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Phosphorus-31 Nuclear Magnetic Resonance Studies of the Effect of Oxygen upon Glycolysis in Yeast†

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ABSTRACT: ^{31}P NMR spectra were obtained at 145.7 MHz on suspensions of *Saccharomyces cerevisiae* cells grown on glucose, raffinose, acetate, and ethanol carbon sources. The NMR spectra were measured either under the growth conditions (i.e., using cells suspended in the growth media in the presence of the growth carbon source and O_2) or after feeding glucose under anaerobic and aerobic conditions. The NMR spectra allowed determination of intracellular pH (pH^{in}), as well as the intracellular concentrations of P_i (P_i^{in}), ATP, and other phosphorylated metabolites. In measurements performed during the steady state of glycolysis after addition of glucose, changes in P_i^{in} levels or pH^{in} were not observed upon oxygenation with glucose-repressed cells; however, in response to oxygen, derepressed cells showed a severalfold reduction in P_i^{in} concentration and an increase of pH^{in} by ~0.2–0.4 pH unit. According to in vitro data [Banuelos, M., Gancedo, C., & Gancedo, J. M. (1977) *J. Biol. Chem. 252*, 6394–6398], these particular changes of intracellular conditions should decrease the V_{max} of phosphofructokinase by a factor of ~3 and could thereby contribute appreciably to the regulation of this enzyme in the presence of oxygen. The pH^{in} values of *Saccharomyces cerevisiae* cells supplied with O_2 and the growth carbon source were found to be between 7.3 and 7.5; measurements made at extracellular pH values between 3.5 and 7.2 showed that under these conditions the pH^{in} values of these cells vary only by 0.1 pH unit. The ^{31}P NMR spectra were also measured during derepression of glucose-repressed cells; it was observed that initially intracellular pH values were ~6.7 and increased to ~7.2 slowly over a period of ~60 min. The time course of a concentrated suspension of yeast cells showed a sudden increase in pH^{in} approximately 4 min after glucose addition and time-dependent changes in P_i, fructose 1,6-bisphosphate, and ATP concentrations.

Recent experiments have demonstrated the usefulness of high-resolution ^{31}P nuclear magnetic resonance (NMR) spectroscopy for studying a variety of problems related to bioenergetics and metabolism in intact cells and tissue (Hoult et al., 1974; Salhany et al., 1975; Ugurbil et al., 1979a; Shulman et al., 1979). From the ^{31}P NMR chemical shifts of P_i and other phosphorylated metabolites with p*K*s near physiological pH values, it has been possible to determine the intracellular pH (pH^{in}). In addition, ^{31}P NMR has been used to measure the concentration of the more abundant phosphorylated metabolites (Navon et al., 1977) and to obtain information about their physical state (Ugurbil et al., 1979b) and about the rate of enzymatic reactions in vivo (Brown et al., 1977). Because all this information can be obtained simultaneously, it is possible to make time correlations between concentrations of intermediates and pH^{in}.

It is well documented that the type of carbon source used for growth by yeast cells plays an important role in determining the levels of many enzymes within these organisms [see Slonimski (1955), Perlman & Mahler (1974) and references cited therein, Mahler et al. (1975), Gancedo et al. (1965), and Rickarol & Hogan (1978)]. For example, in the presence of more than 4 mM glucose, yeast cells display enhanced levels of glycolytic enzymes and rapid rates of glycolysis while respiration is repressed; under these conditions, ethanol, the

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major end product of glucose catabolism in yeast, accumulates in the cultures. However, when yeast cells are grown exclusively on gluconeogenic carbon sources such as ethanol and acetate, respiratory activity is derepressed, the levels of certain enzymes which are necessary for gluconeogenesis are elevated, and the levels of glycolytic enzymes are decreased. Carbohydrates, such as raffinose and galactose, induce responses between these two extremes; with these carbon sources, glycolysis is still a necessary step, but its rate is lower than in the case of glucose, and the cells grown on these molecules exhibit higher respiratory activity than glucose-grown cells.

The short-time reduction of glycolytic rates in response to the availability of oxygen (which is the Pasteur effect) is believed to be accomplished by the regulation of the activities of the glycolytic enzymes, and not by changing their levels. Glucose-repressed cells may not respond strongly to the availability of oxygen because their respiration is repressed; these cells derive their metabolic energy mainly from fermentation without significant use of oxygen even when it is available to them. On the other hand, if ethanol- or acetate-grown cells are supplied with glucose, they will not use this carbon source by fermentation alone, and because of their active respiration, they are expected to be influenced greatly by the availability of oxygen.

This paper follows upon our recent report on glycolysis in *Saccharomyces cerevisiae*, where the catabolism of ^{13}C-labeled glucose was monitored by ^{13}C NMR (den Hollander et al., 1979). It was demonstrated that glucose transport by these cells can be described by a competitive Michaelis–Menten kinetic scheme for the α and β anomers of glucose, with the α anomer being the preferred substrate. From the scrambling of the ^{13}C label between C-1 and C-6 positions in fructose 1,6-bisphosphate (Fru-P_2), it was also concluded that the enzymatic reactions through Fru-P_2 aldolase and triosephosphate isomerase (TPI) were close to being in equilibrium under those experimental conditions.

In this paper, we present the results of a study by ^{31}P NMR which has simultaneously determined intracellular pH and the intracellular concentration of P_i, phosphomonoesters, and nucleotides for yeast cells grown with glucose, raffinose, glycerol, ethanol, and acetate. These carbon sources represent a spectrum from the highly glycolytic to the exclusively gluconeogenic. These parameters were also measured during derepression of glucose-grown cells.

Since pH (Ugurbil et al., 1978) and concentrations of P_i, nucleotides, and metabolic intermediates of glycolysis have all been implicated in regulation of the glycolytic rates (Sols, 1967; Krebs, 1972; Erecinska et al., 1977; Banuelos et al., 1977), ^{31}P NMR measurements were carried out on glycolyzing cells which had been grown on the different carbon sources and subsequently supplied with glucose. These NMR experiments were done in the presence and absence of oxygen to help clarify the molecular basis of oxygen regulation.

Materials and Methods

Saccharomyces cerevisiae, strain NCYC 239, was grown aerobically in a rotatory shaker at 30 °C in one of the following media: (1) 3% glucose, 2% bactopeptone (BP), and 1% yeast extract (YE); (2) 2% raffinose, 2% BP, and 1% YE; (3) 3% ethanol, 2% BP, and 1% YE; (4) 1% potassium acetate, 0.67% yeast nitrogen base (YNB), 0.1% YE, 40 mg/L adenine, and 50 mM potassium biphthalate (Shilo et al., 1976); (5) 3% glycerol, 0.67% YNB, 0.1% YE, and 40 mg/L adenine; (6) 2% raffinose, 0.67% YNB, 0.1% YE, and 40 mg/L adenine. The liquid growth cultures were 1 L in size and were contained in 4-L flasks. Cell growth was monitored by measuring the turbidity with a Klett-Summerson colorimeter. Cells were harvested at mid-exponential phase (50% of saturation; those grown on glucose at 25% of saturation). Before the cells were collected, cultures were chilled in an ice bath to 5 °C under continuous shaking. The cells were harvested by low-speed centrifugation at 4 °C and washed twice in the ice-cold resuspension medium. The resuspension medium was either a buffered salt solution [0.85 g/L KH_2PO_4, 0.15 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, and 0.1 g/L NaCl in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer, pH adjusted to 6 with NaOH (minimal medium)], or the growth medium without the carbon source plus 50 mM Mes, pH adjusted to 6 (enriched medium). When the dependence of the intracellular conditions on the extracellular pH was investigated, the Mes buffer in the two suspension media was replaced by mesotartrate or tartrate. Unless otherwise noted, the cells were resuspended so that the cell pellet volume was 10% of the total sample volume. This cell density corresponds to approximately 6×10^8 yeast cells/cm^3. The samples were kept on ice until they were used. ^{31}P NMR spectra were obtained in the Fourier-transform mode at 145.7 MHz on a Bruker HX-360 NMR spectrometer. The spectra were accumulated in consecutive 4-min blocks (800 transients) using 30° pulses and 0.34-s repetition time, and the free induction decays (FID's) were sequentially stored on disk. The chemical shifts were measured with glycerophosphorylcholine (GPC) as an internal standard; the GPC chemical shift was taken to be −0.49 ppm (downfield) from 85% phosphoric acid. The samples consisted of 3 mL of the cell suspension in 10-mm o.d. NMR sample tubes. In the aerobic experiments, a 95% O_2/5% CO_2 gas mixture was bubbled through the suspension; in the anaerobic experiments, this gas mixture was replaced with 95% N_2/5% CO_2. In all experiments, the gas mixture was bubbled through the suspension by using the two-bubbler scheme described previously (Ugurbil et al., 1981). One bubbler at the bottom of the NMR tube generated small bubbles at a rate of 20–30 cm^3/min, while a second bubbler generated bubbles above the level of the NMR detection coil at 100–200 cm^3/min. Oxygen levels in these suspensions were measured with a Yellow Springs Instruments oxygen electrode, under conditions similar to those during the NMR experiments. For these measurements, a vessel was constructed with the dimensions of the 10-mm o.d. NMR tube; the oxygen electrode was mounted on this vessel at the level of the NMR detection coils. Under these conditions, oxygen levels in the yeast suspensions were found to be 50–80% of O_2 saturation levels; this is many orders of magnitude higher than the K_m of oxygen usage by yeast cells. Immediately before the start of the NMR experiments, the samples were warmed up to 20 °C, and the carbon source was added. Cell extracts were prepared by perchloric acid digestion as described previously (Navon et al., 1977).

The intracellular pH was determined by using the ^{31}P NMR chemical shifts of the intracellular P_i resonance and by using a NMR titration curve obtained for a sample containing 30 mM NH_4Cl, 200 mM KCl, 20 mM $MgCl_2$, and 10 mM KH_2PO_4 (Gancedo & Gancedo, 1973; Conway & Armstrong, 1961).

Results

^{31}P NMR spectra were obtained of *Saccharomyces cerevisiae* cells grown on various carbon sources to determine the intracellular pH and the intracellular concentration of P_i and other phosphorylated metabolites. The carbon sources used for growth were glucose, raffinose, glycerol, ethanol, and acetate. The experiments were done under the following conditions: first, in the presence of the carbon source used

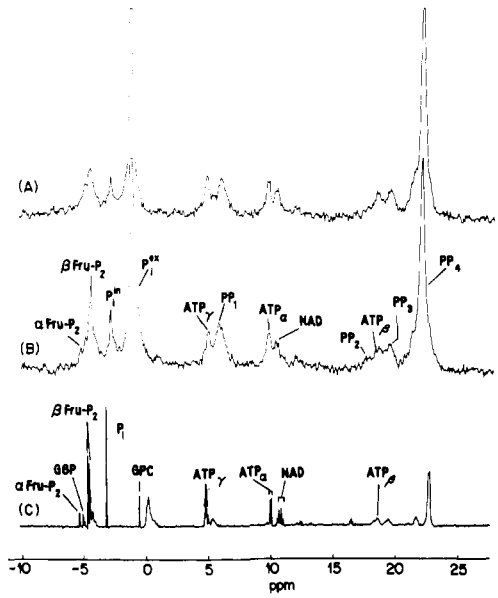

FIGURE 1: ^{31}P NMR spectra of aerobic and anaerobic suspensions of yeast cells during glycolysis. Glucose-repressed yeast cells were resuspended in the minimal medium (see Materials and Methods) at a density of 10% wet weight. Glucose was added, and the NMR spectra were recorded subsequently: (A) 16-min accumulation obtained between 10 and 26 min after glucose addition to the yeast suspension, while O_2 was bubbled; (B) same as (A) except here N_2 was bubbled instead of O_2; (C) ^{31}P NMR spectra of a percholoric acid extract of an anaerobic suspension of yeast cells taken about 10 min after, glucose addition. In (B), peaks labeled PP_1–PP_4 stem from polyphosphate resonances [see Navon et al. (1979)]; P_i^{ex} is extracellular P_i. In (C), unlabeled resonances in the 5–25-ppm range stem from polyphosphate.

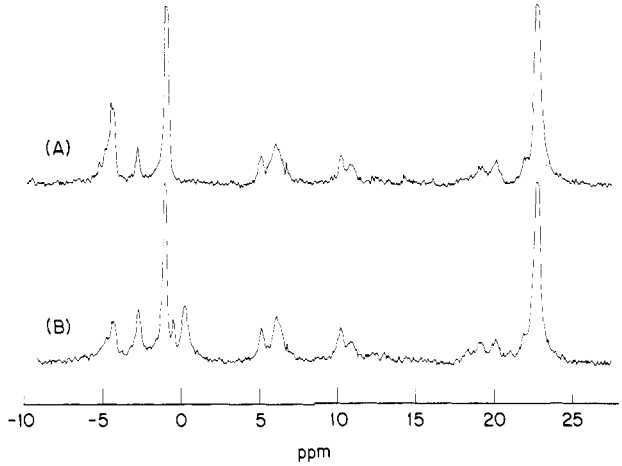

FIGURE 2: ^{31}P NMR spectra of glucose-repressed cells accumulated between 10 and 26 min after addition of glucose. (A) Cells were suspended in the *minimal medium* (pH 6.0) (see Materials and Methods). (B) Cells were suspended in the *enriched medium* (pH 6.0) (see Materials and Methods).

for growth; second, under aerobic and anaerobic conditions in the presence of glucose; and third, as a function of time after transferring glucose-repressed cells to a medium where catabolite derepression occurred.

As will be discussed below, immediately after glucose addition, the ^{31}P NMR spectra were time dependent, reflecting changing intracellular conditions. Steady-state conditions were established 5–10 min after glucose addition and maintained until the glucose was exhausted. During this steady state, the intracellular pH and the concentrations of P_i, sugar phosphates, and nucleotides in these cells were relatively constant. To study the steady-state interval, we used yeast cell concentrations of 6×10^8 cells/cm^3, which could easily be oxygenated and which was not unusually high compared to levels in many reported metabolic studies. At these cell densities and with the concentrations of carbon sources used, the steady-state period lasted at least 30 min. During this time, NMR spectra were accumulated in 4-min blocks. Subsequently, four such blocks were summed to increase the signal to noise ratio. Figures 1–5 show these summed spectra during the steady-state periods for cells grown under different conditions, resuspended in different media and supplied with the indicated carbon sources.

Figure 1 shows the ^{31}P NMR spectra obtained during the steady-state period starting 10 min after feeding 100 mM glucose to a suspension of glucose-grown cells harvested in mid-log phase and suspended in the minimal medium (see Materials and Methods). Parts A and B of Figure 1 compare the ^{31}P NMR spectra obtained under aerobic and anaerobic conditions. Figure 1C is a spectrum obtained from the perchloric acid extract of an anaerobic cell suspension, taken approximately 10 min after glucose feeding. The resonances observed in the sugar phosphate region of the extract spectrum consist almost exclusively of Fru-P_2 with a small contribution from glucose 6-phosphate (G6P). The nucleotide resonances were assigned to ATP α-, β-, and γ-phosphates. At the signal to noise ratio of this extract spectrum, resonances from other nucleotide triphosphates were not observed; it can also be concluded that ADP, if present, must be at least 10 times lower in concentration than ATP. Assignments of G6P, Fru-P_2, and ATP were checked by observing the effect of phosphoglucose isomerase and phosphofructokinase on the extract spectrum; the combined effect of these two enzymes is to convert G6P and ATP into Fru-P_2 and ADP. In Figure 1B, the resonance labeled PP_1 is the sum of several terminal phosphate resonances from phosphate chains, including $P_2O_7^{4-}$ (Navon et al., 1979); peaks PP_2 and PP_3 are penultimate phosphates from polyphosphate chains, including the middle phosphate of tripolyphosphate $P_3O_{10}^{5-}$. Finally, PP_4 comes from the inner peaks of longer polyphosphates (Navon et al., 1979). The cell spectrum shows a sugar phosphate region, which, apart from the line widths, is remarkably similar to that of the extract spectrum. This suggests that in the intact cell Fru-P_2 and G6P are present in about the same ratio as in the extract. In addition, it shows that intracellular Fru-P_2 is essentially in anomeric equilibrium during anaerobic glycolysis, since we are able to distinguish one of the ^{31}P resonances of αFru-P_2 as a shoulder on the main peaks of βFru-P_2. The comparison between the aerobic and anaerobic experiment reveals only small differences. The intracellular pH, measured from P_i chemical shifts, is identical in the two cases within experimental error. The levels of P_i, sugar phosphates, and ATP are also very similar in the two cases, although in the aerobic sample the P_i and Fru-P_2 levels appear a little lower. The αFru-P_2 peak is not clearly discernible in this spectrum.

A pronounced effect is observed when comparing glucose-grown cells resuspended in the minimal medium and in the enriched medium, respectively. Inspection of Figure 2 shows that the Fru-P_2 level is much lower in the presence of a full complement of nutrients than it is in their absence. Intracellular pH and the ATP levels are the same in the two cases, while the intracellular P_i level is slightly higher in the enriched medium than in the minimal medium. One major difference between the composition of the minimal and the enriched media is the lack of nitrogen sources in the former. In order to investigate the possible role of NH_4^+, an additional edperiment was performed in which the cells were resuspended in the minimal medium supplemented with 5 g/L $(NH_4)_2SO_4$. This gave a result very similar to that without $(NH_4)_2SO_4$. Since the composition of the suspension medium clearly per-

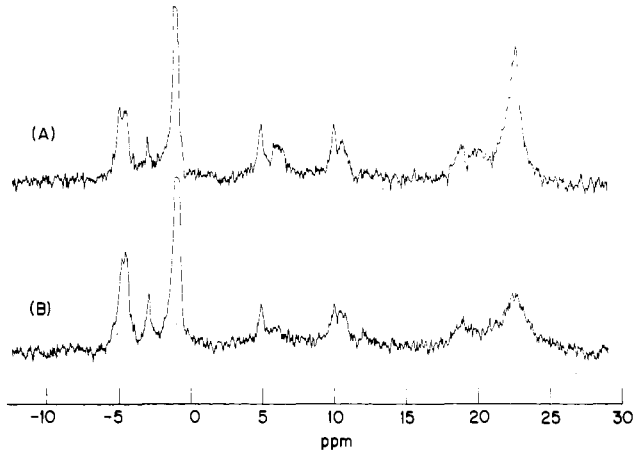

FIGURE 3: Yeast cells were grown to saturation in 2% bactopeptone, 1% yeast extract, and 2% glucose. The cells were resuspended in the minimal medium (see Materials and Methods). (A) Spectrum obtained between 10 and 26 min after glucose addition to an aerobic suspension; (B) Spectrum obtained for an anaerobic suspension under otherwise identical conditions.

turbs sugar phosphate levels and therefore the glycolysis pathway, we have examined the effect of oxygen on the ^{31}P NMR spectra of glucose-grown cells suspended in the enriched medium as well as in the minimal medium. This experiment showed once again that for glucose-grown cells the intracellular conditions are very insensitive to the presence of oxygen during glycolysis in spite of variations caused by the differences in the resuspension media.

The spectra shown in Figure 3 were obtained from suspensions of yeast cells which had been grown for 24 h at 30 °C on 2% glucose, 2% BP, and 1% YE. Under these conditions, the glucose is exhausted long before harvesting, so that the cells are catabolite derepressed when harvested. Figure 3 illustrates the ^{31}P NMR spectra obtained during the steady state after glucose addition to a suspension of these cells in the minimal medium. The spectra show that the intracellular pH under anaerobic conditions was 7.3, which is very similar to that observed for glucose-repressed cells under the same conditions. In the presence of O_2, however, the intracellular pH was 0.2 pH unit higher. In addition, the level of P_i^{in} was substantially lower in the aerobic case compared to the anaerobic case, and there were small differences in the sugar phosphate region between the two spectra. Another interesting feature is that in this series of experiments the polyphosphate level changed appreciably. Both in the aerobic and in the anaerobic experiments, the polyphosphate signal decreased upon the addition of glucose. In the anaerobic case, however, it decreased much more than in the aerobic case, leading to the different peak heights for polyphosphate shown in Figure 3. In these derepressed cells, the amount of polyphosphate immediately after harvesting was somewhat greater than in the cells harvested during exponential growth.

Derepressed cells obtained under different growth conditions showed qualitatively similar results to the cells grown to saturation on glucose. Raffinose-grown cells, harvested and resuspended in the minimal medium (Figure 4), showed an intracellular pH of ~7.3 after either raffinose or glucose feeding under aerobic conditions. However, after glucose addition to an anaerobic suspension, the intracellular pH was 0.15 unit lower. Again, the intracellular P_i level was lower in glucose-fed cells under aerobic conditions than under anaerobic conditions. It was also observed that the overall sugar phosphate level was lower after raffinose feeding than it was after glucose feeding. Raffinose-grown cells resuspended

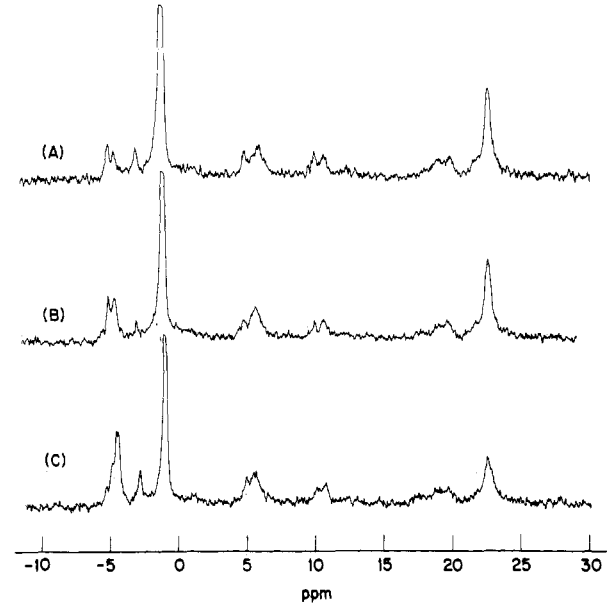

FIGURE 4: Cells were grown with raffinose as the carbon source and resuspended in the minimal medium at a density of 10% wet weight. Each ^{31}P NMR spectrum is a 16-min accumulation obtained between 10 and 26 min after the addition of the carbon source to the yeast suspension. (A) Raffinose was a carbon source, and the cell suspension was aerobic; (B) after glucose addition; the suspension was maintained aerobic; (C) after glucose addition; the suspension was maintained aerobic.

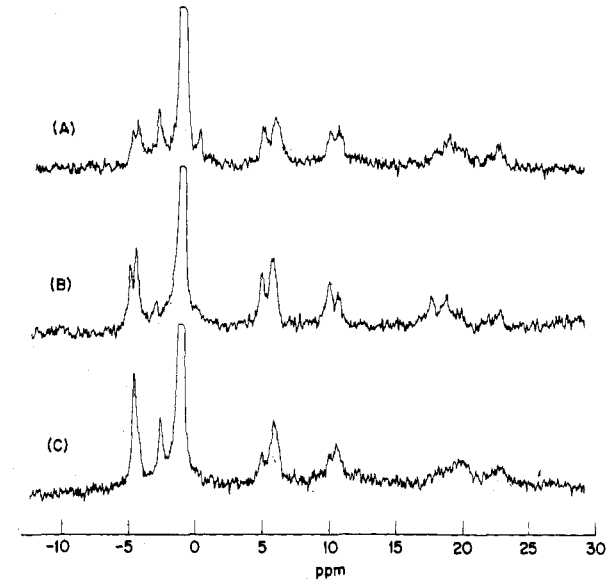

FIGURE 5: Cells grown with acetate as a carbon source were resuspended in the enriched medium at a density of 10% wet weight. Each spectrum is a 16-min accumulation obtained between 10 and 26 min after addition of the carbon source. (A) ^{31}P NMR spectrum obtained from aerobic cells after the addition of acetate; (B) spectrum during aerobic glycolysis after glucose addition; (C) spectrum obtained during anaerobic glycolysis after glucose addition.

in the enriched medium showed a similar behavior to those suspended in the minimal medium except, as in the case of glucose-grown cells (see Figure 2), the sugar phosphate level was lower after raffinose feeding in the enriched medium than it was in the minimal medium.

The greatest pH differences between the aerobic and anaerobic conditions during glucose metabolism were found for cells grown on gluconeogenic carbon sources. Acetate-grown cells (Figure 5), resuspended in the enriched medium, showed a pH^{in} of 7.5 after glucose feeding aerobically, but 7.0

Table I: Intracellular pH of *Saccharomyces cerevisiae* Cells at an External pH of 6.0 and in the Presence of O_2 and the Carbon Source Used for Growth[a]

carbon source	intracellular pH: enriched medium	intracellular pH: minimal medium
glucose	7.30	7.28
raffinose	7.25	7.37
glycerol		7.55
ethanol	7.38	7.35
acetate	7.41	7.51

[a] Cells were grown, harvested, and resuspended in either the enriched medium or the minimal medium as described under Materials and Methods. Subsequently, they were fed their growth carbon source and oxygenated. Intracellular pH measurements were made after a steady state of intracellular conditions, as indicated by the ^{31}P NMR spectra, was attained.

Table II: Dependence of Intracellular pH on Extracellular pH in Acetate- and Glucose-Grown *Saccharomyces cerevisiae* Cells[a]

carbon source	extracellular pH	intracellular pH
glucose	7.2	7.23
glucose	6.0	7.28
glucose	5.2	7.32
glucose	3.0	7.09
acetate	6.0	7.51
acetate	3.5	7.51

[a] Cells were resuspended in the minimal medium and supplied their growth carbon source which was either glucose or acetate. Measurements with glucose-grown cells were performed under anaerobic conditions. Acetate-grown cells were oxygenated as described under Materials and Methods. Suspension media were buffered with 50 mM Mes for measurements at pH 6.0 and 7.2, with 50 mM mesotartrate for pH 5.2 and with 50 mM tartrate for pH 3.5.

Table III: Effect of Oxygen on the Intracellular pH of *Saccharomyces cerevisiae* Cells Grown on Different Carbon Sources and Fed Glucose[a]

carbon source	intracellular pH: aerobic	intracellular pH: anaerobic
glucose (repressed)[b,d]	7.28	7.25
glucose (repressed)[c,d]	7.30	7.30
glucose (derepressed)[b,e]	7.52	7.23
raffinose[b]	7.34	7.21
raffinose[c]	7.35	7.19
glycerol[b]	7.51	7.38
ethanol[b]	7.35	6.99
acetate[c]	7.45	7.03
acetate[f]	7.58	7.13
acetate[g]	7.50	7.15

[a] Cells were grown, harvested, and resuspended in either the minimal or the enriched medium. Subsequently, they were fed glucose in the presence of either O_2 (aerobic) or N_2 (anaerobic). [b] Suspension in minimal medium at pH 6.0. [c] Suspension in enriched medium at pH 6.0. [d] Cells were harvested at a density which was ~25% of the full saturation value. At the time of harvest, these cultures still contain unused exogenous glucose and are therefore *catabolite repressed*. [e] Cells were harvested after 24 h of growth and were fully saturated; these cells are *catabolite derepressed*. [f] Suspension in minimal medium buffered with 50 mM mesotartrate at pH 5.3. [g] Suspension in minimal medium buffered with 50 mM tartrate at pH 3.5.

anaerobically. In the presence of oxygen and acetate, which is the growth carbon source, the intracellular pH of these cells was ~7.5; this pH^{in} is the same as that observed during aerobic glycolysis. Major differences were observed in the sugar phosphate, P_i, and ATP + ADP resonances between the anaerobic and aerobic glucose-fed cells. The P_i level is much lower aerobically than it is anaerobically, and the ATP + ADP levels are higher. In cell spectra, the terminal and α-phosphates of ATP and ADP are poorly resolved. Normally, the ATP_β resonance can be used to measure ATP concentration while either the α- or the terminal phosphate peaks can be used to measure ATP + ADP concentration. Unfortunately, in yeast cells, ATP_β is obscured by the presence of resonances from polyphosphate. Thus, only ATP + ADP levels can be measured from intact cell spectra.

The types of sugar phosphates present in acetate-grown and glucose-fed cells are also different in the anerobic and aerobic cases. The specific assignments of these sugar phosphate resonances have not yet been made. Also, note that the acetate-grown cells have a very low polyphosphate level.

Results obtained with cells grown on ethanol were generally very similar to those obtained from acetate-grown cells; in the presence of ethanol and O_2, ethanol-grown cells displayed a pH^{in} of 7.3 and an intracellular P_i level comparable to that of acetate-grown cells respiring in the presence of acetate. When presented with *glucose*, the ^{31}P NMR spectra of ethanol-grown cells looked very much like those of the acetate-grown cells under the same conditions; in response to oxygenation, the pH^{in} increased from 7.0 to 7.3, the intracellular P_i level decreased drastically, the ATP + ADP level increased, and the sugar phosphate peak split into two main components, as yet unidentified.

All of the measurements presented above were performed at an extracellular pH of ~6.0. In all cases, yeast cells displayed a constant intracellular pH of greater than or equal to 7 when a source of metabolic energy was available to them. Yeast cells are known to grow at external pH values as low as ~3.0. In order to examine to what extent yeast regulates its intracellular pH when challenged with such acidic external pHs, we obtained ^{31}P NMR spectra of glucose-grown and acetate-grown cells in the presence of their respective carbon source of growth at different external pH values. In an aerobic suspension of acetate-grown cells, fed acetate, the value of the intracellular pH of 7.4 decreased by less than 0.1 pH unit as the extracellular pH was changed from 6.0 to 3.5. Similar constancy of pH^{in} was observed for glucose-grown cells resuspended in minimal medium after being fed glucose under anaerobic conditions. When pH^{ex} was changed from pH 7.2 to 5.2, the intracellular pH remained unchanged at 7.25. Decreasing the external pH further to 3.0 slightly lowered the pH^{in} to 7.1.

A summary of the pH measurements is given in Tables I–III. Table I shows the intracellular pH values of *Saccharomyces cerevisiae* cells supplied with the growth carbon source and O_2. Table II shows pH^{in} in glucose-grown and acetate-grown cells as a function of external pH. Table III presents pH^{in} measured in cells grown on several different carbon sources and fed glucose in the presence or absence of O_2. Relative changes in the P_i^{in} and ATP + ADP concentrations in going from anaerobic to aerobic conditions are given in Table IV. These data were derived from spectra shown in Figures 1–5 and others obtained under identical conditions; it is assumed that the T_1 of P_i^{in} did not change upon oxygenation. The absolute values of the intracellular concentrations were measured separately in a few cases (Table V). This required slower pulse repetition rates than those used in the spectra presented in the figures in order to allow full recovery of the magnetization. To estimate intracellular concentrations, we assumed that 1.67 g of wet yeast contains 1 mL of cell sap (Gancedo & Gancedo, 1973).

Table IV: Ratio of the Intensities of P_i and ATP + ADP during Steady-State Glycolysis[a]

carbon source	resuspension medium[b]	aerobic/anaerobic[c] intensity ratio	
		P_i	ATP + ADP[d]
glucose (repressed)[e]	enriched	0.7	0.9
glucose (repressed)[e]	minimal	0.7	1.0
glucose (saturated)[f]	minimal	0.4	1.2
ethanol	minimal	0.4	1.2
acetate	enriched	0.3	2.9
acetate	minimal	0.1	2.5
raffinose	enriched	0.4	1.4
raffinose	minimal	0.3	1.07

[a] Cells were grown with the indicated carbon sources. They were harvested and resuspended in either the enriched or the minimal medium (see Materials and Methods). NMR measurements were performed during steady-state glycolysis after glucose feeding on two samples obtained from the same growth culture, one under anaerobic conditions, the other under aerobic conditions. [b] Definitions of enriched and minimal media are given under Materials and Methods. [c] Calculated errors due to base-line noise are approximately ±25%. [d] In yeast spectra, ATP and ADP concentrations are not measured independently because of interference from the polyphosphate peaks; therefore, ATP + ADP concentration is measured from the overlapping α-phosphate peaks of ATP and ADP. However, an extract obtained from glucose-grown cells during steady-state glycolysis showed that [ADP] $\lesssim$ 0.1[ATP]. [e] Cells were harvested at ~25% of the full saturation density. At the time of harvest, these cultures still contain a substantial fraction of the exogenous glucose and are catabolite repressed. [f] Cells were harvested after growing to full saturation; these cells are catabolite derepressed.

Table V: Intracellular Concentrations of P_i, Total Sugar Phosphates, and ATP + ADP[a]

	intracellular concn (mM)		
	P_i	SP	ATP + ADP[b]
glucose-repressed cells (anaerobic)[c]			
before addition of glucose	26	2.3	<0.1
during glycolysis steady state	5	23[d]	4[e]
after glucose is exhausted	36	2.6	<0.1
glucose-repressed cells (aerobic)[f]	7		
acetate-grown cells[f]			
with acetate (aerobic)	13		
with glucose (anaerobic)	17		
with glucose (aerobic)	$\lesssim$2[g]		

[a] Intracellular concentrations were calculated either from fully relaxed spectra obtained with 2-s repetition time and 30° pulses or from spectra obtained with 0.34-s repetition time and 30° pulses but after correcting the ^{31}P resonances for saturation. Intensities were calibrated by adding a known concentration of P_i into the suspension. Calculation of *intracellular* concentrations assumed that 1.67 g of wet yeast contained 1-mL intracellular volume (Gancedo & Gancedo, 1973). Errors due to base-line noise are calculated to be ~±15%. [b] In yeast spectra, ATP and ADP concentrations are not measured independently because of interference from the polyphosphate peaks: therefore, ATP + ADP concentration is measured from the overlapping α-phosphate peaks of ATP and ADP. [c] Obtained from the experiment shown in Figure 7, after correcting for the saturation of intensities due to rapid pulsing. [d] Extract spectra (Figure 1) show that this is predominantly ($\gtrsim$80%) Fru-P_2. [e] Extract spectra (Figure 1) shown that this is predominantly ($\gtrsim$90%) ATP. [f] In minimal medium. [g] Too low to be measured accurately from the current ^{31}P NMR spectra.

It is possible to derepress catabolite-repressed cells in times which can be followed by NMR. We have measured the ^{31}P NMR spectra as a function of time after transferring glucose-repressed cells to a medium where derepression occurred. Glucose-grown cells, harvested in the log phase of growth, were washed and resuspended in a medium consisting of 2% BP, 1% YE, and 3% ethanol in 50 mM Mes buffer (pH 6) at a density of 10% wet weight. A gas consisting of 95% O_2/5% CO_2 was bubbled through the suspension, and ^{31}P NMR spectra were accumulated for 10-min periods (2000 FID's for each spectrum). The time course of the intracellular pH, as determined from the chemical shift of the intracellular P_i signal, is plotted in Figure 6. The figure shows that the intracellular pH, which was 6.8 in the first spectrum, obtained after starting the experiment remained rather constant during the first 0.5 h and then gradually increased to 7.2 over a period of ~60 min. Subsequently, the intracellular pH remained constant.

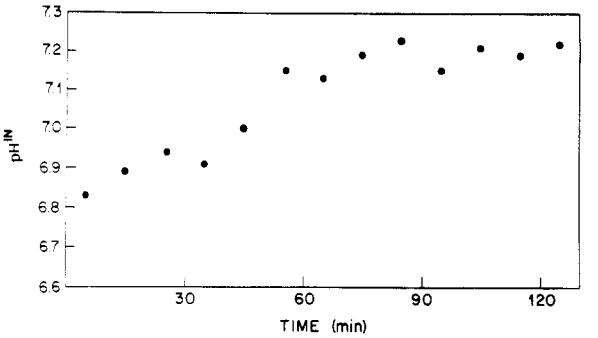

FIGURE 6: Time course of the intracellular pH of glucose-repressed cells during derepression. Glucose-repressed yeast cells were resuspended at a density of 10% wet weight in a derepression medium consisting of 2% bactopeptone, 1% yeast extract, and 3% ethanol. ^{31}P NMR spectra were collected in consecutive 10-min blocks after the onset of oxygenation. The intracellular pH was determined from the peak position of the intracellular P_i peak from each of these spectra.

The data presented in Tables I–III were taken during the steady-state condition starting ~10 min after the introduction of the carbon source. Now we turn to measurements made during the first few minutes after feeding glucose to glucose-grown cells in order to show the time-dependent changes. To increase the signal to noise ratio and thereby allow useful NMR spectra to be accumulated in 0.5-min accumulation times (100 FID's), we used a high concentration of cells (~3 × 10^9 cells/cm^3). These samples were prepared by resuspending a solid pellet of glucose-grown cells in an equal volume of the standard minimal medium which had been supplemented with an additional 15 mM P_i (KH_2PO_4:K_2HPO_4 1:1). Figure 7 shows the time courses of the information obtained from these spectra with 0.5-min time resolution. The cells were anaerobic during the course of this experiment (95% N_2/5% CO_2 gas mixture was bubbled through the cell suspension). The first two points in the time course were obtained before adding glucose, to show the original steady-state values. After 1 min, 75 mM glucose was added to the cell suspension. Subsequent to glucose addition, the sugar phosphate region increased in intensity, reaching a maximum after approximately 4 min, then decreased to a minimum, and subsequently increased again, leveling off at a constant value approximately 7 min after glucose addition. After about 15 min, the glucose was exhausted, and the sugar phosphate concentration returned to its original low value. The duration of the constant period at about 7 min after glucose addition was only limited by the supply of glucose. In the steady-state experiments shown in Figures 1–5, the cell concentrations were 5 times lower than those in Figure 7 so that this constant period lasted at least 30 min, allowing longer accumulations. ^{31}P NMR spectra of a cell extract of glucose-grown cells taken during the constant period (~10 min after glucose addition) showed that the sugar phosphate region during this period consisted predominantly of Fru-P_2 (see Figure 1A). The time course of Fru-P_2 observed previously in a ^{13}C NMR experiment after adding [1-^{13}C]-

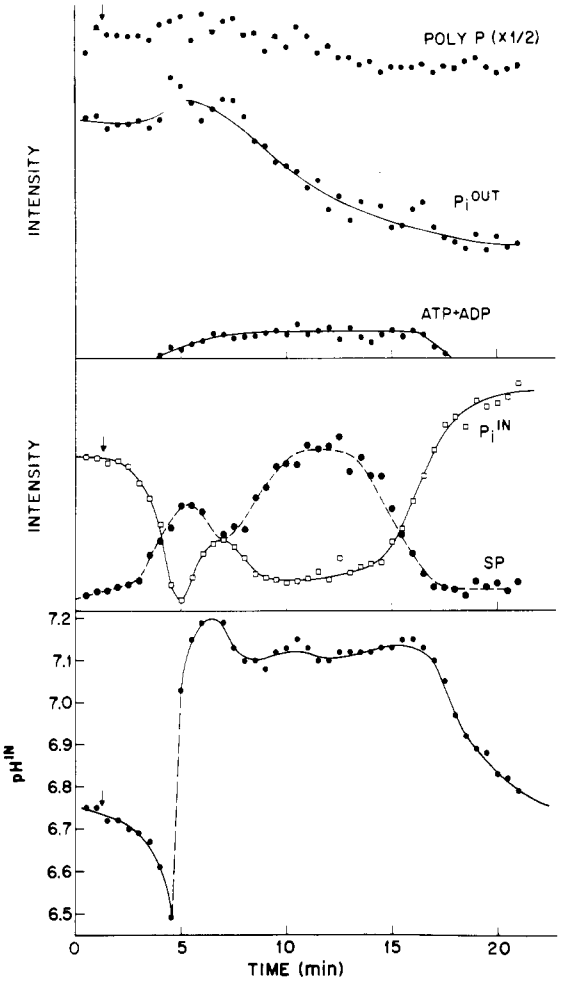

FIGURE 7: Glucose-repressed cells were resuspended in the minimal medium at a density of 50% wet weight. Glucose was added to the yeast suspension, and ^{31}P NMR spectra were accumulated in consecutive 0.5-min blocks. The first two points in these time courses were obtained before adding glucose to the suspension. Time courses measured from these spectra given for pH^{in} (determined from the peak position of P_i^{in}) and the concentration levels of P_i^{in}, P_i^{ex}, polyphosphate, and sugar phosphate.

glucose showed an oscillatory behavior similar to that of the sugar phosphate in the present ^{31}P NMR experiment. The ^{31}P NMR experiment also shows that the P_i^{in} level follows a time course which is complementary to the time course of Fru-P_2: it is high before glucose is added and then decreases to zero ~4 min after glucose addition. After that, the P_i^{in} level increases again, goes through a maximum, and finally reaches a constant value ~8 min after glucose addition. When the glucose is exhausted, the P_i^{in} level returns to its original high value. The intracellular pH as determined from the ^{31}P chemical shift of the P_i^{in} peak follows a very peculiar time course; originally, the pH is 6.75, and during the first few minutes after glucose addition, it acidifies slightly to pH 6.5. Then, at the same moment that the P_i^{in} level reaches a minimum, it jumps to pH 7.0, goes up to 7.2, and then settles down to a value of 7.15. When the glucose is exhausted, it drifts gradually back to its initial value.

In the present experiments, as previously mentioned, we are unfortunately unable to see the ATP and ADP signals separately and are only able to give the time course of ATP plus ADP. This signal appears approximately 3 min after glucose addition, remains quite constant until the glucose is exhausted, and subsequently decreases to zero. An extract taken during this period showed ATP concentration to be at least 10 times higher than ADP concentration (see Figure 1). The extracellular P_i level diminished during the period of a low intracellular level of P_i and high intracellular pH. There is no sign of P_i outflow after glucose exhaustion, when the intracellular P_i level attained a high value. Even in an experiment in which no outside orthophosphate was added, there was no P_i outflow. In this experiment, the polyphosphate signal was essentially constant; this is an contrast to what was observed with cells grown on glucose to full saturation. Note that there is a definite sequence of events after glucose exhaustion: first, the sugar phosphate signal decreases; second, the ATP + ADP signal disappears; and finally, the intracellular pH drifts back to its slightly acidic original value.

Discussion

It is known that the pK of P_i, and therefore the ^{31}P NMR titration curve, depends upon the ionic strength of the solution being measured. In order to minimize systematic errors from this source in intracellular pH determinations, we measured the pH dependence of the P_i chemical shift in a medium which was made up to approximate the concentrations of the major intracellular cationic components in yeast (see Materials and Methods). All the pH values quoted in this paper were determined by using the titration curve for P_i thus obtained. The inaccuracies in determining intracellular pH are only limited by the uncertainty in determining the NMR peak positions, upon assuming this titration curve. The error in chemical shift determinations is estimated to be ±0.05 ppm, with the stronger peaks somewhat more accurately measured and the weaker somewhat less. At pH 7.5, this leads to an error of ±0.1 pH unit and at pH 6.7 to an error of ±0.05. The line width of the intracellular P_i resonance gives an upper limit to the distribution of pH^{in} in the suspension. For the steady-state conditions reported above, the measured P_i line widths [full width at half-maximum (fwhm)] were approximately 40 Hz, which, at pH 7.2, leads to a maximum standard deviation in the distribution of intracellular pH of ±0.2 pH unit. The actual pH distribution is probably considerably less than this upper limit because the line width of the P_i^{in} peak is largely determined by other contributions, e.g., magnetic field inhomogeneity, paramagnetic ions, inhomogeneous magnetic susceptibility of the cell suspension, or chemical shift anisotropy. In support of this statement, we note that the ATP_α peak, which is not pH sensitive in the present intracellular pH range, is ~60 Hz wide.

In the past, the intracellular pH in yeast has been determined with a conventional glass electrode in the cell suspension after rendering the cells permeable by freezing and thawing, boiling, or treating the cells with acetone. A review of this method has been given by Borst-Pauwels & Dobbelmann (1972). These authors found by this technique that in the absence of an exogenous metabolic energy source, the intracellular pH of these cells was 6.7, and after glucose addition, it increased to 7.2. Chance et al. (1978) determined the intracellular pH in yeast during anaerobic glycolysis by the alcohol–aldehyde titration method and found a pH of 7.0. In agreement with these results, a ^{31}P NMR report by Salhany et al. (1975) showed that in yeast cells lacking an exogenous metabolic energy source, the intracellular pH is restricted to a range of 5.7–6.9 as the extracellular pH was varied from 3 to 10, while a more recent ^{31}P NMR study showed that the intracellular pH rose to approximately 7.3 in the presence of glucose and oxygen (Navon et al., 1979).

In a series of preliminary experiments, we measured the intracellular pH under anaerobic conditions without bubbling any gas through the yeast suspension. Under this condition,

we found that after the addition of glucose the intracellular pH increased initially to above 7.0 and subsequently decreased below 7.0 in a few minutes even though glucose was still present in the suspension. When the N_2/CO_2 gas mixture was bubbled through the suspension, the pH^{in} remained constant above 7.0 during glucose catabolism and only decreased after glucose was exhausted. The decrease of pH^{in} in the absence of bubbling is probably due to the accumulation of CO_2 in the cell suspension during glycolysis. Carbonic acid is a weak acid, and its presence will destroy transmembrane pH gradients. This suggests that in yeast cultures where the CO_2 is allowed to accumulate unperturbed, the intracellular pH is actually below 7.0. The well-documented effect of CO_2 and agitation upon growth and fermentation of yeast (Rice et al., 1974; Chen & Gutmanis, 1976) is most probably related to this lower intracellular pH.

One purpose of the present investigations was to determine the intracellular properties under different growth conditions and to see whether or not the pH^{in} responds to the carbon source used in the growth medium. The present ^{31}P NMR measurements of pH^{in} under the growth conditions are listed in Table I. In the enriched medium, with O_2 and the growth carbon source, the steady-state values of pH^{in} were 7.3, 7.3, 7.4, and 7.4 for glucose-, raffinose-, ethanol-, and acetate-grown cells, respectively. These were all measured while the external pH was buffered at 6.0. It is interesting to note that the pH^{in} is rather independent of the carbon source, although it might be slightly higher for the gluconeogenic carbon sources. Several measurements under similar conditions have demonstrated that these values of pH^{in} remain quite constant as pH^{ex} is lowered (Table II), in contrast to earlier reports that pH^{in} depended upon pH^{ex} (Pĕna et al., 1972).

Under the growth conditions, the intracellular concentrations of P_i are in the range of 5–20 mM, and the intracellular levels of ATP + ADP are 5–10 mM (Tables IV and V). The integrated intensities of the sugar phosphate peaks are higher in the minimal medium than in the enriched medium. This is most dramatically demonstrated for glucose-grown cells (Figure 2). Similar differences in the intensities of the sugar phosphate levels are observed for raffinose-grown cells. Since the ^{31}P NMR spectrum of a percholoric acid extract of glucose-grown cells during anaerobic glycolysis showed that the sugar phosphates consist predominantly of Fru-P_2 (90%) and to a lesser extent of G6P (10%), this change in the sugar phosphate level predominantly shows a change in the Fru-P_2 level. Our previous ^{13}C NMR study of glycolysis in yeast showed that the reactions catalyzed by Fru-P_2 aldolase and triosephosphate isomerase (TPI) are near equilibrium under these conditions; this indicates that a rate-limiting step exists in the glycolytic pathway below the TPI step, i.e., at the glyceraldehyde-3-phosphate dehydrogenase step or lower. The decrease of the Fru-P_2 level in the enriched medium can therefore be interpreted either as an increase of the rate through this rate-limiting step or as a decrease in the rate of formation of Fru-P_2. We showed that this effect is not related to the availability of NH_4^+, which could be an allosteric effector of the enzymes involved. Further experiments are needed to distinguish between the various possible explanations.

Another objective of this study was to investigate the effect of O_2 upon the intracellular conditions of yeast cells during glucose metabolism. As discussed in the introduction, yeast cells obtained from various growth conditions were used for this purpose. This was necessary since the rate of respiration of yeast depends upon the carbon source used in the growth medium (Rickarol & Hogan, 1978). Figure 1 compares the ^{31}P NMR spectra obtained during aerobic and anaerobic glycolysis of glucose-repressed cells resuspended in the starvation medium. The comparison shows that oxygen has practically no effect on pH^{in}, P_i^{in}, sugar phosphates, and nucleotide tri- and diphosphates. This result is in agreement with the earlier reports by Serrano & Dela Fuente (1974), and by Utter et al. (1967), that glucose-repressed cells have a negligible Pasteur effect; i.e., the rate of fermentation is virtually independent of the presence of oxygen.

In contrast, important differences were observed between anaerobic and aerobic glycolysis in derepressed cells. In catabolite-derepressed cells grown on acetate and resuspended in enriched medium with glucose, the pH^{in} went from 7.03 to 7.45 upon bubbling oxygen. Similar large pH^{in} changes upon oxygenation were observed for acetate-grown cells in minimal medium when the external pH values were 5.3 and 3.5. In these samples, pH^{in} went from 7.13 to 7.58 and from 7.15 to 7.50, respectively, when oxygen was bubbled. In other derepressed cells grown on glucose (to saturation), ethanol, or glycerol, the pH^{in} values all increased upon oxygenation in the presence of glucose, although the increases were not as large as in the acetate-grown cells. By contrast, we repeat, only the glucose-repressed cells did not show an increase in pH^{in} upon oxygenation. In all cases where the pH^{in} increased upon oxygenation, the concentrations of P_i^{in} decreased. This decrease in P_i^{in} concentration was most dramatic in acetate-grown cells.

The systematic changes reported here for intracellular pH and the level of P_i^{in} are possibly related to the control of glycolysis since oxygen does decrease the glycolytic rate in derepressed cells (the Pasteur effect). It is known that the kinetics of hexokinase, phosphofructokinase (PFK), and glyceraldehyde dehydrogenase from *Saccharomyces cerevisiae* depend upon these factors. Banuelos et al. (1977) and Laurent & Seydoux (1977) investigated the effect of P_i concentration and pH upon the rate of yeast phosphofructokinase. They reported that at pH 7.5 the rate of this enzyme was insensitive to the addition of 10 mM P_i. In the absence of P_i, decreasing the pH to 6.5 reduced the enzymatic rate to ~25% of its value at pH 7.5; however, at pH 6.5, addition of 10 mM P_i increased the PFK rate approximately 100-fold. In the absence of P_i at pH 7, the PFK rate was ~70% of its value at pH 7.5 while addition of 10 mM P_i at pH 7 increased the rate ~10-fold. The range of intracellular P_i concentrations and pH^{in} values reported here falls in the range where PFK activity is affected according to these in vitro studies. Thus, the concerted effect of the increased P_i levels and lower pH^{in} values observed in going from aerobic to anaerobic conditions would be to increase the PFK activity. This could contribute to the enhanced glycolysis rate observed under anaerobic conditions relative to the aerobic condition (Stickland, 1956). The effect of pH and P_i concentration on yeast hexokinase (HK) is similar to that observed for yeast PFK (Kosow & Rose, 1971; Wilkinson & Rose, 1979; Viola & Cleland, 1978; Peters & Neet 1977). At pH 7.4, P_i has no affect on the HK activity; at pH 7.0, the enzyme activity is ~30% of its value at pH 7.5, and P_i in excess of ~5 mM increases the enzyme activity by 2.5-fold. A major difference from PFK is that at acidic pHs addition of P_i does not increase the HK rate above the pH 7.4 level. Thus, if the in vitro data are applicable to the in vivo conditions, our measurements indicate that in derepressed cells fed glucose under anaerobic conditions increased P_i levels almost counteract the inhibitory effect of lower pH^{in} values on yeast HK. For this enzyme, it was also shown that at pH ≥7.3, ATP is an activator in addition to being a substrate. Activation by ATP is small at pH 7.3 but is much stronger at pH 6.9. Under

most of the conditions reported in this paper, the intracellular pH of yeast is in the 7.0–7.3 range. In some cases, it is as high as ~7.6. However, the only time it decreases below 7 is when an exogenous source for metabolic energy is not available. The activity of yeast PFK is also affected by ATP as well as AMP concentrations. From the in vitro work of Atzpodien & Bode (1970), it appears that ATP inhibits PFK at ATP levels above 1 mM; however, the extent of this inhibition is quite insensitive to changes in ATP concentration when the ATP concentration is near 5 mM, where these cells are operating once they are supplied with a carbon source. Hence, the observed changes of the ATP levels upon oxygenation will not appreciably change the PFK rates in these cells. However, the rates of both PFK and HK are also influenced by other effectors such as ADP, NH_4^+, and citrate which are not determined by our spectra. Therefore, further studies are needed to elucidate the complete control of these enzymes upon oxygenation. Also, it is very important to realize that while the present NMR data show a possible explanation of the Pasteur effect in terms of pH and P_i^{in} control of PFK activity, they have not shown that the PFK reaction is in fact the rate-limiting step of glycolysis. This question will be discussed more fully in a subsequent report where the Pasteur effect is investigated more thoroughly.

An interesting observation comes from the behavior of polyphosphate levels in derepressed cells after the addition of glucose. For instance, for cells grown into saturation (Figure 3), the level of polyphosphate went down by a factor of 2 during the first 5 min of aerobic glycolysis. During the same period of anaerobic glycolysis, however, the polyphosphate level went down by a factor of 4. This higher usage of polyphosphate during the first period of anaerobic glycolysis shows up in Figure 3 (which represents the spectra obtained after the polyphosphate attained a stable level) as a smaller polyphosphate peak. This decrease of the polyphosphate level is observed in all experiments where glucose was added to a suspension of derepressed cells. On the other hand, the time courses given in Figure 7 show that for glucose-repressed cells there is only a small decrease in the polyphosphate peak after the addition of glucose. The decrease of polyphosphate levels in derepressed cells seems to be correlated with the buildup of high sugar phosphate levels in these cells during glycolysis, which occurs during the first 5–10 min after glucose addition. The sugar phosphate builds up to a higher level anaerobically than aerobically, which would correlate to the higher mobilization of polyphosphate in anaerobic conditions. These results suggest that polyphosphate serves as a phosphate store, which serves to meet a sudden demand for intracellular P_i. In this way, polyphosphate mobilization could be a controlling factor for the level of intracellular P_i. It is not clear from these considerations why less polyphosphate is being mobilized when glucose is added to glucose-repressed cells in the minimal medium. Our results demonstrate, however, that ^{31}P NMR is an ideal technique for further studies of phosphate metabolism in yeast.

Figure 7 illustrates the intensities as a function of time of the ^{31}P NMR peaks in a concentrated (3×10^9 cells/cm^3) suspension of glucose-repressed cells, fed glucose at time zero. The overall flow of phosphate can be followed from these peak intensities. Absolute concentrations were obtained by comparison with an experiment in which 2-s pulse intervals were used. Certain semiquantitative conclusions about the phosphate flux can be drawn from the data. The sum of P_i^{in}, sugar phosphate (SP), and ATP concentrations appears to be approximately constant. It is particularly clear from the middle panel of Figure 7 that phosphate flows back and forth between P_i^{in} and SP. In the absence of glucose, i.e., before and after glucose feeding, P_i^{in} concentration is very high (approximately 30 mM at the beginning and approximately 45 mM at the end), and SP concentrations are low. The variations in the first 7 min are presumably related to the long-term oscillations which have been maintained in the glycolytic pathway of yeast (Hess, 1973). The out-of-phase relation between P_i^{in} and SP during the entire experiment supports the hypothesis that one flows into the other. In contrast to these temporal correlations, note that there is a long-term decrease of P_i^{ex} concentration and a small decrease of polyphosphate concentration which to a first approximation is not correlated with the temporal fluctuations of the P_i^{in} and SP. There are, however, some fluctuations in the P_i^{ex} levels which may be correlated with the P_i^{in} levels, but these are at present barely outside the noise levels. In any case, to a first approximation, it is clear that P_i^{ex} and polyphosphate concentrations decrease gradually during the time of the experiment, while the orthophosphate and the sugar phosphate pools exchange phosphate between each other. Similar results have been seen in acetate-grown cells, fed glucose anaerobically, although in that case the amount of polyphosphate decreases almost to zero. In general, these changes agree quite well with those obtained with chemical methods of analysis (Solomos, 1970). The most striking feature in Figure 7 is the discontinuous change in pH^{in} at 4 min after glucose addition. This behavior of pH^{in} may be related to the pH profile of PFK and HK discussed before. A possible explanation is that at the slightly acidic initial pH^{in} and the high P_i^{in} level both PFK and HK have a very high activity; PFK activity is particularly enhanced under these conditions, thus providing an efficient sink for ATP. This results in the accumulation of Fru-P_2 and the depletion of P_i^{in}. After the P_i^{in} level decreases, both PFK and HK activities diminish, allowing the buildup of a higher ATP level and the reduction of intracellular buffering by P_i^{in}. Once the ATP level is high enough, it can be used by the cell membrane ATPase for proton-pump activity, leading to a higher pH^{in}. At this higher pH^{in}, PFK is regulated at a lower activity, allowing the establishment of a constant glycolytic rate, and constant levels of ATP and P_i. After the ATP level diminishes because of glucose exhaustion, the proton-pump activity ceases, and the intracellular pH drifts back to a slightly acidic value.

It is clearly seen from Figure 7 that when glucose-repressed cells deplete the available glucose, major changes in intracellular conditions occur; the ATP levels decrease below the level of detection in our spectra, the intracellular P_i concentration increases to approximately 40 mM, and the intracellular pH decreases to ~6.7. Thus, in the derepression experiment (Figure 6), when glucose-grown cells were resuspended in enriched medium without glucose and supplied ethanol, their initial intracellular conditions were very similar to those observed upon depletion of glucose in Figure 7; their pH^{in} is acidic (~6.7), and they have high concentrations of intracellular P_i and low concentrations of ATP. Upon oxygenation, pH^{in} initially increased from 6.7 to 6.9, and they continued to maintain high P_i concentrations and low but observable ATP levels. As shown in Figure 6, ~60 min after the onset of oxygenation, the intracellular pH increased to levels observed in ethanol-grown cells. During this period of low intracellular pH, the glucose-repressed cells develop a more active respiration and thus become derepressed.

To summarize this ^{31}P NMR study, we have measured pH^{in} and P_i^{in} concentration under a variety of conditions in *Saccharomyces cerevisiae*. We observed that in the presence of an exogenous carbon source the pH^{in} values of these cells are

between 7.0 and 7.5, and the intracellular P_i concentrations are in the 2–20 mM range. In the absence of such a carbon source, pH^{in} was found to be ~6.7. While glucose is being catabolized, the most dramatic response of the cells to the presence or absence of O_2 is observed with derepressed cells. Such cells display a decrease in pH^{in} of almost 0.5 pH unit and an increase in the concentration of P_i^{in} of severalfold when going from aerobic to anaerobic conditions.

The effects of these changing intracellular conditions upon the activities of PFK and HK have been estimated by referring to the published in vitro studies. From these, it is possible to infer that the combined changes in P_i^{in} concentration and pH^{in} in the presence of oxygen would decrease the activity of PFK in vivo. From the present data, we cannot, however, tell what the role of other possible effectors is in the control of PFK activity following oxygenation. More importantly, we cannot tell to what extent control of PFK or HK activity in general affects the rate of glycolysis; therefore, we cannot say that pH^{in} or P_i^{in} controls the glycolysis rate and is responsible for the Pasteur effect. However, the effects of these changes in pH^{in} and $P_{i_n}^{i}$ concentration must be taken into account in any effort to understand the Pasteur effect. While control of the Pasteur effect by P_i^{in} concentration was suggested long ago by Lynen (1941) and Johnson (1941) and more recently by Banuelos et al. (1977), there have been no suggestions that pH^{in} might be changing upon oxygenation, and consequently might be significantly affecting the activity of PFK.

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