quickly diminishes, and the spectra show a simultaneous increase in the other phosphate peaks, in particular ATP. Because the more intense low-field P_i peak, assigned to the spores, disappears upon germination, the epiplasmic P_i peak no longer appears as a shoulder but is a well-resolved resonance and, within experimental error, remains constant in intensity and position. Consistent with these assignments, we have also found that the single P_i peak present in single spores disappears completely during the first 10 min of germination.

It is important to stress that measurements of proton activity by P_i chemical shift, by the distribution of a weak acid, and even by a pH electrode depend upon the ionic strength of the medium. In the compartments of the ascus the ionic strengths are

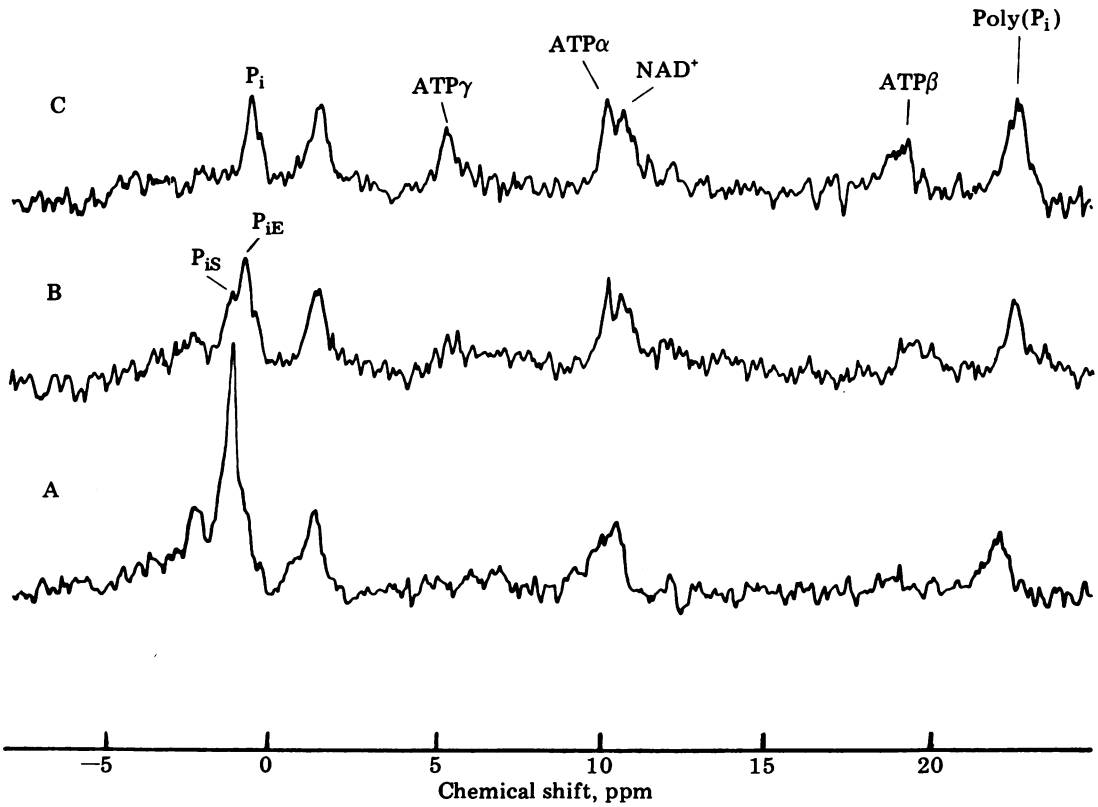

FIG. 3. ^{31}P NMR spectra of a suspension of asci (2×10^9 cells per ml) before and during germination at 20°C. P_{iS} and P_{iE} indicate the inorganic phosphate in spore and epiplasmic compartments, respectively. The asci had been freshly harvested, washed, and suspended in water (pH 5.8) before spectra were taken. Each spectrum represents a sum of 1000 scans. A, Asci before glucose addition. B, Accumulations between 2 and 7 min after glucose addition. C, Accumulations between 7 and 12 min.

not known and, indeed, large ionic strength differences between compartments may contribute to the P_i chemical shift differences we observe. These chemical shifts, however, clearly indicate the internal pH of the yeast ascus to be acidic and show a different chemical potential of the proton in the two compartments.

Assuming the chemical shift anisotropy of 200 ppm to be the major source of line broadening, we calculate from the 60-Hz linewidths of the P_i peaks that within the spore the rotational correlation time, τ_c, is 28 nsec. The diffusion coefficient calculated from this shows that the P_i within the spore is not immobilized. In fact, both the spore and the yeast cell have the same widths of the P_i peaks and therefore the same calculated values of τ_c. We determine, however, only an upper limit for τ_c. Partial binding of phosphate to macromolecules or membranes may also account for this apparent τ_c. The observed line widths could be explained by 0.2% binding in the limit of fast exchange. Other factors, such as magnetic field inhomogeneities within the sample or additional compartmentation, may also contribute to this peak width.

In summary, we have been able to observe the P_i peaks in the yeast ascus. The line widths were comparable to those in yeast cell spectra and indicate a liquid interior. Assuming a normal physiological ionic strength, we have derived the pH from the observed ^{31}P chemical shifts. In the ascus two P_i peaks corresponding to two compartments were observed and were assigned to spore and epiplasm. In support for these assignments, only one of the peaks (at higher pH) was observed in single spores and in both cases only this peak disappeared upon germination. Another observation was that ATP peaks, missing in the spores and asci, appeared upon germination. Finally, we have seen that the internal pH values in the ascus are acidic (in the vicinity of pH 6) and, although they are not tightly regulated, they did not go above pH 6.3. Because low pH has been shown to decrease glycolytic rates in *E. coli*, it is likely that the acidic interior plays some role in the spore's dormancy. However, further studies are needed to see how important the pH is as a controlling factor.

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