^{31}P and ^{13}C NMR Studies of Intermediates of Aerobic and Anaerobic Glycolysis in *Saccharomyces cerevisiae*†

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ABSTRACT: The levels of intermediates of aerobic and anaerobic glycolysis were determined in perchloric acid extracts prepared from glycolyzing suspensions of *Saccharomyces cerevisiae* by ^{31}P and ^{13}C NMR spectroscopy. From ^{31}P NMR measurements a small increase in the level of nucleoside triphosphates was found in derepressed cells upon oxygenation, while the ratio of nucleoside diphosphates to nucleoside triphosphates was a factor of 3 lower aerobically. Combined with the previous observation that the level of intracellular P_i is lower by a factor of 3 aerobically, this leads to the conclusion that the phosphate potential $[NTP]/([NDP][P_i])$ is lower by an order of magnitude during anaerobic glycolysis than during aerobic glycolysis. There was no correlation between the level of glucose 6-phosphate and the rate of glucose utilization. We used ^{13}C NMR to determine the scrambling of the ^{13}C label from C_1 to C_6 in fructose 1,6-bisphosphate (Fru-P_2). There was more scrambling of the label during aerobic than during anaerobic glycolysis. Since the level of Fru-P_2 did not change much upon oxygenation, this suggests that in aerobic glycolysis there is control of at least one enzyme in the lower part of the Embden–Meyerhof–Parnas pathway, below Fru-P_2, which gives the ^{13}C level more time to equilibrate between C_1 and C_6 of Fru-P_2. Previous ^{13}C NMR measurements of glucose utilization rates had shown a 2-fold reduction upon oxygenation, reflecting control in the early stages of the pathway.

Over the past several decades numerous studies have been made of the Pasteur effect in yeast and in other organisms (Sols, 1967, 1976; Racker, 1974; Ramaiah, 1974; Krebs, 1972; Sols et al., 1971; Rose, 1971). Notwithstanding this considerable effort, many questions about the nature of the Pasteur effect and its mechanism are still unanswered or controversial. This is clearly illustrated by recent reports (Lagunas, 1981, 1979) that stated that there is no Pasteur effect in yeast, in defiance of the vast body of literature existing on this subject.

This paper addresses the question: Is there a Pasteur effect in yeast, and if so, how large is it, and how does it manifest itself? In our recent studies on this subject (den Hollander et al., 1981, 1985) we have emphasized that a meaningful answer to these questions can only be obtained for a well-defined set of conditions. For a versatile organism like yeast, it is important that the growth conditions of the yeast cells are well-defined, since it has been known for a long time that the levels of many enzymes are under control of catabolite repression or are inducible (Mahler et al., 1981; Wales et al., 1980; Funayama et al., 1980; Muratsubaki & Katsume, 1979; Mazon, 1978; Holzer, 1976; Perlman & Mahler, 1974; Duntze et al., 1969; Cartledge & Lloyd, 1972a–c; Vary et al., 1969; Criddle & Schatz, 1969; Schatz, 1965; Polakis & Bartley, 1965).

We have been able to show by ^{13}C NMR[1] measurements of glucose utilization rates (den Hollander et al., 1985) that the effects of oxygen upon glucose consumption depend critically upon whether or not the cells are respiratory-competent, a finding that helps to explain some contradictory results found in the literature. Our finding of a factor of 2 difference between aerobic and anaerobic glucose utilization rates in derepressed yeast cells had been found before by direct assays of glucose levels (Stickland, 1956a,b), and a value close to this had been reported by Lynen (1958). However, this difference is much smaller than had been calculated from Warburg manometer measurements (Serrano & DelaFuente, 1974). The discrepancy between direct measurements of glucose utilization and Warburg experiments can be partially explained by the observed increase of trehalose formation under aerobic conditions, by differences in the yields of glycerol, and by an increased biosynthetic activity [see den Hollander et al. (1985)].

A significant contribution toward the explanation of the Pasteur effect was the identification of an inhibitory site for ATP in phosphofructokinase (PFK) (Passonneau & Lowry, 1962, 1963; Vinuela et al., 1963). Since then, possible explanations of the Pasteur effect have focussed upon allosteric and feedback control of glycolytic enzymes. From the study of glycolytic oscillations in yeast, it was established that PFK is being regulated under physiological conditions (Hess, 1973; Ghosh & Chance, 1964; Betz & Chance, 1965a,b). However, in vitro studies of PFK (Sols, 1981; Banuelos et al., 1977; Atzpodien & Bode, 1970) and of other enzymes of the gly-

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[1] Abbreviations: Fru-P_2 and Fru-1,6-P_2, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; P_i, inorganic phosphate; ATP, adenosine triphosphate; NMR, nuclear magnetic resonance; PFK, phosphofructo-1-kinase; ADP, adenosine diphosphate; AMP, adenosine monophosphate; G6P, glucose 6-phosphate; TPI, triosephosphate isomerase; NAD^+, oxidized nicotinamide adenine dinucleotide; α-GP, α-glycerophosphate; 3PGA, 3-phosphoglyceric acid; PK, pyruvate kinase; UDPG, uridine diphosphoglucose; F6P, fructose 6-phosphate; GAPDH, glyceraldehyde-phosphate dehydrogenase.

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colytic pathway have shown that glycolysis can be regulated in a complex fashion by many effectors. Consequently, a great deal of uncertainty still exists about the importance of the various control sites and about the regulatory role of many effectors of the glycolytic pathway in the switch from anaerobic to aerobic glycolysis.

A generally accepted picture of the regulation of glucose utilization in the Pasteur effect has been that PFK is controlled by ATP and citrate, reinforced by allosteric activation by P_i, ADP, and AMP (Tejwani, 1978; Banuelos et al., 1977; Sols, 1976; Ramaiah, 1974; Chance, 1959). The consequent increase in the concentration of glucose 6-phosphate (G6P) then supposedly would result in the inhibition of the transport of glucose across the cell membrane (Serrano & DelaFuente, 1974; Azam & Kotyk, 1969; Kotyk & Kleinzeller, 1967; Sols, 1967). This mode of control of the rate of glucose utilization has been proposed because yeast hexokinase is insensitive to G6P inhibition, while in animal cells feedback inhibition of hexokinase has been well established (Sols, 1976). This model, however, is inconsistent with the observations that there is little change in the level of ATP in changing from anaerobiosis to aerobiosis (Holzer, 1961; Lynen et al., 1959) and in the level of G6P (Salas et al., 1965). Furthermore, there is no effect of 2-deoxyglucose 6-phosphate upon sugar transport, which was expected if G6P acts as an inhibitor of transport (Serrano & DelaFuente, 1974).

An alternative picture is based upon the observation that in ascites tumor cells inorganic phosphate plays an important role (Racker, 1974). Here a coordinated action of P_i has been proposed at the level of at least three enzymes, glyceraldehyde-phosphate dehydrogenase, PFK, and hexokinase (Racker, 1965). Such a coordinated control of several enzymes of the glycolytic pathway (Buecher & Ruessmann, 1963) removes the need of feedback control by G6P in order to explain the change in the rate of glucose utilization and explains the constant level of G6P in the change from anaerobic to aerobic conditions. The role of P_i as a regulating factor was first proposed by Lynen (1941) and by Johnson (1941). In the case of yeast, it has been shown that P_i can act as an effector of PFK (Banuelos et al., 1977; Laurent & Seydoux, 1977).

To help answer these questions about control during the Pasteur effect, we studied in a recent paper the intracellular conditions in glycolyzing yeast by ^{31}P NMR spectroscopy (den Hollander et al., 1981). That study showed that in glucose-repressed cells there was no change of intracellular pH, intracellular P_i, or nucleoside triphosphates upon oxygenation. On the other hand, in derepressed cells the intracellular pH reproducibly increased by 0.2–0.4 pH unit upon oxygenation (depending upon growth conditions) while the level of intracellular P_i diminished by a factor of 3 or more. These changes in intracellular conditions influence the kinetics of various enzymes of the Embden–Meyerhof–Parnas pathway, particularly PFK (Banuelos et al., 1977), in a way that is consistent with the observed reduced flow under aerobic conditions. It is interesting to note that in erythrocytes an increase of internal pH stimulates glycolysis (Rose, 1971), whereas we have found that in yeast a decrease in internal pH corresponds to a higher glycolytic rate.

In the ^{31}P studies of intact yeast cells we were not able to evaluate the effect of oxygen upon the levels of glycolytic intermediates because the NMR lines in the spectra from suspensions of intact cells were too broad to allow separation and assignment of the sugar phosphate peaks. In addition, from the ^{31}P NMR spectra we were not able to measure the nucleoside triphosphate to nucleoside diphosphate ratios. In this study we investigate the concentrations of these metabolic intermediates by ^{31}P and ^{13}C NMR so as to extend our studies of intact cells. This allows us to make correlations between the measured rates of glucose metabolism as determined by ^{13}C NMR and the levels of metabolites. We have prepared perchloric acid extracts of suspensions of yeast cells glycolyzing under aerobic and anaerobic conditions and have studied the levels of intermediates in those extracts.

We previously showed that when [1-^{13}C]glucose is fed to yeast cells the ^{13}C label appears in both the C_1 and the C_6 positions of fructose 1,6-bisphosphate (Fru-P_2) (den Hollander et al., 1979). This scrambling of the ^{13}C label was used to determine the relative forward and backward rates through Fru-P_2 aldolase and triosephosphate isomerase (TPI). We have now prepared extracts of aerobic and anaerobic suspensions of yeast cells after feeding [1-^{13}C]glucose or [6-^{13}C]glucose and studied the label distribution by ^{13}C NMR. In this way we have been able to study the effect of oxygen upon the relative rates of the enzymes of the aldolase–TPI part of the glycolytic pathway. The combined measurement in extracts of metabolite levels (by ^{31}P NMR) and of the degree of scrambling in Fru-P_2 (by ^{13}C NMR) supplement our NMR measurements of the whole yeast cells, reported in the previous paper. By putting together these NMR measurements, we have obtained a measurement of the glycolytic controls and of their changes during the Pasteur effect.

Experimental Procedures

The *Saccharomyces cerevisiae* strain NCYC 239 was grown and harvested as described previously (den Hollander et al., 1981, 1985), with glucose, raffinose, or acetate as a carbon source. After harvesting, the cells were resuspended to a density of 20% wet weight, in a resuspension medium consisting of 6 mM KH_2PO_4, 1 mM K_2HPO_4, 4 mM $MgSO_4$, and 1.7 mM NaCl per liter. For the ^{31}P NMR studies the medium was buffered with 50 mM MES and for the ^{13}C NMR studies with 50 mM $Na_2H_2P_2O_7$; in both cases the pH of the solution was adjusted to 6.0 with NaOH. The cell suspensions were kept in an ice bath until used.

In preparing the perchloric acid extracts we followed the procedure outlined by Saez & Lagunas (1976). The advantage of this method is that the cell suspension is cooled down as quickly as possible, by liquid nitrogen. When instead of this method the suspensions were added to a precooled test tube containing perchloric acid (Weibel et al., 1974; Navon et al., 1979), we noticed that it sometimes took up to 1 min before the suspension was frozen solidly. The ^{31}P NMR spectra of the extracts prepared in that way were sometimes very different from the ^{31}P NMR spectra of the whole cells obtained immediately before extraction. We attributed these differences to artifacts of the extraction procedure.

With the ^{31}P NMR spectra as assay, the following procedure was established: 5 mL of the yeast suspension were warmed to 20 °C under continuous bubbling of either N_2 or O_2. After equilibration, 75–100 mM glucose was added to the suspension, while bubbling continued. After 10–12 min of incubation with glucose, the suspension was poured into a mortar that had been precooled and filled with liquid nitrogen. then, 1 mL of 85% perchloric acid was added to the frozen yeast suspension, which was kept immersed in liquid nitrogen. The frozen mixture was ground to a powder with a pestle and transferred to a test tube, which had been precooled in dry ice/ethanol. Then, the sample was thawed in melting ince, after which it was frozen again in the cold mixture. This freeze–thaw cycle was repeated 3 times, after which the residue was centrifuged at 4 °C. The

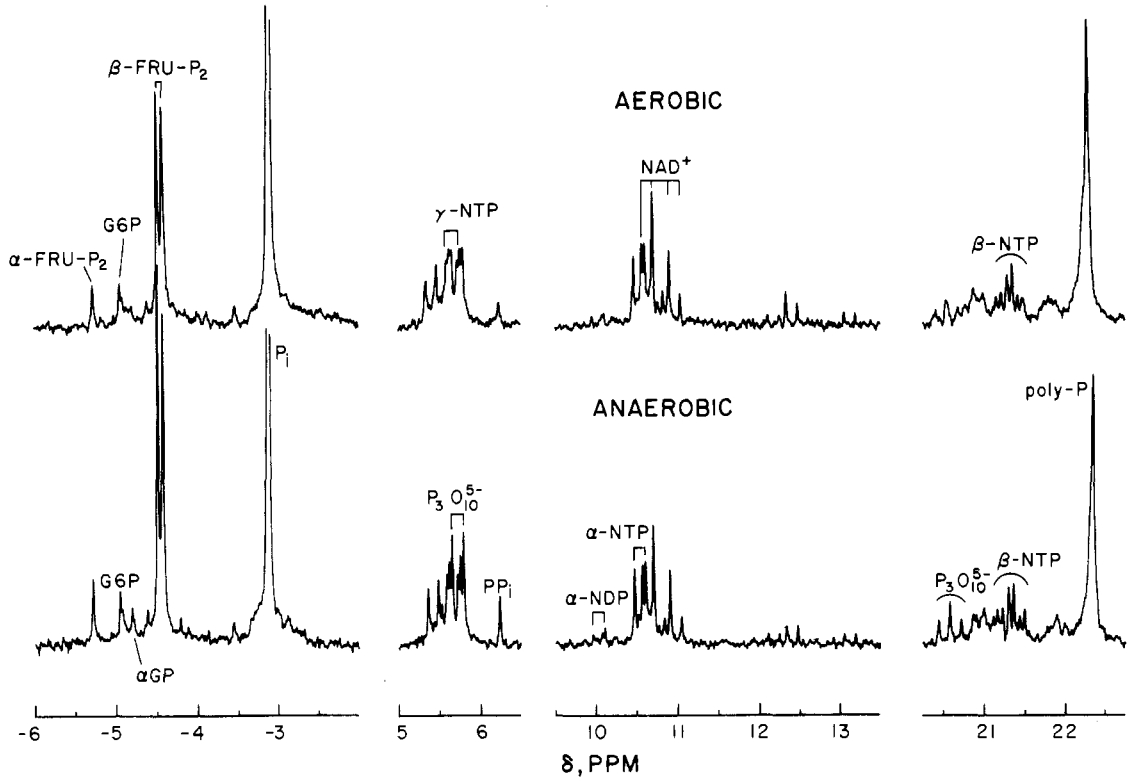

FIGURE 1: ^{31}P NMR spectra of perchloric acid extracts of *S. cerevisiae* taken during aerobic and anaerobic glycolysis. The cells were grown with glucose as carbon source and harvested in log phase, so that they are catabolite repressed. The intermediates Fru-P_2, G6P, αGP, NTP, NDP, and NMP were assigned on the basis of their chemical shifts and titration behavior and in some cases by adding the suspected source to the extract and remeasuring the ^{31}P NMR.

Table I: Concentrations of Intermediates (mM) Measured in Glycolyzing Cells[a]

	glucose grown repressed		glucose saturated		acetate grown		raffinose grown	
	anaerobic	aerobic	anaerobic	aerobic	anaerobic aerobic	aerobic	anaerobic	aerobic
G6P	1.8	1.6	1.0	4.1	0.8	6.9	3.6	4.0
αGP	0.7	0.3	3.2	1.8	5.7	2.0	3.6	1.1
3PGA	0.4	0.4	<0.2	0.7	0.3	0.8	0.9	1.1
Fru-P_2	10.1	6.2	3.1	4.2	1.9	2.2	10.3	6.8
NDP	0.5	0.4	0.9	0.4	1.1	0.6	0.9	0.2
NTP	3.7	3.4	3.7	4.8	3.2	4.7	2.7	2.2
NAD^+	2.5	2.6	2.3	2.8	2.4	2.7	2.7	2.4

[a] These are the estimated concentrations of the levels of intermediates, determined from the ^{31}P NMR spectra, in nanomolar intracellular concentrations. The estimates were made by assuming that 1.6 g of wet cells contains 1.0 mL of cell sap (Gancedo & Gancedo, 1973). The values are arranged according to growth conditions and compare the levels during aerobic and anaerobic glycolysis. The estimated error in these measurements is ±0.2 mM or ±10%, which ever is larger.

supernatant was neutralized with $KHCO_3$, and the precipitate was removed by centrifugation. Then, the NMR spectra were measured. For the ^{31}P NMR spectra it was necessary to remove paramagnetic cations by treating the extracts with Chelex. After the extracts were passed through a Chelex column they were lyophilized and redissolved, after which the NMR spectrum was again measured. By measurement of the NMR spectra before and after treatment with Chelex, possible changes in metabolite concentrations due to this treatment would have been detected. Extracts prepared from aerobic and anaerobic suspensions were prepared in parallel, and the NMR spectra were obtained under identical conditions. In this way the NMR spectra from parallel experiments can be compared directly. This is supported by the observation that the NAD^+ signals are of equal intensity in parallel experiments (±10% variation). The calibration to obtain intracellular concentrations was made by comparison with the ^{31}P NMR spectra of the whole cells (den Hollander et al., 1981).

NMR spectra were obtained on a Bruker WH 360 WB NMR spectrometer, operating in the FT mode. ^{31}P NMR spectra were obtained at 145.8 g MHz and ^{13}C NMR spectra at 90.55 MHz. The pulse interval was 2 s, and the flip angle was 45°. For the ^{13}C NMR spectra, gated decoupling of protons was employed to suppress Overhauser enhancement, with a decoupling power during aquisition of 12 W. For ^{31}P NMR, continuous decoupling was used, with a radio-frequency power of 1 W.

Results

Figure 1 shows the ^{31}P NMR spectra of chelexed perchloric acid extracts of suspensions of glucose-repressed cells taken during aerobic and anaerobic glycolysis. In both spectra, the major component in the sugar phosphate region is Fru-P_2. The intracellular concentration of this intermediate is about 10 mM anerobically and 6 mM aerobically (see Table I). A minor component in the sugar phosphate region is glucose 6-phosphate (G6P); the level of G6P is about 1.5–2.0 mM in the two spectra. In the extract obtained during anaerobic glycolysis a small peak is observed that has been assigned to α-glycerophosphate (αGP). The level of this intermediate is lower in

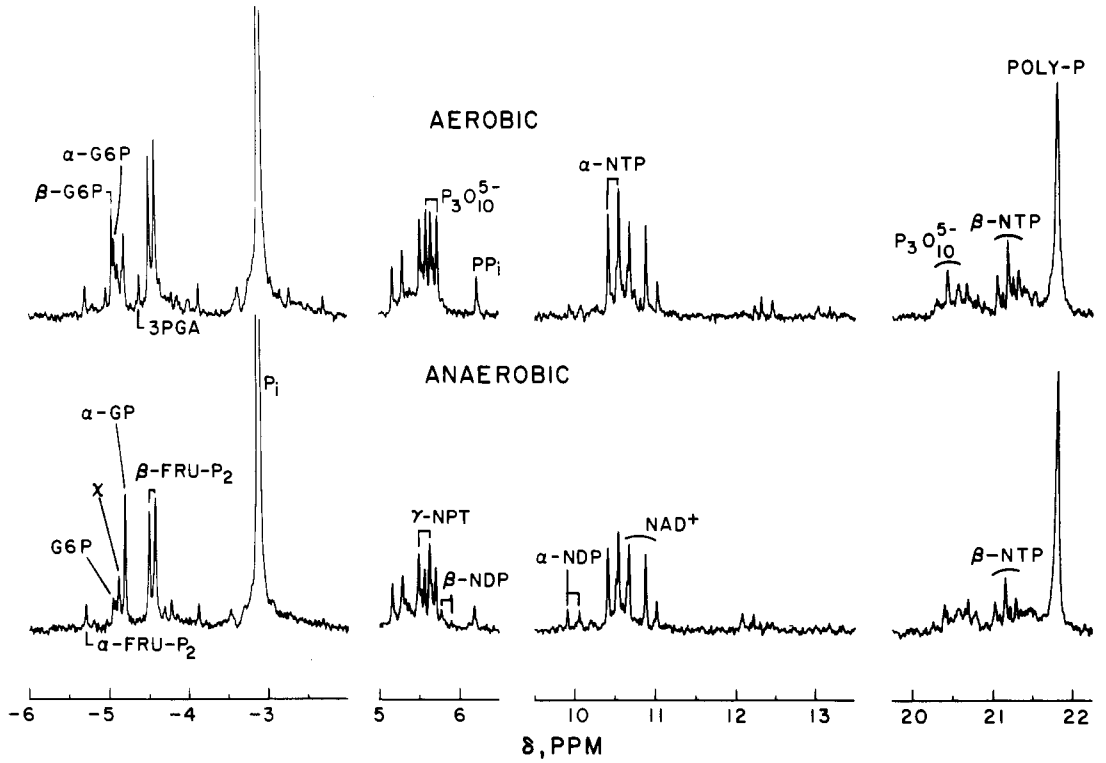

FIGURE 2: ^{31}P NMR spectra of extracts of suspensions of cells grown on glucose that were derepressed by growing them into saturation for 24 h. The extracts taken during aerobic and anaerobic glycolysis are compared. Note that the level of G6P is about 4 times higher aerobically than anaerobically, while NDP signals are higher anaerobically.

the extract obtained under aerobic conditions. The level of 3-phosphoglyceric acid (3PGA) is similar in the two spectra (about 0.5 mM). Because 3PGA is used to indicate PK control (see below), it was identified by adding this compound to the extract and remeasuring the NMR spectrum.

The upfield region of the spectra shows that two nucleoside triphosphates (NTP) are present with about the same concentration. The α-phosphate signals of these two nucleoside triphosphates overlap, but the β and γ signals of these compounds are clearly resolved. The α-phosphate signal of nucleoside diphosphate is observed near 10 ppm; the level of NDP is estimated at 0.5 mM, while NTP is about 3.5 mM intracellular concentration. In the region of 5–6 ppm, additional signals are obsered that have been assigned to pyrophosphate, the terminal phosphate of $P_3O_{10}^{5-}$, and the terminal phosphates of higher polyphosphates (Navon et al., 1979). The middle phosphate of $P_3O_{10}^{5-}$ is observed as a triplet at 20.5 ppm. In the region of 10.5–11 ppm, we observe the quartet of NAD^+, and further upfield are the signals of UDPG near 12.4 and 13.2 ppm. For all these signals little or no differences are observed between the aerobic and anaerobic samples.

Figure 2 shows a similar comparison of ^{31}P NMR spectra of extracts of suspensions of derepressed cells, taken during aerobic and anaerobic glycolysis. The cells were obtained by growing them into saturation in a medium consisting of 2% bactopeptone, 1% yeast extrct, and 2% glucose for 24 h. The major differences between these and the spectra obtained from glucose-repressed cells are in the sugar phosphate region. The level of Fru-P_2 is ~2 times lower then it was for repressed cells; estimated intracellular concentrations are 3 mM anaerobically and 4 mM aerobically (see Table I). In the extract obtained during anaerobic glycolysis the αGP peak is the highest peak in the sugar phosphate region (about 3 mM), while in the aerobic case the αGP signal is decreased and the G6P signals are increased from 1 to about 4 mM. The level of 3PGA is 0.7 mM aerobically, whereas it was unobservable anaerobically (i.e., <0.2 mM). The nucleoside phosphate peaks at 10 to 11 ppm show that under both aerobic and anaerobic conditions the NTP levels are much higher than the NDP levels. However, note that the NDP peaks are about 2 times higher in the anaerobic sample while its NTP levels are about 20% lower.

The ^{31}P NMR spectra of extracts obtained of yeast grown with acetate as a carbon source were qualitatively similar to those shown in Figure 2. The major difference was that the Fru-P_2 level was somewhat lower, and in the anaerobic case the αGP peak was even higher. The increase in G6P level upon oxygenation in this sample was a factor of 10, whereas is was a factor of 4 in Figure 2. The level of 3PGA increased by a factor of 3 upon oxygenation. All these values are listed in Table I.

Table I also lists data taken from the ^{31}P NMR spectra of extracts of cells that were grown with raffinose as a carbon source. Most of the differences between the aerobic and anaerobic cases are similar to those already shown in Figure 2. There is an important difference from both Figure 2 and the acetate-grown cell data which is that in the raffinose grown cells the G6P and 3PGA levels are quite similar aerobically and anaerobically (See Table I).

Next we turn to the ^{13}C NMR spectra of perchloric acid extracts taken during the utilization of ^{13}C-labeled glucose. Figure 3 shows the spectra of extracts of aerobic and anaerobic suspensions of yeast cells, after they had been grown with raffinose as a carbon source, while they were glycolyzing [1-^{13}C]glucose. The extract obtained during aerobic glycolysis shows a higher level of residual [1-^{13}C]glucose, because of the lower rate of glucose utilization. The inset shows an expanded view of the part of the spectrum from 64–69 ppm. It is clearly observed that Fru-P_2 is labeled in both the C_1 and the C_6 positions. In the extract taken under anaerobic conditions the ratio of the Fru-P_2 labeled at C_6 to Fru-P_2 labeled at C_1 is observed to be 0.5 (see Table II). In the aerobic case this ratio is 0.84, which is substantially higher. In the aerobic

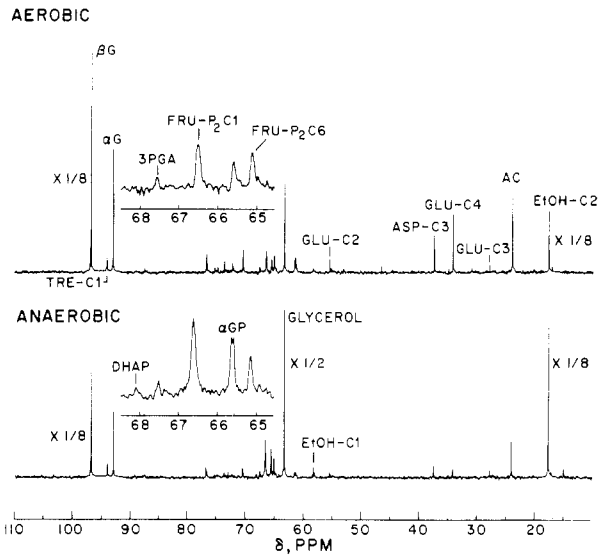

FIGURE 3: ^{13}C NMR spectra of extracts of suspensions of raffinose-grown cells taken during glycolysis on [1-^{13}C]glucose. The upper trace shows the spectrum of the extract obtained under aerobic conditions, while the lower trace is of the extract obtained anaerobically. The ratio [6-^{13}C]Fru-P_2/[1-^{13}C]Fru-P_2 is higher aerobically than anaerobically.

Table II: Measured ^{13}C Scrambling Ratios of Fru-1,6-P_2[a]

substrate	measured ratio	anaerobic glycolysis	aerobic glycolysis
[1-^{13}C]glucose	[6-^{13}C]Fru-1,6-P_2/[1-^{13}C]-Fru-1,6-P_2	0.5	0.84
[6-^{13}C]glucose	[1-^{13}C]Fru-1,6-P_2/[6-^{13}C]-Fru-1,6-P_2	0.24	0.33

[a] Scrambling ratios in Fru-1,6-P_2 of the ^{13}C label from C_1 to C_6 (for [1-^{13}C]glucose) and from C_6 to C_1 (for [6-^{13}C]glucose) during anaerobic and aerobic glycolysis in raffinose-grown cells. The scrambling ratios in Fru-1,6-P_2 were determined in perchloric acid extracts taken 10 min after the addition of ^{13}C-labeled glucose to suspensions of yeast cells.

spectra we also observe the ^{13}C label in glutamate and aspartate. It is interesting to note that aspartate is preferentially labeled in the C_3 position, reflecting its direct formation from oxaloacetate, without having passed through the TCA cycle.

Figure 4 shows the expanded region between 60 and 70 ppm of the ^{13}C NMR spectrum of extracts obtained from raffinose-grown cells under conditions similar to those of Figure 3, but here [6-^{13}C]glucose was used instead of [1-^{13}C]glucose. These spectra again show that both C_6 and C_1 of Fru-P_2 are labeled. In the anaerobic case the ratio of Fru-P_2 labeled at C_1 to Fru-P_2 labeled at C_6 is 0.24, while in the aerobic case it is 0.33. It is interesting that the fractions scrambled starting from [6-^{13}C]glucose are about 2 times lower than the corresponding ratios with [1-^{13}C]glucose, but again the observed scrambling was higher in the aerobic sample. In Figure 4 we also observe that the G6P is labeled at C_6.

In the ^{13}C NMR spectra the [6-^{13}C]Fru-P_2 signal happens to be quite close to the signal of [6-^{13}C]F6P, while the [6-^{13}C]G6P interferes with [1-^{13}C]F6P. In the raffinose-grown cells the Fru-P_2 level is considerably higher than the G6P and F_6P levels, so that these overlaps do not seriously affect our measurements of the scrambling ratios in Fru-P_2. When we repeated these experiments with cells grown on acetate, we were not able to measure reliable scrambling ratios because the high levels of F6P and G6P in the aerobic case interfered with our measurements. However, for these cells we once again observed a higher scrambling ratio in Fru-P_2 under aerobic conditions.

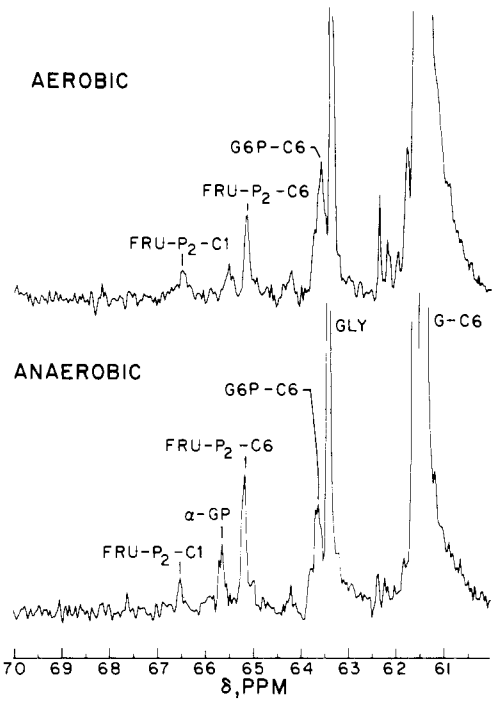

FIGURE 4: Expanded view of the 60–70 ppm part of the ^{13}C NMR spectrum of extracts prepared during aerobic and anaerobic glycolysis of [6-^{13}C]glucose. The ratio [1-^{13}C]Fru-P_2/[6-^{13}C]Fru-P_2 is higher aerobically. Note that the scrambling of the label is about a factor of 2 less than it was for [1-^{13}C]glucose.

DISCUSSION

This study was undertaken to measure and compare the levels of intermediates of the hexose pathway during aerobic and anaerobic glycolysis. The ^{31}P NMR spectra show the hexose phosphates and the pools of nucleoside di- and triphosphates, while the ^{13}C NMR studies allow us to follow the label in the intermediates and end products. The levels of intermediates as determined from the ^{31}P spectra are collected in Table I. Qualitatively, there is a good agreement between these values and those reported in literature, which were measured by using various assay techniques (Gancedo & Gancedo, 1973; Betz & Chance, 1965a,b; Ghosh & Chance, 1964; Barwell et al., 1971) although significant quantitative differences exist with previous comparisons of aerobic and anaerobic glycolysis (Holzer et al., 1958; Holzer & Freytag-Hilf, 1959). Two questions were addressed in this study. First, by use of the crossover theorem (Chance et al., 1958; Buecher & Ruessmann, 1963) it should be possible to determine which enzymatic steps control the glycolytic flux by comparing the changes in the levels of intermediates upon oxygenation. Second, we have tried to determine the factors responsible for the change of the glucose utilization rate when going from anaerobic to aerobic conditions.

In the previous paper (den Hollander et al.), we discussed the changes in glucose utilization rates under the conditions used in this study. It was shown that in glucose-repressed cells the rate of glucose utilization is the same aerobically and anaerobically. However, in derepressed cells under aerobic conditions the rate of glucose utilization (i.e., the apparent V_{max}) was about 2 times slower than anaerobically. For glucose-repressed cells there is no difference in the level of G6P between aerobic and anaerobic glycolysis (Table I), which corresponds with the observation that their rate of glucose utilization is the same (den Hollander et al., 1985). However, for derepressed cells we obtain inconsistent correlations between G6P levels and the rate of glucose utilization. For

acetate-grown cells (See Table I) we observe an increase in G6P level of almost 1 order of magnitude, while for cells grown into saturation (Figure 2 and Table I) there is an increase of about a factor of 4. These observations are in agreement with the notion that PFK is slowed down in the aerobic case and are also in apparent agreement with the idea of feedback inhibition of glucose uptake by G6P. However, for raffinose-grown cells, we do not observe any change in the level of G6P when comparing aerobic and anaerobic glycolysis; in both cases the level is about 4 mM intracellular. We have established separately (den Hollander et al., 1985) that the change in the rate of glucose utilization is about the same for cells grown into saturation, for acetate-grown cells, and for raffinose-grown cells. We therefore conclude that for raffinose-grown cells G6P is not controlling the rate of glucose utilization, but that instead the rate of glucose utilization is controlled by a different, independent mechanism. Hence, it seems likely but not definite that for cells grown under different conditions the same mechanism independent of G6P is responsible for the control of glucose utilization. The changes in G6P level in these systems apparently reflect the changes in the rates of enzymes lower down in the hexose pathway, particularly PFK as discussed in the following paper by Reibstein et al. (1985). Beyond this independence of G6P levels, we have not studied and do not understand the mechanism responsible for the control of glucose utilization.

In all systems we studied we found only small increases of 30% in the levels of nucleoside triphosphates going from anaerobic to aerobic conditions. Therefore, we concluded that the nucleoside triphosphates do not play a significant role in the control of the glycolytic flow, since the ATP level is always in a range where it hardly affects the rate of PFK (Banuelos et al., 1977; Holzer et al., 1958).

For the derepressed cells, however, we do observe a significant change in the level of the nucleoside diphosphates upon oxygenation. Aerobically, this level is about a factor of 3 lower than that anaerobically. In a separate paper (den Hollander et al., 1981) we have reported that the level of intracellular P_i also decreased by at least a factor of 3 upon oxygenation of suspensions of derepressed cells while glycolyzing. Therefore, $[ATP]/([ADP][P_i])$ is increased by more than an order of magnitude upon oxygenation, with only a small fraction coming from the small increase in ATP.

If we assume that the adenylate kinase reaction is close to equilibrium we have to conclude that the level of AMP should decrease by almost an order of magnitude upon oxygenation (from about 0.3 to about 0.05 mM). Direct measurement of AMP levels in the extracts is not very reliable, because any hydrolytic activity occurring during the extraction procedure will greatly increase the levels of AMP. It is interesting that these predicted levels of AMP are in the range where they should greatly influence the rate of PFK (Banuelos et al., 1977). Therefore the changes were have observed for P_i and pH (den Hollander et al., 1981) and infer here for AMP all are in the range where they could affect the rate of PFK in a way that is consistent with observed changes in the levels of G6P and the glycolytic flux. On the other hand, changes in the level of ATP are small and hardly affect the rate of PFK. This conclusion about the role of ATP is consistent with findings of Holzer et al. (1958) but is commonly ignored in discussion about the Pasteur effect in yeast. The roles of these different effectors of PFK are analyzed experimentally in the following paper by Reibstein et al.

The spectra also show that the level of 3PGA is about 3 times higher in the aerobic condition, both for cells grown into saturation and for acetate-grown cells. This observation suggests that there is also a control in the triose part of the pathway, presumably pyruvate kinase (PK). We do not know which metabolite is responsible for this particular control of PK although a more detailed study is possible by the present methods.

As mentioned, the level of Fru-P_2 in the systems we studied is not very responsive to the presence of oxygen. For acetate-grown cells and for cells grown into saturation there is a 30% increase in the level of Fru-P_2, while for glucose-repressed and for raffinose-grown cells there is a 30% decrease upon oxygenation. This relatively constant level of Fru-P_2 must be reconciled with the fact that in derepressed cells the rate of glucose utilization decreased upon oxygenation. Furthermore, in acetate-grown cells, and cells grown into saturation, the G6P level increases in the presence of O_2 when the flow through G6P decreases, indicating that the rate of PFK is controlled. The crossover theorem (Chance et al., 1958) would have predicted a *decrease* in the level of Fru-P_2 when we observe an increase in the level of G6P where we actually observe a relatively small *increase* in Fru-P_2 (Buecher & Ruessmann, 1963). This observation therefore indicates that there is another control point in the lower part of the pathway, which is responsible for an increased level of Fru-P_2 upon oxygenation. One reason for the decreased flow through the lower part of the patway in aerobic conditions may come about because of the observation that in derepressed cells under anaerobic conditions there is an appreciable formation of glycerol (Holzer et al., 1963). In derepressed cells this provides an additional flow from DHAP through αGP dehydrogenase, which is essentially turned off aerobically.

Further evidence for control in the triose part of the pathway comes from the observation of "scrambling" of the ^{13}C label in Fru-P_2 in the ^{13}C NMR experiments, and its dependence upon oxygenation. In an early paper we showed that the scrambling of the ^{13}C label in Fru-P_2 could be used to obtain quantitative information about the kinetics of the Fru-P_2–aldolase–TPI triangle (den Hollander et al., 1979). Using the kinetic scheme used for that study, we have performed model calculations to evaluate the present measurements of the scrambling of the ^{13}C label in Fru-P_2 during aerobic and anaerobic glycolysis in terms of the various enzymatic rates involved. In the model calculations this is expressed as a smaller apparent rate constant of GAPDH. Such a lower rate constant leads to a higher probability that the ^{13}C label, once it has appeared in GAP, will backup and reappear in Fru-P_2 through aldolase activity, leading to more scrambling in Fru-P_2. Therefore, both the constant level of Fru-P_2 and the higher scrambling of the ^{13}C label in Fru-P_2 in aerobic glycolysis are consistent with the existence of another control point in the triose part of the pathway.

The present data for raffinose-grown cells show a factor of 2 difference between the scrambling of the label in the [1-^{13}C]- and the [6-^{13}C]glucose experiments. It should be noted that for [6-^{13}C]glucose the pentose cycle cannot contribute to appearance of label in the C_1 position of Fru-P_2. Therefore, the observation that in the experiments with [6-^{13}C]glucose the amount of label appearing in the C_1 position did increase upon oxygenation indicates an increased flow through aldolase to Fru-P_2 from the trioses, regardless of the flow through transaldolase. However, the existence of an appreciable flow through transaldolase greatly complicated a quantitative treatment, and additional information is required for such a calculation. Information about the absolute enrichment of glycerol and ethanol would provide such information.

In conclusion, the present results indicate that there are several independent control sites in the Embden–Meyerhof–Parnas pathway, a conclusion that is similar to the coordinated control proposed by Racker for ascites tumor cells (Racker, 1974, 1965). The first control site, either the glucose transport system or hexokinase, determines the rate of glucose utilization. Our study indicates that the control of the utilization rate is independent of feedback control by G6P, which is contrary to previous results (Serrano & DelaFuente, 1974; Azam & Kotyk, 1969; Kotyk & Kleinzeller, 1967; Sols, 1965).

The second control site is PFK, which is responsible for the increase in G6P levels we have observed under certain conditions. Here we have shown that the levels of intracellular P_i and AMP, as well as the intracellular pH, are changed in the transition of aerobic to anaerobic glycolysis while ATP is almost constant. The quantitative effects of these changes upon flux through PFK are investigated in the following paper.

A third control site is in the triose pathway and is probably PK, as concluded from our observation of an increased level of 3PGA under aerobic conditions and the increased scrambling of 1-^{13}C to 6-^{13}C in Fru-P_2. Given the high and relatively constant levels of Fru-P_2 and ATP, we infer that they are not the most likely relevant effectors of PK during the switch to aerobic glycolysis. The present measurements supplement our NMR measurements of intact cells, and from these combined measurements we have found systematic changes in going from anaerobic to aerobic glycolysis. The quantitative determination of control at PFK is the next step to be studied, and this is done in the following paper.

Registry No. NAD, 53-84-9; D-glucose, 50-99-7; D-glucose 6-phosphate, 56-73-5; α-glycerophosphate, 57-03-4; 3-phosphoglyceric acid, 820-11-1; D-fructose 1,6-bisphosphate, 488-69-7.

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Studies on the Regulation of Yeast Phosphofructo-1-kinase: Its Role in Aerobic and Anaerobic Glycolysis†

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ABSTRACT: The kinetics of yeast phosphofructo-1-kinase has been studied in vitro. Effector concentrations (Fru-6-P, ATP, ADP, AMP, P_i, Fru-1,6-P_2, and Fru-2,6-P_2) and pH were adjusted so as to mimic intracellular concentrations in yeast. Under these conditions we were able to reproduce the measured in vivo rate of PFK. In addition, by reconstituting the intracellular conditions existing during aerobic and anaerobic glycolysis, we were able to reproduce in vitro the changes in the rate of PFK observed under these conditions. Without the addition of the newly discovered effector Fru-2,6-P_2, in vitro rates of PFK are much lower than its in vivo rate. Changes in Fru-2,6-P_2, Fru-1,6-P_2, ATP, AMP, P_i, and pH in going from aerobic to anaerobic conditions all contributed somewhat to the change in the rate of PFK observed during the Pasteur effect, with no contribution coming from ADP. These studies show that the control of PFK under the condition of the Pasteur effect cannot be ascribed to changes in any one particular effector but rather to contributions from a variety of effectors. Also, the *net* change in the rate of PFK in the switch from anaerobic to aerobic glycolysis is small compared with the change in its dependence upon its substrate Fru-6-P, indicating a compensation mechanism.

Phosphofructo-1-kinase (PFK)[1] has been implicated as an important control point in the glycolytic pathway (Passonneau & Lowry, 1962, 1963; Vinuela et al., 1963; Tejwani, 1978; Banuelos et al., 1977; Sols, 1976; Ramaiah, 1974; Chance, 1959), in particular in explanations of the Pasteur effect (Krebs, 1972; Racker, 1974; Rose, 1971; Sols et al., 1971). The activity of PFK from yeast is known to be altered in vitro by many physiologically relevant effectors. Among these are ATP, ADP, AMP, NH_4^+, fructose 1,6-bisphosphate (Fru-1,6-P_2), fructose 2,6-bisphosphate (Fru-2,6-P_2), pH, P_i, and possibly citrate (Banuelos et al., 1977; Atzpodien & Bode, 1970; Hess, 1973; Ghosh & Chance, 1964; Moore et al., 1965; Betz & Chance, 1965a,b; Hofmann & Kopperschlaeger, 1982; Salas et al., 1965). In addition, PFK shows sigmoidal kinetics with respect to its substrate Fru-6-P (Sols, 1981). It is clearly possible that any of these effectors might be important in changing the activity of PFK in response to changing environmental and metabolic conditions. In fact, the literature abounds in claims that one or another of these effectors is *the* controller of PFK activity, and there is a history of conflict between proponents of different effectors.

Two questions emerge from these facts: First, why are there so many effectors of PFK? Second, how important is each of these effectors in vivo? In order to answer these questions, at least two requirements must be satisfied: (1) the in vivo

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[1] Abbreviations: Fru-6-P, fructose 6-phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; P_i, inorganic phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK, phosphofructokinase; NADH, reduced nicotinamide adenine dinucleotide; PMSF, phenylmethanesulfonyl fluoride; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; G6P, glucose 6-phosphate.

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