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High resolution ^{31}P nuclear magnetic resonance studies of intact yeast cells

(polyphosphates/intracellular pH)

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ABSTRACT **High resolution ^{31}P nuclear magnetic resonance (NMR) spectra at 145.7 MHz are presented for intact yeast cells. Several peaks are resolved and assigned. They include the middle phosphate peaks from long chain or cyclic polyphosphates. Our results are consistent with the suggestion that these polyphosphates act as a phosphate store in the cell. We have also been able to measure cytoplasmic pH using the orthophosphate peak inside the cell, as compared with outside the cell. The results show that yeast cells maintain their cytoplasmic pH around 6.3. This value is considerably higher than the acidic extracellular pH at which they normally live. These preliminary results indicate that ^{31}P NMR at 145.7 MHz can be a rapid, informative, and non-invasive method for probing biochemical events within living cells.**

The high sensitivity and resolution presently available in Fourier transform (FT) nuclear magnetic resonance (NMR) spectrometers has recently allowed the detection of ^{31}P resonances from metabolites in intact cells. Moon and Richards (1) have shown that with FT NMR techniques it is possible to observe ^{31}P resonances of diphosphoglycerate and inorganic phosphates in erythrocytes at 40.5 MHz, and have used the measured resonance positions to determine intracellular pH. Henderson *et al.* (2) have measured the concentrations of diphosphoglycerate and ATP in the erythrocyte during metabolic processes at frequencies of 36.4 MHz. Recently Hoult *et al.* (3) with the high sensitivity available from an FT NMR spectrometer operating at 129 MHz have been able to follow the reactions of ATP added to intact rat muscle and glycogen particles.

In the present paper we present the results of a ^{31}P NMR study of intact baker's yeast cells. The measurements have mainly been made at 145.7 MHz with a Bruker HX-360 NMR spectrometer. Yeast represent a convenient system for the study of ^{31}P NMR because, as has been known, they actively transport orthophosphate (4) and store phosphates as polymers in volutin granules (5). These stored polyphosphates are in a reversible equilibrium with orthophosphate. There is a question as to whether these stored polyphosphates are a phosphate or an energy store (6), but polyphosphates can accumulate to over 10% of the dry weight in yeast cells, which, as will be shown, leads to very intense NMR signals. From the NMR measurements we have been able to follow the hydrolysis of the polyphosphate reserve in yeast cells and to measure the intracellular pH as the pH of the medium is changed.

Abbreviations: NMR, nuclear magnetic resonance; FT, Fourier transform; ppm, parts per million.

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MATERIALS AND METHODS

Cultures of commercial baker's yeast (standard brands) were obtained by single cell isolation. The cells were grown in medium containing, on a per liter basis: 10 g of bactopeptone; 5 g of yeast extract; 4 g of $(NH_4)_2SO_4$; 1 g of KH_2PO_4; 0.5 g of $MgSO_4$; 0.5 g of $CaCl_2$; and 50 g of glucose. The pH was adjusted to 5 at the beginning of growth. The cells were grown to stationary phase. Cells grown on intermediate- and low-phosphate media had the same initial composition as above except for the amount of KH_2PO_4 added (intermediate = 10^{-2} g and low = 10^{-4} g). When the stationary phase of growth (determined by the number of cells per ml) was reached, the cultures of yeast cells were harvested by low-speed centrifugation at 4° in a Sorvall high-speed centrifuge. The cells, always in the cold, were packed to about ≥50% by volume and brought to room temperature when the extracellular pH was measured. D_2O was added to these cells to about a 10% level and the sample was placed in the NMR sample tube. When the extracellular pH was changed from the normal acidic level to more alkaline values NaOH was used. The sodium tripolyphosphate used for titration in these experiments was obtained as a gift from Monsanto in New Jersey.

The ^{31}P NMR spectra were measured on a Bruker HX-360 spectrometer at 145.7 MHz (84.5 kG) although one spectrum taken at 40.5 MHz on a Varian XL-100 NMR spectrometer is presented for comparison. In the XL-100 12 mm diameter tubes were used, whereas in the HX-360 10 mm tubes were used and both samples were 1.0 cm high. Unless otherwise specified, spectra were measured at 25°. The pulsed Fourier transform technique was used on both instruments. In the HX-360 the field homogeneity was adjusted on 10% D_2O added to the cell suspensions and the field was locked on that signal. In some cases, D_2O in a 4 mm capillary at the center of a 10 mm sample tube was used for the field lock. For the spectrum observed with the XL-100 the field was locked on the H_2O signal. In our experience with yeast and other cells, spinning the sample did not measurably sharpen most of the ^{31}P lines observed, although it did sometimes sharpen the narrow lines observed from extracellular orthophosphate (see below). Nevertheless, sample spinning was usually employed. All spectra are reported relative to 85% phosphoric acid at 0 parts per million ppm.

RESULTS

The ^{31}P NMR spectra at two different magnetic fields of intact yeast cells in stationary growth phase are shown in Fig. 1. Spectrum A shows an FT NMR spectrum at 40.5 MHz after 2924 pulses. Spectrum B shows an FT NMR spectrum

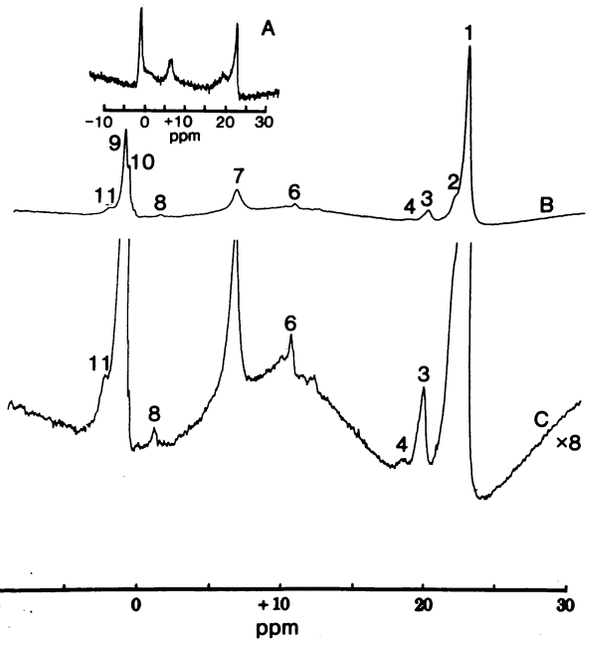

FIG. 1. Field dependence of 50% suspension of yeast cells in their stationary phase of growth. (A) Spectrum taken at 40.5 MHz by pulsed FT NMR; 12 mm sample tube, pH 6.4. This spectrum was recorded after 2924 accumulations with a repetition time of 2 sec per pulse, an H_2O lock, and without proton decoupling. (B) Spectrum taken at 145.7 MHz by pulsed FT NMR; 10 mm sample tube, pH 3.5, 4800 accumulations with a repetition time of 0.6 sec. The sample contained about 10% D_2O to lock the magnetic field. (C) Same spectrum as B, after turning up the gain a factor of 8.

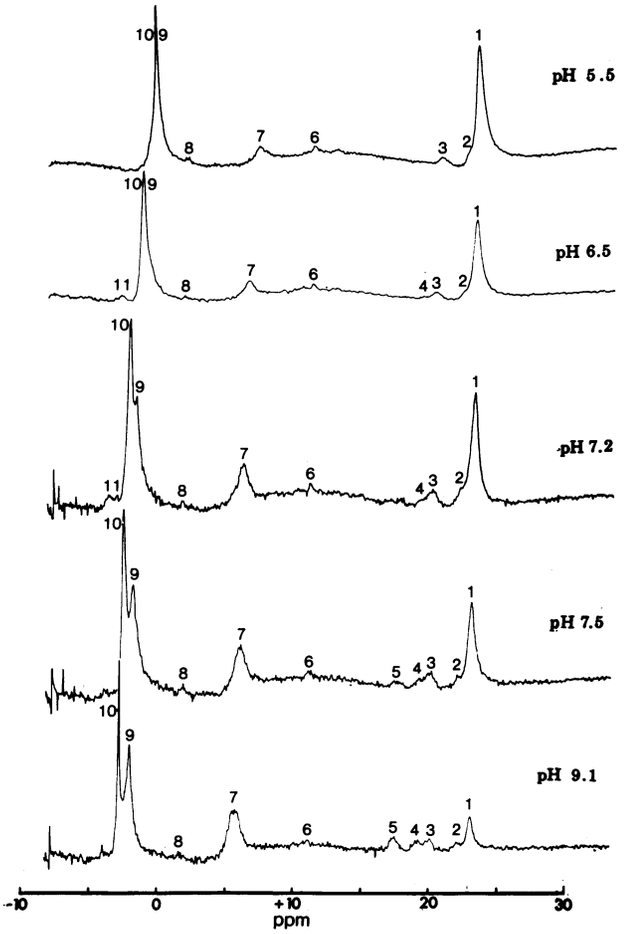

FIG. 2. ^{31}P NMR spectra of yeast cells at 145.7 MHz; 1000 pulses of 1 sec repetition time. The sample volume was variable because of dilution, thus only relative intensities within each spectrum should be compared. The yeast cells were from the stationary phase of growth and had the initial "normal" spectrum shown in Fig. 1 (B and C).

at 145.7 MHz after 4800 pulses. It is clear that the higher frequency gives better resolution because the larger magnetic field does not significantly broaden the prominent peaks of the yeast spectrum, as can be seen by comparison with spectrum A taken at the lower field. Additionally, and perhaps most significantly, the higher frequency spectrometer has a greater sensitivity. We estimate that the signal to noise ratio is about six times greater with the HX-360 than with the XL-100. This can be concluded from a comparison of the three spectra in Fig. 1 where spectrum C shows the same spectrum as B but with the display gain increased by a factor of 8.

Turning now to the positions, peak 1 in spectrum B of Fig. 1 resonates at 22.5 ppm upfield from 85% phosphoric acid. Peaks 2, 3, and 4 occur at about 20, 19, and 17.4 ppm upfield, respectively. These positions are consistent with those reported for nonterminal phosphates of polyphosphates (7, 8). By comparing the integrated area of peak 1 with measurements of a known orthophosphate concentration at similar conditions, we estimate that peak 1 corresponds to a ^{31}P concentration of 0.1 M within the cells.

Peak 6 resonates at +10.9 ppm and does not titrate. This is the position of an NAD resonance (1). This peak was estimated to represent about 2 mM of phosphate in the cell. The weak peak 8, which resonates at about +1.4 ppm, has not been assigned but it does coincide with ^{31}P resonances from phosphodiesters such as are found in tRNA (9). Peaks 9 and 10 are identified as orthophosphate inside and outside the cells, respectively. More will be mentioned about the pH dependence of these peaks. Peak 11 occurs between −1.9 to −4.1 ppm depending upon the pH, and coincides with peaks of phosphomonoesters such as the sugar phosphates.

Fig. 2 shows the pH dependence of normal yeast cells in the stationary phase of growth as the extracellular pH was raised by adding sodium hydroxide to the concentrated cell suspension in the NMR tubes. As can be seen, peak 1 does not titrate, consistent with it's being the resonance from central phosphates in polyphosphate chains. Note that peaks 2, 3, 4, 6, and 8 do not titrate, while peaks 7, 9, 10, and 11 do. Another interesting phenomena was that as the pH was raised the relative intensity of peak 1 decreased while those of 4 and 7 increased. A new peak, peak 5, was formed when the extracellular pH was raised above pH 7.0. The pH dependencies of these peaks are shown in Fig. 3.

Fig. 4 shows yeast cells which were grown on normal- (spectrum 1), intermediate- (spectrum 2), and low- (spectrum 3) phosphate media but which were otherwise the same. The extracellular pH was 3.5 for each set of cells. As can be seen, the intensities of peaks 1 and 9 are greatly influenced by the phosphate content of the growth medium. Note also that peak 5 in Fig. 4-3, usually seen in the normal cell at alkaline pH but not seen at acidic extracellular pH (Fig. 1), is also present. Even though the extracellular pH is the same for all three cells, it can be readily seen that peaks 7, 9, and 11 move downfield with decreasing intracellular phosphate, indicative of an increasing pH. The area of peak

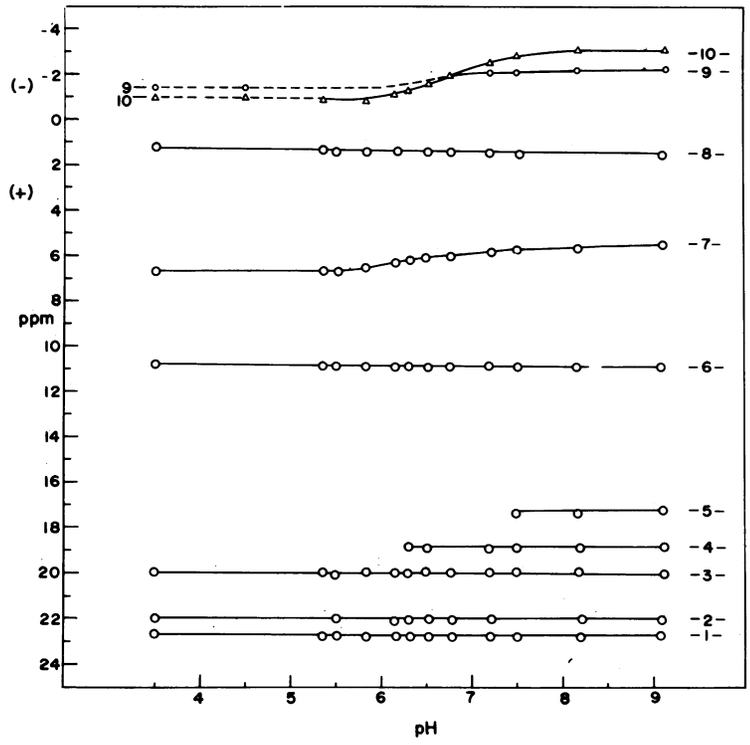

FIG. 3. Position (in ppm) of the various ^{31}P NMR lines seen in Figs. 1–2 at 145.7 MHz. Lines 1–6 and 8 do not titrate, while lines 7, 9, 10, and 11 do.

1 in spectrum 3 corresponds to a concentration of about 14 mM, down seven fold from normal.

The spectra shown in Figs. 1, 2, and 4 were measured on yeast samples which were harvested from the stationary phase of growth at 37°, and then brought to 25° where they were washed, centrifuged, and concentrated to about 50% cells by volume in the NMR tubes. The pH of the concentrated suspension was then adjusted to the indicated values, and the spectra were measured within twenty minutes. Subsequent spectra (and pH measurements) showed that the pH-dependent NMR peaks moved after several hours in the NMR tube, as did the pH. For the samples at low pH such as 3.5 both the NMR positions and the direct measurements indicated that the extracellular pH increased towards pH 6. Since these changes occurred under poorly defined conditions we performed another series of experiments, under better-specified conditions, with the results shown by the broken lines in Fig. 3. In these experiments after harvesting of the cells from the growth medium they were incubated for an additional hour at 37°, at the pH specified, in growth media without phosphate. The temperature was then lowered to 4°, the cells were centrifuged and made into a concentrated suspension, and the NMR spectra were measured within the first 14 min at 8°. Two lines were observed in the orthophosphate region with the stronger line attributed to intracellular phosphate, because the incubation in the absence of phosphate had reduced the extracellular phosphate considerably. In these experiments it was possible to follow the titration in the low pH region because it took several hours at 8° before the pH changed from the value used during incubation. Fig. 5 shows the *in vitro* titration curves of pyrophosphate and tripolyphosphate. The upfield line centered around +21 ppm is the center phosphate of tripolyphosphate and, as reported (7), is split into a 1:2:1 triplet. The downfield peaks are the end group phosphates and are doublets. Both end and center phosphates are titratable. The end phosphates show a very similar titration when compared with pyrophosphate. The titrations of peak 7 and 1 from yeast cells are also shown on this figure for comparison.

From the titratable orthophosphate lines inside and outside of the yeast cell, and knowing the titration of orthophosphate ^{31}P NMR positions, we can gain information about the pH within the cell at the location of orthophosphate. Fig. 6 shows results from such measurements using data from Figs. 2 and 3. The open circles show the movement of orthophosphate outside the yeast cell with pH (line 10). There is a linear relationship between the pH calculated from that line and the measured extracellular pH within experimental accuracy. When the same calculations are made for line 9, it is clear that this line represents orthophosphate in a different pH environment. The titration of this line suggests that the intracellular pH varies by only one pH unit as the extracellular pH varies from 3.5 to 9.1. At extracellular pH values below about 6, the intracellular *pH in the environment of orthophosphate* is at about 5.8 to 6. As the extracellular pH rises, the intracellular pH does respond by rising, but in a more attenuated fashion. Above pH ~6.3 the intracellular pH falls below the extracellular value.

Also shown in this figure is the calculated intracellular pH from line 9 in Fig. 4 for yeast cells grown on media containing various amounts of orthophosphate initially. As can be seen, all of these cells have a higher intracellular pH than the extracellular pH, which was ~3.5. However, cells grown on a depleted phosphate medium have a higher intracellular pH than those grown on the normal phosphate medium by nearly one pH unit. When orthophosphate is added to the cells whose spectra are shown in Fig. 4, a well-resolved line is seen at a higher field whose position is consistent with the measured extracellular pH. This shows that the environment of the orthophosphate responsible for line 9 has a higher pH.

DISCUSSION

Several peaks of the NMR spectra can be assigned with considerable confidence from the measured positions and pH dependences, and by comparing their measured intensities with those of known concentrations.

Peak 1 is at the same position (22.5 ppm) as resonances from the central phosphates of long chain or cyclic polyphosphates. Its insensitivity to pH changes means that it is from neither the terminal nor the penultimate phosphate. The pH-insensitive peak at +10.9 ppm (peak 6) is quite probably NAD or possibly an α-phosphate, bound on one side to another phosphate and on the other to an aliphatic carbon such as the α phosphate of ADP, ATP, or FAD. It is quite possible that peaks 6, 3, and 7 contain, amongst others, contributions from the α, β, and γ phosphates of ATP, respectively. Fig. 5 shows that while peak 7 titrates in the same region as the terminal phsophates in pyro- and tripolyphosphate it is not specifically either of these two exclusively.

Peak 8 is at the characteristic position of a phosphodiester bond such as has been found in tRNA. It does not move with pH, as expected from this assignment. It is interesting to note that the intensity of this resonance corresponds to about 1 mM phosphodiester bonds, while it is known (10) that in unicellular organisms like *Escherichia coli* the concentration of the tRNA phosphodiester bonds is about 30 mM. Hence we are seeing at most 3% of these phosphates, even though recent NMR studies of purified tRNA molecules showed that these ^{31}P resonances are narrow enough to be observed (9). The most probable explanation of these intensities is that the tRNA molecules in yeast are predominantly complexed

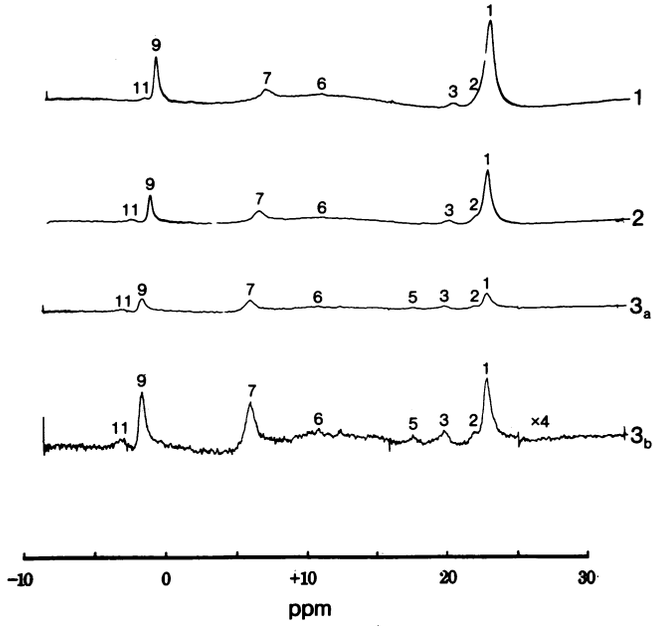

FIG. 4. ^{31}P NMR spectra at 145.7 MHz of yeast cells grown on media containing different initial orthophosphate concentrations. Spectra 1, 2, and 3_a represent yeast grown on normal- (1), intermediate- (2), and low- (3_a) phosphate media. The concentrations of orthophosphate in the initial growth media of these cells are given in the text. The number of scans (1000) and all other instrumental settings are identical for these first three spectra, as is the total cell volume of the sample tube and the measured extracellular pH (3.5). Spectrum 3_b is the same as 3_a, except that the gain was increased by a factor of 4. Concentrations were determined by comparison with an orthophosphate sample. The concentration of ^{31}P attributable to peak 1 of spectrum 1 (normal) is about 0.1 M per total cell volume. Peak 9 of the same spectrum is due to 20 mM of material and peak 7 corresponds to about 12 mM. In the spectrum of the cells grown on orthophosphate-depleted medium, the concentration of P accounting for peak 1 is reduced to about 14 mM.

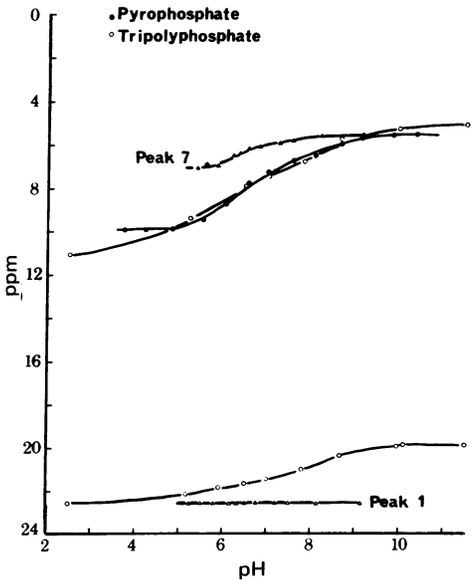

FIG. 5. Position in ppm from an external H_3PO_4 sample of the ^{31}P NMR lines from sodium pyrophosphate (●) and tripolyphosphate (○) as a function of pH. Also included are the pH dependences of lines 1 and 7 from yeast cells (Fig. 4) for comparison.

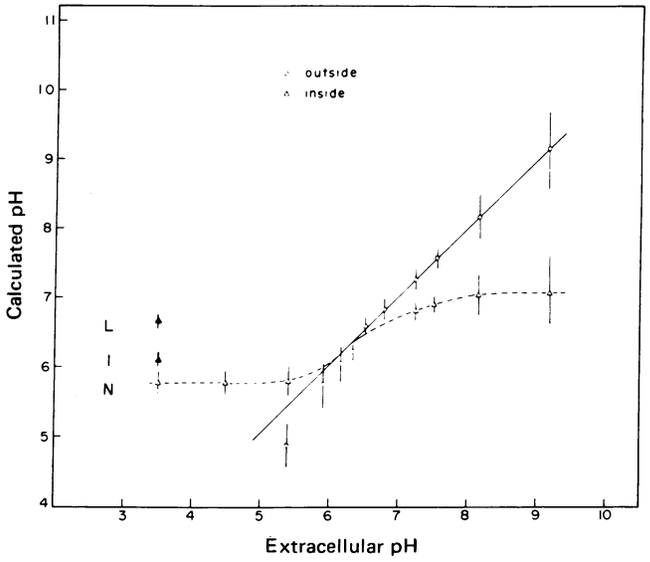

FIG. 6. Plot of the calculated pH from the orthophosphate lines of yeast (lines 9 and 10) versus the measured extracellular pH. The open circles come from line 10 (or 9 and 10 when they are not distinguishable), which is assigned to orthophosphate outside the cell. The open triangles are from line 9, which is assigned to orthophosphate inside the normal cell. The points at pH 3.5 come from calculations using line 9 in Fig. 3 for yeast grown in normal- (N; spectrum 1); intermediate- (I; spectrum 2); and low- (L; spectra 3_a and 3_b) phosphate media. It should be noted that at pH 5.35 it was possible to see line 9 as an upfield shoulder on line 10, thus allowing the approximate intracellular pH to be calculated. The pH values were calculated from a separate titration curve collected for orthophosphate. The vertical bars represent the error (range) in measuring pH on this titration curve. The pH measurements are most accurate when the pH of the microenvironment is near the orthophosphate pK (~6.8).

with other structures so that their rotation is slowed several-fold and their resonances are too broad to be observed.

Peaks 9 and 10 are assigned to intra- and extracellular orthophosphates, respectively. The position and pH dependence of peak 10 agrees very well with the behavior expected of orthophosphate in the measured extracellular pH. Peak 9 is assigned to intracellular orthophosphate on the basis of its position which, as shown in Figs. 3 and 6, is in the middle of the range expected for this ion. Another reason for this assignment is that peak 9, like peak 10, is unusually narrow, as would be expected for this small molecule (see Fig. 2). The position of peak 9 (given in Fig. 3) has been used as an indicator of the intracellular pH with the results given in Fig. 6. This shows that as the extracellular pH varies from 3.5 to 9.1 the intracellular pH varies only from ~5.8 to 6.8. While the aerobic growth condition lowered the medium pH to 3.5 it is clear that the yeast cells maintain an intracellular pH closer to that normal for life processes.

It is well known that yeast cells grown to the stationary phase accumulate polyphosphates (6). The amount of polyphosphates can be as much as 10% of total dry weight of yeast cells. This corresponds to about 0.3 M polyphosphates concentration in cells. The observed polyphosphates concentrations (0.1 M) is a good part of the accumulated polyphosphates. When yeast cells are grown in a phosphate-deficient medium, the polyphosphate content is, as expected, much lower than in the normal medium (Fig. 4). Furthermore, the ratio of end group phosphate (peak 7 in Fig. 4) to middle group phosphate (peak 1) is increased, indicating that polyphosphates with shorter chain length becomes abundant. It is also of considerable interest to see that after incubation at higher pH's the polyphosphate supply in normally grown cells is hydrolyzed and the concentrations of terminal phosphates (peak 7) and orthophosphates (peak 9 and 10) are increased. In summary, low pH and high concentration of inorganic phosphate in the medium lead to high intracellular polyphosphate concentrations, presumably because the phosphate is being stored in that form. Raising the pH results in hydrolysis of the polyphosphates, possibly by activating alkaline phosphatases. Lowering the phosphate concentrations in the growth medium also results in the hydrolysis of the stored polyphosphates, showing that they do indeed act as a phosphate store.

It is clear from these preliminary results that ^{31}P NMR can be a very useful monitor of intracellular metabolism.

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