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Use of ^{31}P Nuclear Magnetic Resonance Spectroscopy and ^{14}C Fluorography in Studies of Glycolysis and Regulation of Pyruvate Kinase in *Streptococcus lactis*

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High-resolution ^{31}P nuclear magnetic resonance spectroscopy and ^{14}C fluorography have been used to identify and quantitate intermediates of the Embden-Meyerhof pathway in intact cells and cell extracts of *Streptococcus lactis*. Glycolysing cells contained high levels of fructose 1,6-bisphosphate (a positive effector of pyruvate kinase) but comparatively low concentrations of other glycolytic metabolites. By contrast, starved organisms contained only high levels of 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate. The concentration of P_i (a negative effector of pyruvate kinase) in starved cells was fourfold greater than that maintained by glycolysing cells. The following results suggest that retention of the phosphoenolpyruvate pool by starved cells is a consequence of P_i-mediated inhibition of pyruvate kinase: (i) the increase in the phosphoenolpyruvate pool (and P_i) preceded depletion of fructose 1,6-bisphosphate, and (ii) reduction in intracellular P_i (by a maltose-plus-arginine phosphate trap) caused the restoration of pyruvate kinase activity in starved cells. Time course studies showed that P_i was conserved by formation of fructose 1,6-bisphosphate during glycolysis. Conversely, during starvation high levels of P_i were generated concomitant with depletion of intracellular fructose 1,6-bisphosphate. The concentrations of P_i and fructose 1,6-bisphosphate present in starved and glycolysing cells of *S. lactis* varied inversely. The activity of pyruvate kinase in the growing cell may be modulated by the relative concentrations of the two antagonistic effectors.

Pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) from *Streptococcus lactis* is a tetrameric enzyme (240,000 M_r) composed of four subunits of equal molecular weight (8). Data from in vitro (7, 8, 35) and in vivo (19, 36, 38, 41) studies suggest that the allosteric enzyme may play an important role in the regulation of glycolysis in group N streptococci and other lactic acid bacteria (1, 2, 34). Kinetic analyses by Collins and Thomas (7) and by Crow and Pritchard (8) show that pyruvate kinase from *S. lactis*, like that from other organisms, is activated by fructose 1,6-bisphosphate (Fru-P_2) and inhibited by P_i (1, 7). Enzyme activity was a sigmoidal function of Fru-P_2 concentration, and the positive effector increased the apparent V_{max} and decreased the K_m for the two substrates phosphoenolpyruvate (PEP) and ADP. Although the Fru-P_2 concentration required for half-maximum velocity in vitro was only 0.06 to 0.2 mM (8, 35), the intracellular concentration of Fru-P_2 (15 to 30 mM; 7, 38, 41) in glycolysing cells of *S. lactis* was several hundredfold greater than the Fru-P_2 concentration required for half-maximum velocity. In comparison with Fru-P_2, the concentrations of other potential activators (35) of pyruvate kinase (e.g., glucose 6-phosphate [G6P], fructose 6-phosphate, and dihydroxyacetone phosphate) and of 2-phosphoglycerate (2-PG), 3-phosphoglycerate (3-PG), and PEP were low (38, 41).

In 1977 Thompson and Thomas (41) discovered that starved cells of *S. lactis* contained fourfold-greater concentrations of 2-PG, 3-PG, and PEP (total, ca. 30 to 40 mM) than did glycolysing cells. Significantly, starved cells were depleted of other glycolytic intermediates, including Fru-P_2. These findings (subsequently confirmed by Mason et al. [19]) were important for three reasons. First, since the conversion of PEP to pyruvate by pyruvate kinase is thermodynamically very favorable ($K = 2 \times 10^3$ to 20×10^3 [24]), the retention of the PEP pool showed that the enzyme was catalytically inactive. Second, the concentration of PEP changed in a direction opposite to that of glycolytic flux, and the PEP crossover (17, 29) provided in vitro confirmation of the regulatory function of pyruvate kinase. Finally, the maintenance of an endogenous PEP pool permitted the study of PEP-dependent sugar-phosphotransferase systems (PTS; 12, 28) in physiologically intact cells (6, 30, 38, 40, 41). In our original report (41), we suggested that inactivation of pyruvate kinase was a consequence of depletion of positive enzyme effectors, primarily of Fru-P_2. However, Mason et al. (19) proposed that the high intracellular P_i concentration was responsible for enzyme inhibition.

In this investigation we have reexamined and attempted to reconcile the two mechanisms proposed for pyruvate kinase inhibition in *S. lactis*. To define the properties of a regulatory enzyme (24, 29) such as pyruvate kinase, one would ideally monitor changes in enzyme activity simultaneously with variation of effector concentrations in the intact cell. This in vivo approach is now feasible with high-field Fourier transform-nuclear magnetic resonance spectroscopy (NMR) and the application of this noninvasive technique (notably by Shulman and colleagues; 22, 42, 43) has significantly advanced our understanding of microbial energetics. Most NMR studies have been conducted with yeasts (23) and gram-negative bacteria, including *Escherichia coli* (22, 42, 43), *Chromatium vinosum* (25, 27), and *Rhodopseudomonas sphaeroides* (26). With the exception of two recent investigations with *Staphylococcus aureus* (9) and *Streptococcus faecalis* (33), there have been no reports of the application of ^{31}P NMR to study carbohydrate transport and metabolism in

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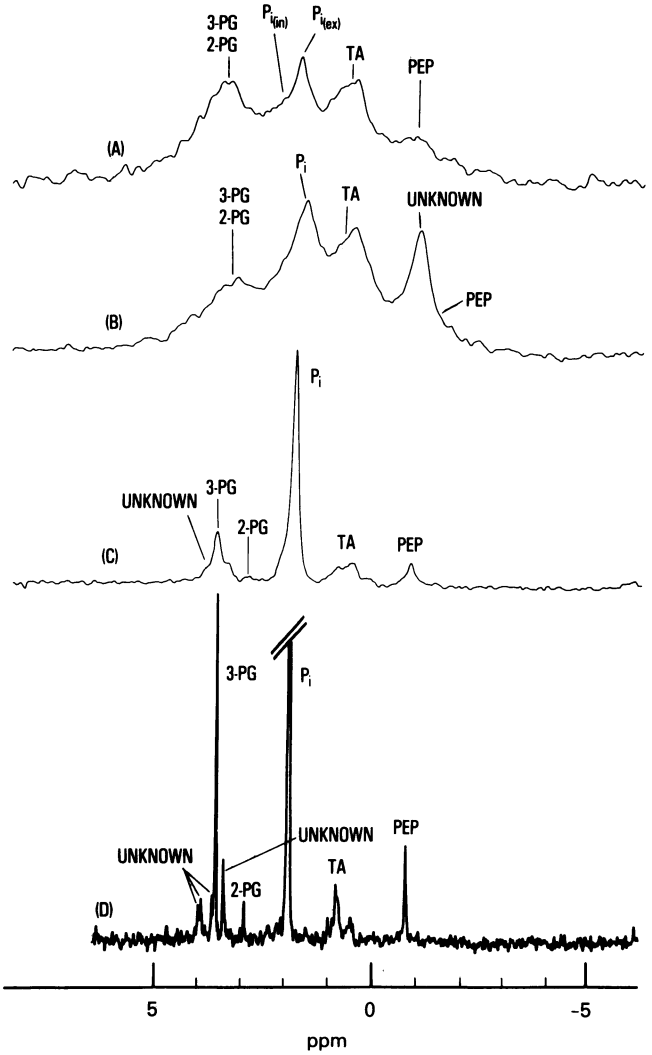

FIG. 1. ^{31}P NMR spectra of (A) starved cells of *S. lactis* 133; (B) starved cells of *S. lactis* ML3. (C) cell extract prepared from *S. lactis* ML3; and (D) cell extract prepared from *S. lactis* ML3 in the −2- to 5-ppm range. All spectra were obtained at 101.4 MHz except (D) which was obtained at 121 MHz. The chemical shift scale is in parts per million relative to external 85% H_3PO_4.

gram-positive organisms. We have used a combination of ^{31}P NMR and ^{14}C fluorography to follow time-course fluctuations in the concentrations of Embden-Meyerhof pathway intermediates (5) in glycolysing and starved cells. Our data provide in vivo evidence for the dual but antagonistic roles of Fru-P_2 and P_i in modulation of pyruvate kinase activity in *Streptococcus lactis*.

MATERIALS AND METHODS

Organisms. *S. lactis* ML3 and *S. lactis* 133 were obtained from the culture collection of the New Zealand Dairy Research Institute, Palmerston North, New Zealand.

Growth of cells. Organisms were grown in a complex medium (40) containing 28 mM galactose or 14 mM maltose, except for studies of sugar transport when cells of *S. lactis* ML3 were grown in a chemically defined medium (37) supplemented with high-purity maltose.

Sugar transport by intact cells. The procedures for the preparation of starved cells and for monitoring sugar uptake have been presented in previous communications (38, 41). In the present study, cells were suspended at a density of 200 μg (dry weight) of cells per ml in 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.2) containing 0.5 mM $MgSO_4$ and 10 mM iodoacetate. Radioactive sugars (specific activity, 0.2 μCi/μmol) were added to the suspension at the following concentrations: glucose and 2-deoxy-D-glucose, 0.2 mM; chromatographically purified maltose, 0.2 mM. Accumulation of sugars was followed by membrane (Millipore Corp., Bedford, Mass.) filtration and liquid scintillation procedures (38, 41). Arginine, when required, was present at a final concentration of 5 mM, and all transport experiments were conducted at 30°C.

Time course of glycolysis; ^{31}P NMR and fluorography analyses. Cells of *S. lactis* ML3 were grown in 1,600 ml of complex medium containing 28 mM galactose and were collected (at the mid-log phase of growth) by centrifugation at 13,000 × *g* for 5 min at 4°C. Supernatant fluid was discarded, and the cell pellet was washed twice by suspension in 200 ml of 0.01 M $MgSO_4$ solution (at 0°C) followed by centrifugation. The packed cell pellet (ca. 3.6 g [wet weight]) was suspended to a final volume of 8 ml with 0.1 M PIPES buffer (pH 7.2) containing 0.5 mM $MgSO_4$. Cell suspension (5 ml) was transferred to a 25-ml Erlenmeyer flask, and, after equilibration to 30°C, 200 μl of [*U*-^{14}C]glucose was added to a final concentration of 20 mM (specific activity, 0.25 μCi/μmol). After glucose addition, samples (0.5 ml) of cell suspension were removed at intervals of 2, 10, 20, and 30 s, and 1, 2, 5, and 10 min, and the samples were immediately injected into 5 ml of boiling water contained in 15-ml Corex tubes. After 5 min of boiling-water extraction, the suspensions were cooled to 0°C and then were clarified by centrifugation at 27,000 × *g* for 30 min at 4°C. Supernatant fluids were removed, lyophilized, and reconstituted to a final volume of 100 μl with distilled water. Radiolabeled glycolytic intermediates present in extracts were separated and quantitatively identified by thin-layer (polyethyleneimine) fluorography as described previously (38, 39). For ^{31}P NMR spectroscopy, each of the reconstituted extracts was made up to a final volume of 0.7 ml in 0.1 M PIPES buffer (pH 7.2) containing 0.5 mM $MgSO_4$ and 5 mM EDTA.

Preparation of cells for ^{31}P NMR studies. Cells of *S. lactis* ML3 and *S. lactis* 133 were grown and harvested as described above. However, the cell pellet was washed twice with 100 ml of 0.1 M PIPES buffer (pH 7.2) containing 0.5 mM $MgSO_4$ (at 0°C). The cell pellet (3.4 to 3.8 g) was then suspended in a final volume of 8 ml with 0.2 M PIPES buffer (pH 7.2) containing 1 mM $MgSO_4$. The cell suspension was maintained on ice until ^{31}P NMR spectra were obtained.

Preparation of cells containing [^{14}C]PEP pool intermediates. Cells of *S. lactis* ML3 were grown in 1,600 ml of complex medium containing 14 mM maltose. At the mid-log phase of growth, the cells were collected by centrifugation and washed twice with 0.01 M $MgSO_4$ as described previously. The washed cell pellet (3.8 g [wet weight]) was suspended in and thoroughly homogenized with 0.1 M PIPES buffer (pH 7.2) containing 0.5 mM $MgSO_4$ to a final volume of 8 ml. [*U*-^{14}C]glucose solution (200 μl) was added to a final concentration of 20 mM (specific activity, 0.25 μCi/μmol). After 10 min of incubation at 30°C, all of the glucose had been metabolized, and the starved cells contained only the [^{14}C]PEP metabolites (3-PG, 2-PG, and PEP). Iodoacetate was then added to the suspension (10 mM final concentration) to block glyceraldehyde 3-phosphate dehydrogenase and subsequent glycolysis (38). The cells were collected by centrifugation (13,000 × *g* for 5 min at 4°C), and the cell

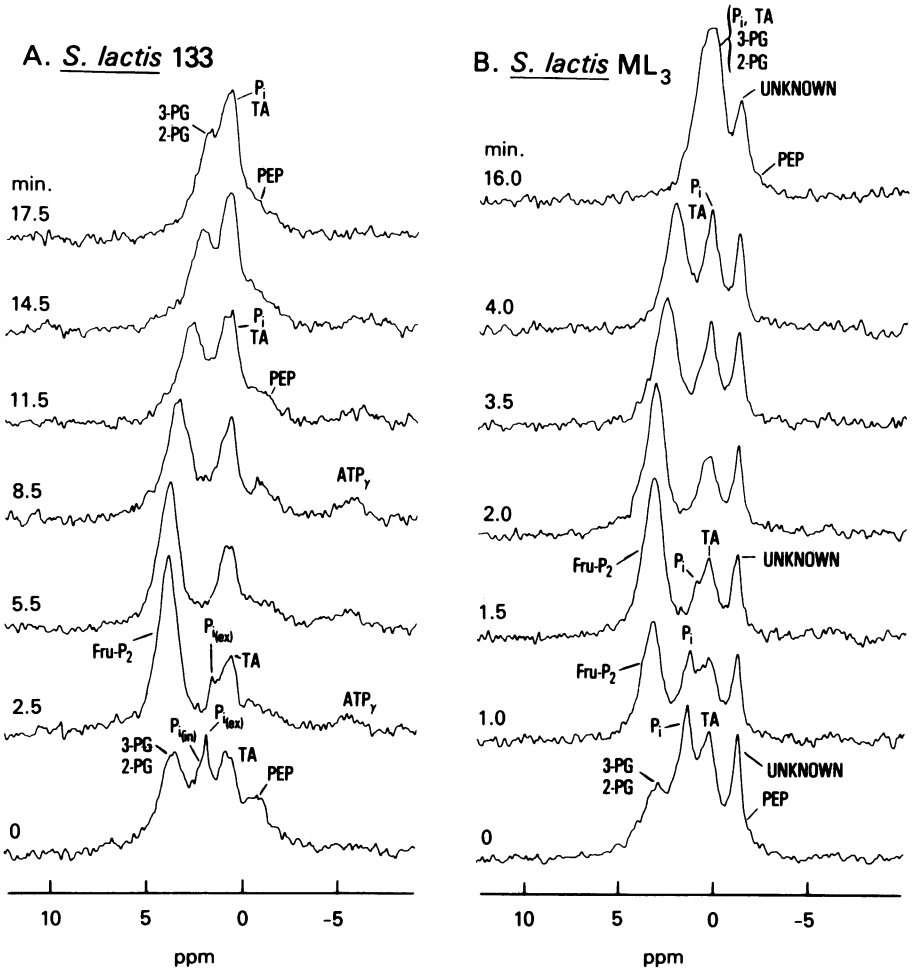

FIG. 2. ^{31}P NMR spectra (101.4 MHz) of time course in glycolysing cells of (A) *S. lactis* 133 and (B) *S. lactis* ML3.

pellet was suspended to 5 ml with 0.1 M PIPES buffer (pH 7.2) containing 0.5 mM $MgSO_4$ plus 5 mM iodoacetate. Thereafter, 1-ml volumes of cell suspension were added in turn to four tubes. Tube 1 contained 0.5 ml PIPES buffer (pH 7.2), 0.5 mM Mg^{2+}, and 5 mM iodoacetate (control). Tube 2 contained contents as in tube 1 plus 30 mM arginine. Tube 3 contained contents as in tube 1 plus 15 mM maltose. Tube 4 contained contents as in tube 1 plus a combination of 30 mM arginine and 15 mM maltose. After 4 min of incubation at 30°C, the tubes were transferred to a boiling-water bath and 5 ml of boiling water was added to each. The suspensions were boiled for 5 min, cooled, and clarified by centrifugation. Supernatant fluids were removed and lyophilized, and the residue was reconstituted to 200 μl with distilled water. Radiolabeled metabolites were identified by thin-layer fluorography. For ^{31}P NMR studies, the four reconstituted extracts were made up to 0.7 ml with 0.1 M PIPES buffer (pH 7.2) containing 5 mM EDTA and 0.5 mM $MgSO_4$.

NMR spectroscopy. ^{31}P NMR spectra were obtained at 101.4 MHz with a spectrometer built at the National Institutes of Health and described elsewhere (32). A probe containing a solenoid coil (8-mm outside diameter; 0.4-ml volume) tuned at the ^{31}P resonance frequency was used since this coil geometry gives better sensitivity than the saddle coil geometry normally used in commercial spectrometers (13). Sample temperature in the probe was maintained at 20 to 22°C by passing nitrogen gas over the NMR tube which was located within the probe Dewar flask. Sample spinning, field frequency locking, and ^{1}H decoupling were not employed since line widths observed in the spectra of the cells (100 to 200 Hz) greatly exceeded both the inhomogeneity in the external field (ca. 8 Hz) and the ^{31}P-^{1}H J couplings (ca. 10 Hz). In a few cases, high-resolution spectra of cell extracts were obtained at 121 MHz on a Bruker WM300 spectrometer with proton decoupling. It was essential to obtain spectra of cells as quickly as possible because of the rapid rate of glycolysis in *S. lactis*. Spectra with adequate signal-to-noise ratios were obtained in 45 s by using a 5-μs (40°) pulse, a pulse recycle time of 0.35 s, and 128 signal aquisitions. The relative intensities of the various signals in these spectra were the same as those observed in cells obtained by using a recycle time of 1 s.

Spectra of cell extracts were obtained with the same spectrometer settings that were used to obtain spectra of whole cells, except that the pulse recycle time was 1 s. The relative signal intensities obtained in these spectra were the same as those obtained by using a 2-s recycle delay time. Chemical shifts are reported in parts per million (ppm) relative to external 85% phosphoric acid. All spectra were obtained by using a 10-kHz total spectral window with a digital resolution of 2.5 Hz/pt. in the transformed spectra. All mathematical operations on the data were performed

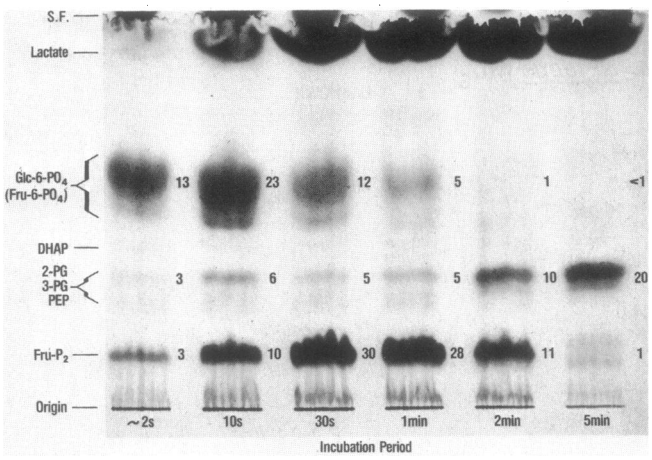

FIG. 3. Time course analysis by ^{14}C fluorography of glycolytic intermediates in *S. lactis* ML3. The cells were incubated with [*U*-^{14}C]glucose, and cell extracts were prepared as described in the text. Samples (5 μl) of each reconstituted extract were applied to the polyethyleneimine thin-layer. S.F., second solvent front (0.5 M LiCl/2 N formic acid [1:1]); DHAP, dihydroxyacetone phosphate. Numerical values are intracellular (mM) concentrations of the intermediates.

with Nicolet NTCFT software. Exponential filters of 5 and 10 Hz were applied to the free induction decay signals of the cell extracts and intact cell samples, respectively, before Fourier transformation. Signal areas were measured by using the integration subroutine in the NTCFT software and have uncertainties of ±10%.

Reagents. Extra-high-purity maltose (<0.25% glucose) was obtained from BDH, Poole, England. [*U*-^{14}C]glucose and [*U*-^{14}C]maltose were purchased from New England Nuclear Corp., Boston, Mass. The radiolabeled disaccharide was purified by paper chromatography (Whatman 3MM paper; solvent, 1-butanol-acetic acid-water [ratio, 50:20:30]) to remove [^{14}C]glucose. 2-Deoxy-D-[*U*-^{14}C]glucose was purchased from ICN, Irvine, Calif. Precoated layers of polyethyleneimine-cellulose (Bakerflex) were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. PIPES and other chemicals were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

^{31}P NMR spectra of starved cells. ^{31}P NMR spectra (101.4-MHz) of *S. lactis* 133 (Fig. 1A) and *S. lactis* ML3 (Fig. 1B) cells have four overlapping signals centered at 3.5, 2.0, 0.7, and −0.85 ppm. Previous enzymatic analyses of cell extracts provide evidence that starved *S. lactis* cells contain high levels of 2-PG, 3-PG, PEP, and P_i (19, 38, 41). Spectra of the metabolites at pH 7.0 lead to the tentative assignment of the signals in the cell spectra as follows: 3-PG plus 2-PG, 3.5 ppm; intracellular P_i, 2.0 ppm; PEP, −0.85 ppm. The signal centered at 0.7 ppm in both strains of *S. lactis* is assigned to teichoic acid for three reasons: (i) a similar signal reported in spectra of *Staphylococcus aureus* (9) has been assigned to teichoic acid; (ii) the cell wall membrane of *Streptococcus lactis* contains teichoic acid consisting of 16 to 17 glycerol phosphate units joined by 1 → 3 phosphodiester linkages (approximately half of which are substituted with β-D-galactosyl residues [44]); (iii) in spectra of cell extracts (Fig. 1C), the 0.7-ppm signal is either absent or appears as a low-intensity doublet. The prominent, relatively narrow (50-Hz line width) signal at −0.8 ppm in the spectrum of *S. lactis* ML3 was also assigned to a flexible cell wall component because this signal was not observed in spectra of extracts, and its position and intensity were unchanged during glycolysis in intact cells. This signal overlays a broad PEP signal at its base.

^{31}P NMR spectra of cell extracts. To confirm the assignments of the signals in intact cells, NMR spectra of cell extracts were obtained. Four signals (line widths, 50 to 100 Hz) were observed in the −2- to 5-ppm region of spectra of extracts which lacked EDTA. In the presence of the chelating agent (1 mM), the signals narrowed, and considerable fine structure was observed in the spectrum (Fig. 1C). However, resolution was limited by the homogeneity of the National Institutes of Health spectrometer. When the spectrum of the extract was obtained at 121 MHz on a Bruker high-resolution spectrometer (Fig. 1D), the triplet centered at 3.6 ppm (Fig. 1C) was resolved into six sharp peaks (line widths, <3 Hz). The major peak at 3.6 and the peak at 2.9 ppm coincided with those of authentic 3-PG and 2-PG, respectively (added as internal standards to the cell extract). The four other peaks have not been identified, but the signal at 3.4 ppm has been tentatively assigned to 5′-AMP. Recently, in studies with membrane vesicles from *E. coli*, Hunt et al. (14) reported that 3-PG is 0.7 ppm upfield of 2-PG. For two reasons we think it likely that these assignments should be reversed. First, upon addition of 3-PG and 2-PG standards to extracts of *S. lactis*, we observed that the 3-PG signal was 0.7 ppm downfield from 2-PG. Second, from consideration of the equilibrium concentrations of the products from phosphoglycerate mutase and enolase in vivo (38), the disappearance of PEP would be accompanied initially by the formation of 2-PG, but 3-PG would predominate at equilibrium. This is, in fact, the sequence shown previously (Fig. 1 of reference 14) when the assignments of the two isomers were reversed. The remaining major peaks at 1.9 ppm and −0.85 ppm in Fig. 1D were those of P_i and PEP, respectively. The ^{31}P NMR cell extract data confirmed the assignment of the intact cell signals and agreed with results obtained previously by enzymatic analysis (38), which showed that 3-PG, 2-PG, and PEP (concentration ratio, 6:1:2) were the predominant glycolytic intermediates in starved cells of *S. lactis*. If we assume a value of 30 mM for the total concentration of metabolites comprising the PEP pool (19, 38, 41), digital integration of peak areas suggests that the intracellular P_i concentration of starved cells must be ca. 60 mM. A similar concentration was determined (by enzymatic analysis) in starved cells of the atypical *S. lactis* 7962 by Mason et al. (19). In our studies, starved cells were suspended in PIPES buffer (pH 7.0) lacking P_i, and, despite large outwardly directed concentration gradients, the high levels of P_i and PEP pool metabolites were retained by the cells for several hours at 0°C.

^{31}P NMR studies of glycolysis in *S. lactis*. Before discussing glycolysis, we should point out that there is a small, narrow peak at 1.8 ppm in the spectra of starved cells (Fig. 1A and 2A, $t = 0$). This peak is 0.2 ppm upfield from the internal P_i peak and is assigned to extracellular P_i since its intensity increases when phosphate buffer is added to the cell suspensions (data not shown). Normally, a difference in chemical shift between internal and external P_i is ascribed to a pH difference across the cytoplasmic membrane. However, we cannot draw this conclusion in the present study because the position of the intracellular P_i peak may be affected by paramagnetic ions within the cell (26).

^{31}P NMR data in Fig. 2A and B show time-course spectra of metabolites present in glycolysing cells of *S. lactis* 133 and

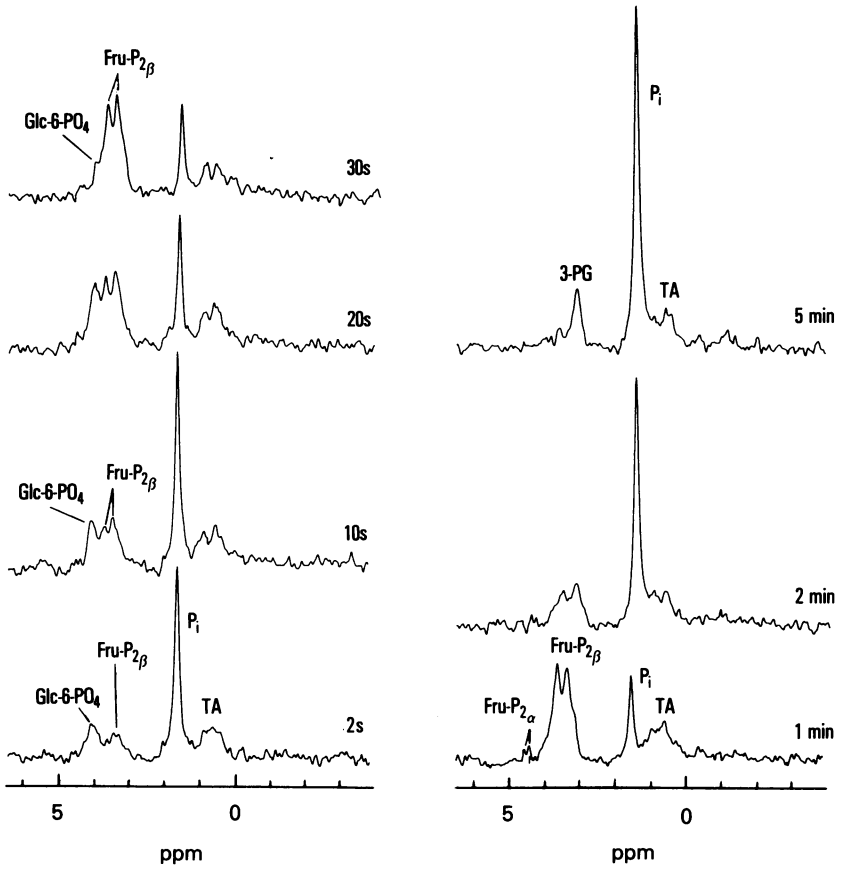

FIG. 4. ^{31}P NMR spectra (101.4 MHz) of cell extracts obtained from the glycolysis time course experiment (Fig. 3).

ML3, respectively. The spectra obtained from starved and glycolysing cells were markedly different. Within 1 to 2 min of glucose addition, the intracellular P_i signal had virtually disappeared from the spectrum, and the broad resonances of 3-PG, 2-PG, and PEP were replaced by a larger signal from the β-anomer of Fru-P_2 at 4 ppm. The generation of ATP in glycolysing cells was evident from the signal at −6 ppm (γ-phosphate) and additional signal intensity from the α- and β-phosphate of the nucleotide triphosphate at −10.5 and ca. −19 ppm, respectively (data not shown). The rapid production of lactic acid and the attendant pH decrease caused the β-Fru-P_2 doublet and P_i peaks to migrate upfield until the point of glucose exhaustion (ca. 12 to 14 min). At the end of glycolysis, the signal from P_i, which increased in intensity, and those from 3-PG and 2-PG (see below) were all superimposed on the resonance peak from teichoic acid at 0.6 to 0.7 ppm. In the intact cell spectra, the teichoic acid peak and the unknown peak in *S. lactis* ML3 at −0.8 ppm did not titrate, and no change in chemical shift occurred during glycolysis (initial pH of the suspension, 7; final pH, 5.5.)

^{31}P NMR and ^{14}C fluorography analyses. The broadness (100 to 200 Hz) of signals observed in spectra of intact cells (Fig. 2) precluded quantitative determination of metabolite levels. To circumvent this problem and to monitor the earliest changes in metabolite levels, we incubated cells of *S. lactis* ML3 with [*U*-^{14}C]glucose, and, at intervals, samples of cell suspension were extracted with boiling water. Radiolabeled glycolytic intermediates present in the extracts were separated and quantitatively determined by thin-layer fluorography (Fig. 3). Within 2 s of glucose addition, the cells contained high levels of [^{14}C]G6P (formed via the PEP-dependent mannose-PTS [38, 40]) but a relatively low concentration of Fru-P_2. The intracellular concentration of G6P was maximum at ca. 10 s, and 3-PG, 2-PG, and PEP were detectable at this time. Between 30 s and 1 min, the concentrations of the intermediates were maintained at the levels determined previously in steady-state glycolysing cells (19, 38, 41). At the point of exhaustion of [^{14}C]glucose (~1 min), the levels of G6P and Fru-P_2 declined with concomitant increases in 3-PG, 2-PG, and PEP concentrations. Starved cells (5 min) contained high levels of the PEP pool intermediates (Fig. 3), and, although still detectable, the intracellular Fru-P_2 concentration was <1 mM. To monitor changes in intracellular P_i and to confirm the identity of resonance signals, we analyzed the extracts by ^{31}P NMR (Fig. 4). In general, the fluorography and ^{31}P NMR results were in excellent agreement: (i) within the first 10 s during pure PEP-PTS cycling (19), the intracellular P_i concentration remained constant as the cells accumulated high levels of [^{14}C]G6P with the (unlabeled) PEP pool metabolites as the phosphoryl donor; (ii) the increase in Fru-P_2 concentration (20 s to 1 min) occurred simultaneously with a decrease of 60 to 70% in intracellular P_i concentration; and (iii) after glucose exhaustion (>1 min), Fru-P_2 declined with a concomitant increase in intensity of the P_i signal. It should be noted (Fig. 3, 2 min) that 3-PG, 2-PG, and PEP levels increased simultaneously with the P_i increase (Fig. 4, 2 min), although the cells still contained an appreciable concentra-

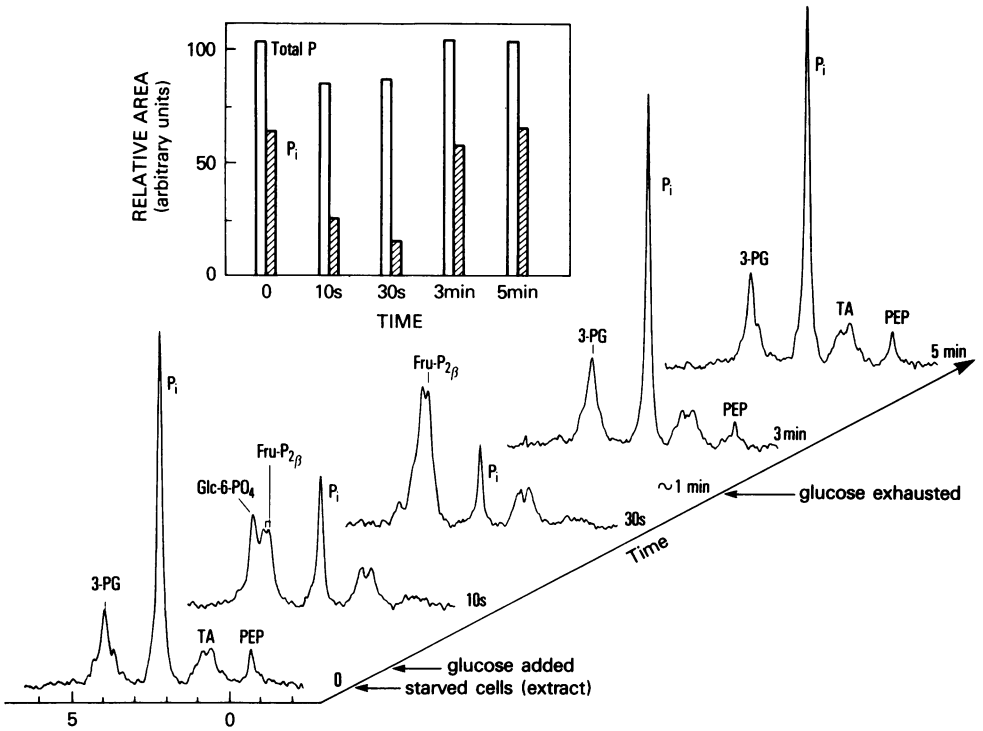

FIG. 5. ^{31}P NMR spectra of cell extracts of *S. lactis* ML3 showing the interconversion and conservation of phosphate during transition from starvation to glycolysing status and return to starvation conditions. Relative peak areas of intracellular P_i and total phosphate signals throughout the metabolic cycle are shown in the histogram (upper left).

tion (~11 mM) of Fru-P_2. The data suggest that partial inhibition of pyruvate kinase (presumably by P_i) must precede the total depletion of intracellular activator (Fru-P_2).

Conservation of intracellular P_i. In the experiment described in Fig. 3, a lower cell density than normal was used, and the cell extracts generated correspondingly smaller signals (Fig. 4). To obtain better signal-to-noise ratios for the quantitation of P_i changes during glycolysis (Fig. 5), we increased the cell concentration twofold. The series of ^{31}P NMR spectra in Fig. 5 were obtained from cells of *S. lactis* ML3 during the cyclic transition from starvation to glycolysis and back to starvation status. The spectra of extracts prepared before glucose addition (time 0) and after exhaustion of sugar from the medium (time, 5 min) were virtually

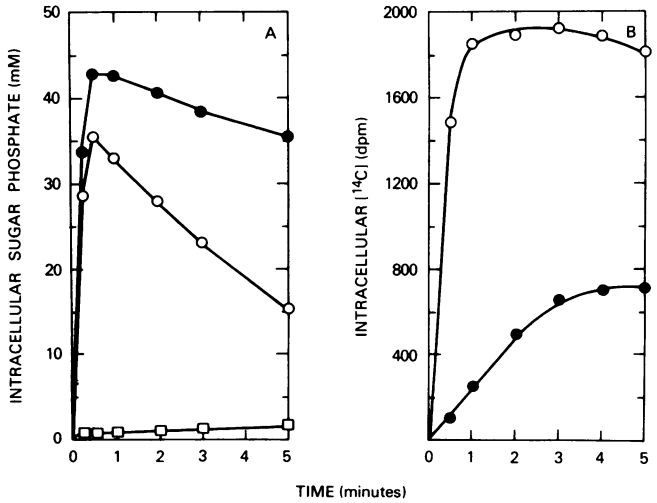

FIG. 6. Accumulation of maltose by *S. lactis* ML3 and requirement for ATP generation. (A) PEP-dependent uptake of sugars by starved cells. The cells were suspended at a final concentration of 200 μg (dry weight) per ml in 0.1 M PIPES buffer (pH 7.2) containing 0.5 mM $MgSO_4$ and 10 mM iodoacetate. Radiolabeled sugars (0.2 μCi/μmol) were added to the desired (mM) concentration as follows: ●, glucose (0.2); ○, 2-deoxy-D-glucose (0.2); □, maltose (0.5). (B) Stimulation of [*U*-^{14}C]maltose uptake by arginine. Starved cells were resuspended as in (A) in the presence (○) or absence (●) of 5 mM arginine. After 5 min of incubation [*U*-^{14}C]maltose (0.5 mM) was added to the suspension and accumulation of radiolabeled sugar was monitored as described in the text.

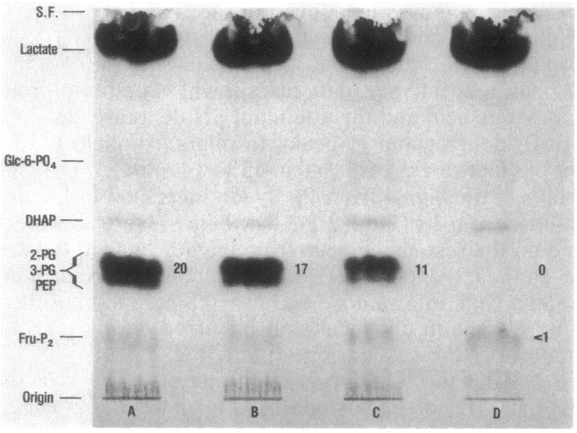

FIG. 7. Demonstration by ^{14}C fluorography of in vivo reactivation of pyruvate kinase in *S. lactis* ML3. Starved cells containing [^{14}C]PEP pool intermediates (2-PG, 3-PG, and PEP) were suspended in buffered medium containing (A) no addition (control), (B) arginine, (C) maltose, and (D) maltose plus arginine. After 4 min of incubation, the cells were extracted and ^{14}C-metabolites were detected by fluorography as described in the text.

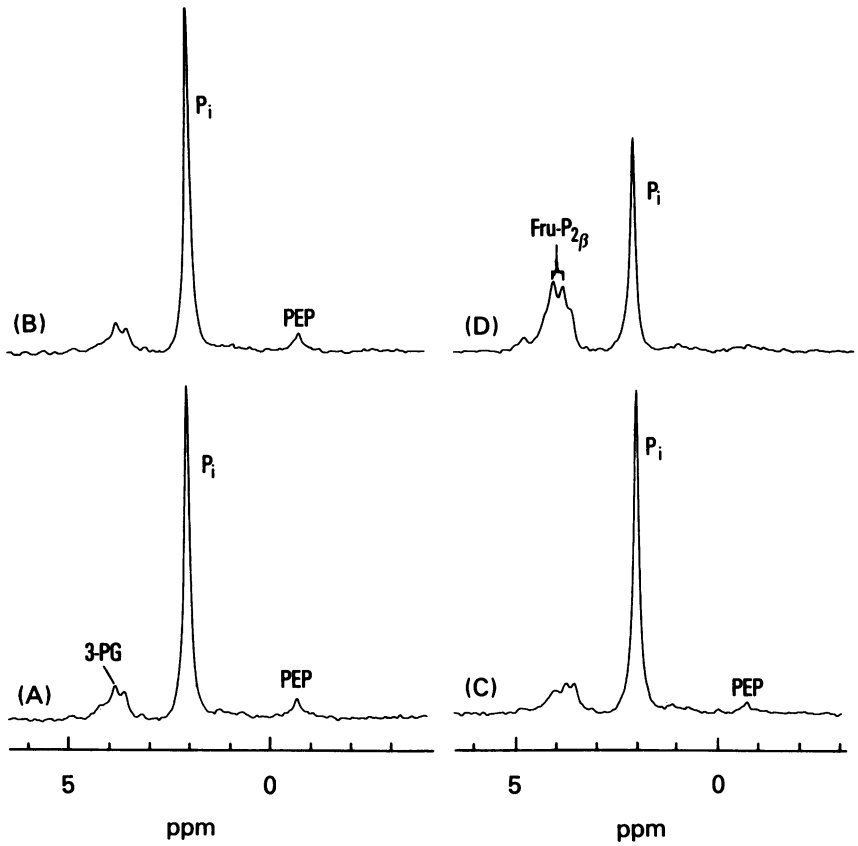

FIG. 8. ^{31}P NMR spectra (101.4 MHz) of P_i and PEP pool intermediates in the cell extracts from the experiment described in Fig. 7: (A) PEP-loaded cells (control), (B) cells incubated with arginine, (C) cells incubated with maltose, and (D) cells incubated with maltose plus arginine.

identical. Digital integration of peak areas (Fig. 5, inset) showed that the total intracellular phosphate (P_i plus phosphorylated metabolites) was conserved by the cells throughout the metabolic cycle. However, the intracellular P_i concentration during glycolysis (ca. 15 mM) was only 20% of that determined in the starved cells because the remainder was conserved by the formation of high levels (ca. 30 mM) of Fru-P_2 (Fig. 5, t = 30 s) and lower concentrations of adenine nucleotides (data not shown). The data of total P_i recovery (Fig. 5, inset) and the correspondence of concentrations of intermediates determined in glycolysing intact cells (Fig. 2A and B) with those in cell extracts show that boiling water provides a reliable and rapid extraction of metabolites from lactic acid bacteria (20).

Reactivation of pyruvate kinase in vivo. The data suggest that high levels of P_i are primarily responsible for inactivation of pyruvate kinase. By corollary, a reduction in P_i concentration should restore activity of the enzyme in starved cells. The procedure used to manipulate the intracellular P_i concentration was based on the following data: (i) maltose is a non-PTS sugar (or a poor PTS substrate) in *S. lactis* ML3, and endogenous PEP, which served as the phosphoryl donor for translocation of glucose and 2-deoxy-D-glucose via the mannose-PTS, was not utilized for maltose transport (Fig. 6A); (ii) maltose-grown cells are induced for the enzymes of the arginine dihydrolase pathway (3), and catabolism of arginine can generate ATP required for maltose transport (Fig. 6B); (iii) iodoacetate may be used to block glycolysis (38) but the sulfhydryl-group reagent does not prevent ATP formation or inhibit maltose transport; and (iv) group N streptococci catalyze the phosphorolysis of maltose to β-D-glucose-1P and glucose (10, 21), and the latter may be further converted to G6P by ATP-dependent glucokinase. We reasoned that a combination of maltose plus arginine would serve as a phosphate trap, thereby decreasing the intracellular P_i concentration in iodoacetate-treated cells. In the experiment illustrated in Fig. 7, cells of *S. lactis* ML3 containing a ^{14}C-labeled PEP pool were incubated with arginine, maltose, or maltose plus arginine. Extracts were prepared, and intracellular metabolites were identified by thin-layer fluorography. The ^{14}C-PEP pool was present in all cell extracts except in the extract prepared from cells incubated with maltose plus arginine (Fig. 7D). These findings were confirmed by ^{31}P NMR (Fig. 8), but in addition, the spectra showed that the intracellular P_i concentration decreased by ca. 40% during incubation of the cells with maltose plus arginine (Fig. 8, lane D). The latter extract contained significant levels of (unlabeled) Fru-P_2, but we believe that the decrease in P_i is the primary cause of pyruvate kinase reactivation.

DISCUSSION

By two independent methods (^{31}P NMR and ^{14}C fluorography), we have identified and determined the concentrations of Embden-Meyerhof pathway metabolites in starved and glycolysing cells of *S. lactis*. The results obtained are in excellent agreement with data obtained previously by enzymatic analysis (19, 38, 41). The major ^{14}C-labeled intermedi-

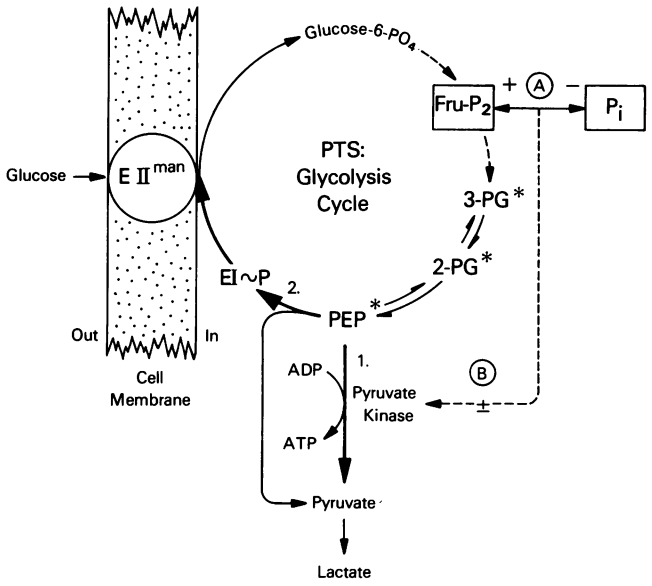

FIG. 9. Proposed mechanism for in vivo modulation of pyruvate kinase activity in *S. lactis*. Symbols: *, metabolite of the PEP pool in starved cells; EI ~ P, phosphorylated form of enzyme I of the multicomponent PTS; A, interconversion (and conservation) of Fru-P_2 and P_i; B, net resultant from the competition between the two antagonistic effectors.

ates and predominant ^{31}P NMR signals in starved cells and extracts were identified as 3-PG, 2-PG, PEP, and P_i. In glycolysing cells, Fru-P_2 was the predominant metabolite, and the intracellular P_i concentration was only 20% of that determined in starved organisms. The combination of ^{31}P NMR and fluorography permitted changes in both P_i and phosphorylated glycolytic intermediates to be monitored simultaneously in vivo. Three lines of evidence support the contention of Waggoner and co-workers (19) that high levels of P_i play a major role in inhibition of pyruvate kinase in starved cells of *S. lactis*. First, at commencement of glycolysis (Fig. 3; 2 s) the concentration of Fru-P_2 (ca. 3 mM) was considerably greater than the concentration required for half-maximum velocity for pyruvate kinase, but the cells continued to accummulate G6P via the PEP-dependent mannose-PTS. Since G6P is also a potent activator of pyruvate kinase (35, 36), the preferential diversion of PEP into the PTS (designated pure PEP-PTS cycling by Mason et al. [19]) and the absence of [^{14}C]lactic acid showed that pyruvate kinase was inhibited in the initial stages of sugar phosphate accumulation. Second, at the point of glucose exhaustion from the medium (Fig. 3, 2 min), the intracellular concentrations of 3-PG, 2-PG, and PEP increased although the cells at that time contained ~11 mM Fru-P_2. Finally, during starvation (Fig. 3, 5 min; Fig. 7), the Fru-P_2 concentration within the cells, although low (~1 mM), was still greater than the concentration (0.06 to 0.2 mM) required for activation and half-maximum velocity of pyruvate kinase in vitro (8, 35). In all three instances, the cells contained high levels of P_i. It seems likely (as suggested by Mason et al. [19]) that it is the increase in cellular P_i which initiates the inhibition of pyruvate kinase at the onset of sugar exhaustion. However, the inhibitory effect of P_i will be augmented by the simultaneous depletion of in vivo activators, as we had envisioned previously (41).

Newsholme and Start (24) emphasize that theories of metabolic control should give rise to predictions which are experimentally testable. If in *S. lactis* high levels of P_i are responsible for inhibition of pyruvate kinase, then by corollary, a decrease in P_i concentration should cause restoration of enzyme activity. We tested this prediction by reducing the intracellular P_i concentration in starved cells by a modified procedure of that described by Harold and Spitz (11). In *Streptococcus faecalis*, the combination of glycerol plus arginine (by promoting formation of glycerol phosphate) served as a trap for intracellular P_i. Glycerol was not phosphorylated by cells of *S. lactis* ML3 (unpublished data) in the presence or absence of arginine, but the combination of arginine plus maltose was an effective P_i trap. When iodoacetate-treated cells, previously loaded with [^{14}C]PEP pool intermediates, were incubated with maltose plus arginine, the intracellular P_i concentration decreased by ca. 40% (Fig. 8), and at the same time, cells were depleted of the intracellular PEP pool (Fig. 7, lane D). Under the prescribed conditions, utilization of PEP via the PEP-dependent PTS was minimal, and we attribute the disappearance of the ^{14}C-labeled PEP pool to reactivation of pyruvate kinase.

The reactions involved in the transport and catabolism of sugars by *S. lactis* constitute a cycle (Fig. 9; 15) in which energy (ATP) and lactic acid are the metabolic products. The central features of the PTS-glycolysis cycle (16, 31) are as follows: (i) the cycle must be tightly regulated so that sugars are transported only as rapidly as they can be fermented; and (ii) PEP, which is both a product and initiator of the cycle, provides the metabolic link between transport and energy-yielding reactions. Metabolic regulation frequently occurs at a branch point of a pathway or at a step responsible for partitioning an intermediate between two or more competing enzyme systems (4, 18). PEP is a branch point metabolite of the cycle illustrated in Fig. 9, and the competing systems (pyruvate kinase and enzyme I of the PTS) commit the high-energy phosphoryl donor into ATP synthesis (route 1) or sugar phosphorylation (route 2), respectively. The distribution of PEP will be dependent upon the activity and hence upon the concentrations of effectors of pyruvate kinase. ^{31}P NMR data (Fig. 2 and 4) showed that during glycolysis the P_i concentration in *S. lactis* (as in anaerobic *E. coli* [42]) decreased by 80% but was conserved by the formation of ca. 30 mM Fru-P_2. Conversely, at the point of glucose exhaustion, P_i increased coincident with a rapid decline in Fru-P_2. It is axiomatic that the concentrations of the two antagonistic effectors of pyruvate kinase are inversely related. Steady-state glycolysis (high Fru-P_2, low P_i) and starvation (low Fru-P_2, high P_i) represent the extreme conditions for activation or inhibition of pyruvate kinase. However, the net direction of the Fru-P_2 $\leftrightarrow$ P_i interconversion (Fig. 9, step A) will fluctuate according to the energetic status of the cell. The resultant from the interconversion of the two effectors (Fig. 9, step B) may modulate pyruvate kinase and, indirectly, the activity of the PTS-glycolysis cycle in the growing cell. The mechanism outlined in Fig. 9 may prove to be an oversimplification; however, it merits consideration because the scheme accommodates much of the data accumulated from in vitro and in vivo experiments.

^{31}P NMR is a powerful and noninvasive probe for the study of bacterial energetics and metabolism, but mention should be made of some practical problems encountered in our studies with intact cells of *S. lactis*. In general, the line widths observed in the ^{31}P NMR spectra of cells of the gram-positive *Streptococcus* strains (Fig. 1A and B) were considerably broader than those described by Shulman et al. (22, 42, 43) in gram-negative *E. coli*. We believe that intracellular

paramagnetic ions (26), rather than cell or field inhomogeneities, are primarily responsible for this peak broadening in *S. lactis*. In a recent study, Ezra et al. (9) found that accumulation of Mn^{2+} during growth was the cause of similar signal broadening in gram-positive *Staphylococcus aureus*. A more general problem, which is encountered in ^{31}P NMR studies with most microorganisms, is the necessity for use of thick cell suspensions to increase the signal-to-noise ratio and to reduce signal aquisition times. This requirement, coupled with the rapid glycolytic activity and acid production by *S. lactis*, results in a rapid pH decrease and upfield migration of chemical shifts. Finally, some cell lysis occurred when suspensions of *S. lactis* were maintained (at 20°C) in the NMR tube for periods of >30 min. A flowthrough system would circumvent some of these problems by maintaining a constant buffering capacity and by permitting continuous addition of sugar and removal of organic acids in the perfusate.

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