The Importance of Inorganic Phosphate in Regulation of Energy Metabolism of *Streptococcus lactis* 

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Peter W. Mason‡, David P. Carbone§, Robert A. Cushman¶, and Alan S. Waggoner

From the Chemistry Department, Amherst College, Amherst, Massachusetts 01002

This paper is concerned with the control of glycolysis in nongrowing *Streptococcus lactis* 7962. Changes were measured in the concentrations of glycolytic intermediates, intracellular inorganic phosphate (Pi), and adenine nucleotides following addition of glucose to cells that were in a starved condition. We find that intracellular Pi is a major factor in the control of glycolysis. In starved cells, the intracellular Pi concentration is high, greater than 40 mM. The large phosphoenolpyruvate pool that exists in starved cells can be explained as a result of inhibition of pyruvate kinase by the high concentration of Pi. On the other hand, in cells that are metabolizing glucose at a steady state rate, the cellular Pi concentration is low and pyruvate kinase is active. Upon depletion of glucose from the medium, the metabolism concentrations return to the values originally found in the starved state. This glucose depletion raises the intracellular Pi, which again leads to inhibition of pyruvate kinase and the consequent buildup of the P-enolpyruvate pool.

The homofermentative lactic acid bacteria, *Streptococcus lactis* and *Streptococcus faecalis*, have provided nearly ideal systems for the study of bacterial membrane energetics and transport. One attractive feature is the relative metabolic simplicity of these organisms. *S. lactis*, for example, has no endogenous polymeric carbohydrate stores (1). Thus, cells isolated from food sources become energy-starved. Nevertheless, the starved bacteria remain capable of metabolizing preferred carbohydrates for up to a day, if the cells are kept at 4°C. Because the organism has no cytochromes, most of the energy available will be converted directly to lactate via the Embden Meyerhoff pathway of glycolysis (2) as illustrated in Fig. 1a. Since some of the reactions interconverting the glycolytic intermediates shown in this figure are in rapid equilibrium, we have lumped these metabolites into the three pools. The glucose 6-phosphate pool (signified by G6P* in Fig. 1) contains glucose 6-phosphate (Glc-6-P) and fructose 6-phosphate (Fru-6-P); the Fru-1,6-P2 pool (FDP*) contains fructose diphosphate, glyceraldehyde 3-phosphate (glyceraldehyde-3-P), and dihydroxyacetone-P; and the P-enolpyruvate pool (PEP*) contains 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate.

When glucose is made available to the bacteria, it is converted to Glc-6-P and transported into the cell by the phosphotransferase system (3, 4). The glucose uptake reaction is driven by simultaneous conversion of P-enolpyruvate to pyruvate at the phosphotransferase system. Starved *S. lactis* cells are well adapted for rapidly scavenging phosphotransferase system sugars like glucose. In the starved state the organism maintains an unusually high intracellular P-enolpyruvate pool which can be rapidly recruited to drive sugar uptake through the phosphotransferase system (4-7). The existence of a large P-enolpyruvate pool in the starved organism is surprising, however, because conversion of P-enolpyruvate to pyruvate by pyruvate kinase is thermodynamically very favorable. Inhibition of pyruvate kinase in the starved cells must account for the high P-enolpyruvate pool. Collins and Thomas (8) and Thompson and Thomas (9) have proposed that the absence in starved cells of early glycolytic intermediates, which are pyruvate kinase activators, accounts for the pyruvate kinase inhibition. On the other hand, Collins and Thomas (8) and Crow and Pritchard (10) show that P is a strong inhibitor of pyruvate kinase and Thomas and Thompson (9) mention that P inhibition may play some role in the regulation of this enzyme. We propose that the exceedingly high concentration of Pi present in the starved cells account for the observed inactivation of pyruvate kinase.

In steady state glycolysis, most of the glucose provided to the cells will be converted directly to lactate (2), and in the process, 2 inorganic phosphate (Pi) molecules and 2 adenosine diphosphate (ADP) molecules will be converted to 2 adenosine triphosphate (ATP) molecules. The ATP synthesized by the glycolytic chain is used in three types of processes. A fraction of the ATP is used for "priming" the glycolytic chain at phosphofructokinase to produce phosphorylated glycolytic intermediates. Another fraction of the ATP is used to phosphorylate cellular metabolites involved in biosynthesis, but not those involved in glycolysis. In a medium free of all nutrients except glucose, cell growth is minimal, and the fraction of ATP used for biosynthetic phosphorylations should be used only for initial recharging of depleted biosynthetic intermediates. The remaining fraction of ATP is used in hydrolysis reactions. The major ATP hydrolysis reaction in *S. lactis* occurs at an ATP-driven proton pump that translocates protons from the cytoplasm to the medium when ATP is converted to ADP and Pi (11-14). In this process, the free energy of ATP hydrolysis is converted to membrane energy that can be used by the cell to exchange and concentrate ions and to drive accumulation of nutrients against their concentration gradients.

1. The equilibrium constant for the pyruvate kinase reaction is reported to be $2 \times 10^7$ (25). The mass action ratio $\Gamma = [\text{pyruvate}] [\text{ATP}]/[\text{P-enolpyruvate}][\text{ADP}]$ for *S. lactis* cells in the starved state is calculated from the data in Fig. 2 to be $<0.04$. 

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‡ Present address, Department of Biochemistry, University of Washington, Seattle, Wash. 98195.

§ Present address, Department of Biochemistry, University of Washington, Seattle, Wash. 98195.

¶ Present address, Department of Biochemistry, University of Washington, Seattle, Wash. 98195.

Present address, School of Medicine, Johns Hopkins University, Baltimore, Md. 21218.

Present address, School of Medicine, Emory University, Atlanta, Ga. 30322.
In this paper, we have examined the regulation of glycolysis in starved and in glucose-consuming *S. lactis* 7962. This was accomplished by measuring changes in the concentrations of each of the glycolytic intermediates, intracellular $P_i$, and adenine nucleotides following addition of glucose to cells that are in a starved condition. Because the changes in metabolite concentrations are somewhat complicated following the addition of glucose to the cells, we will present the results and the discussion in four parts: "Starved State," during which the cells are deprived of an energy source; "Cell Activation," during which a number of transient changes take place in the levels of cellular metabolites after the cells are provided glucose; "Steady State," during which the non-growing cells metabolize glucose at a constant rate; and "Cell Shutdown," during which the cell metabolites return to their starved state levels after the glucose supply is exhausted. In addition, inhibition of pyruvate kinase isolated from *S. lactis* 7962 is presented in a fifth section, "Pyruvate Kinase Kinetics."

**MATERIALS AND METHODS**

**Organism and Culture Conditions—** *S. lactis* ATCC 7962 was grown at 37°C with shaking (at 40 oscillations/min) in complex media until the growth reached late log phase before harvesting and washing in 25 mM Tris-phosphate, pH 7.0, buffer containing 100 mM NaCl (suspension buffer) to a density of 0.0862 g/ml 0°C. After 1% h, the precipitate was removed by centrifugation at 10,000 g for 10 min at 0°C. To this supernatant additional ammonium sulfate was added at a density of 20 to 30 mg dry weight/ml and ruptured by two passages through an Amino French pressure cell at 20,000 p.s.i. The cell debris was removed by centrifugation at 35,000 g for 20 min at 0°C. EDTA was added to a final concentration of 0.01 mM and streptomycin sulfate (10% w/v) was added with stirring at 0°C (to remove DNA) until no further precipitate was formed. The precipitate was removed by centrifugation at 10,000 × g for 10 min at 0°C. This final pellet was resuspended in 1 ml of 10 mM phosphate, pH 6.7, 100 mM KCl, 0.1% mercaptoethanol (MSH) buffer, and dialyzed against 350 ml of the same buffer overnight at 5°C. The sample was then placed on a Whatman DE52 ion exchange column (0.9 × 5 cm) equilibrated with 10 mM phosphate, pH 6.7, 100 mM KCl, 0.1% MSH buffer (5°C). The column was washed with the equilibration buffer until the tryptophan fluorescence peak fractions revealed that most of the pyruvate kinase activity was eluted with the 180 mM KCl buffer.

The concentration of ATP was determined with the firefly luciferase assay of Cole et al. (19).

All NADH and NADPH fluorescence measurements were made on a Perkin-Elmer MFP-3 fluorescence spectrometer with excitation at 340 nm and emission at 440 nm. A 340 nm barrier filter and 360 nm long-pass emission filter were used to eliminate stray light.

**Pyruvate Kinase Purification—** The technique utilized for the purification of pyruvate kinase from *S. lactis* 7962 was a combination of the methods used by Crow and Pritchard (10) and Collins and Thomas (8) with several modifications. The bacteria were harvested by two methods: (a) in 7.0, 10 mg glucose/ml, the bacteria were harvested at a density of 20 to 30 mg dry weight/ml and ruptured by two passages through an Amino French pressure cell at 20,000 p.s.i. The cell debris was removed by centrifugation at 35,000 × g for 20 min at 0°C. EDTA was added to a final concentration of 0.01 mM and streptomycin sulfate (10% w/v) was added with stirring at 0°C (to remove DNA) until no further precipitate was formed. The precipitate was removed by centrifugation at 10,000 × g (0°C) for 10 min. Finely ground ammonium sulfate (0.325 g/ml) was then added to the streptomycin sulfate-treated supernatant, while stirring at 0°C after 1½ h, the precipitate was removed by centrifugation at 10,000 × g for 10 min at 0°C. This supernatant additional ammonium sulfate was added (0.0862 g/ml 0°C. After 1½ h, the precipitate was removed by centrifugation. This final pellet was resuspended in 1 ml of 10 mM phosphate, pH 6.7, 100 mM KCl, 0.1% mercaptoethanol (MSH) buffer, and dialyzed against 350 ml of the same buffer overnight at 5°C. The sample was then placed on a Whatman DE52 ion exchange column (0.9 × 5 cm) equilibrated with 10 mM phosphate, pH 6.7, 100 mM KCl, 0.1% MSH buffer (5°C). The column was washed with the equilibration buffer until the tryptophan fluorescence (excitation at 280 nm, emission at 330 nm) fell to zero. Stepwise elution was then carried out until each successive wash yielded zero tryptophan fluorescence, utilizing 20 mM phosphate, pH 6.6, 0.1% MSH buffer with successive salt concentrations of 150, 180, and 220 mM KCl. Analysis of the tryptophan fluorescence peak fractions revealed that most of the pyruvate kinase activity was eluted with the 180 mM KCl buffer.

**Determination of Glycolytic Intermediates—** Extraction of intermediates was performed by two different methods. In both cases an aliquot of stock of cells on ice at a concentration of 7 mg/ml dry weight (in harvesting buffer) was suspended in 25 mM Tris-HCl, pH 7.5, buffer containing 100 mM NaCl (suspension buffer) to a density of 1 mg/ml. In Method 1, a 1-ml aliquot of cell suspension was added to 1 ml of CHCl3 (held at -10°C in a salt-ice bath), blended on a vortex mixer, and returned to the salt-ice bath. The extracts were then brought to 0°C and centrifuged (0°C) at 20,000 × g for 20 min to remove cell debris. The resulting supernatant fluid was stored at -20°C for up to 3 days; a 0.1-ml aliquot of supernatant fluid was utilized for the determination of lactate and 0.05 ml was utilized for the determination of ATP. In Method 2, A 0.75-ml sample of cell suspension at a density of 1 mg dry weight/ml was pipetted using a Rainin Pipetman onto a Millipore filter (2.5 cm diameter, HA 0.45 μm pore size) and immediately washed with 4 ml of ice-cold suspension buffer. The filter was then added to 2 ml of 21 CHCl3:MeOH solution in a centrifuge tube (held at -10°C in a salt-ice bath), blended on a vortex mixer, and returned to the salt-ice bath. Two and one-half ml of ice-cold suspension buffer was added to each tube and they were centrifuged at 1.100 × g for 20 min at 0°C. Two and one-half ml of the aqueous layer was then removed and centrifuged at 20,000 × g for 10 min at 0°C to remove the cell debris. The resultant supernatant was stored at -20°C. A 0.7-ml aliquot of supernatant fluid was assayed for inorganic phosphate content within 24 h, and two samples of 0.65 ml each were assayed for either P-enolpyruvate, 2-P-glycerate, and pyruvate or Fru-1,6-P2, glyceraldehyde-3-P, and dihydroxyacetone-P within 3 days.

Fru-1,6-P2, glyceraldehyde-3-P, dihydroxyacetone-P, P-enolpyruvate, 3-P-glycerate, and 2-P-glycerate were assayed by quantitative measurement of NADH fluorescence decreases in enzymatic reactions utilizing the respective metabolites, as described by Maitra and Estabrook (16). Glc-6-P was assayed by monitoring the increase in NADPH fluorescence resulting from the oxidation of Glc-6-P to form 6-glucono lactone catalyzed by glucose-6-phosphate dehydrogenase. Inorganic phosphate (Pi) was subsequently determined by the addition of phospho-lase a and phosphoglucomutase to the above mixture catalyzing the conversion of free inorganic phosphate and glycogen to form Glc-6-P (17).

Lactate was assayed by monitoring the increase in NADH fluorescence upon oxidation to pyruvate by lactate dehydrogenase in the presence of semicarbazide at pH 10.6 (18).

The concentration of ATP was determined with the firefly luciferase assay of Cole et al. (19).
The fractions with activity greater than 20 units/mg were combined and concentrated by dialysis in polyethylene glycol (molecular weight 15,000 to 20,000) in 10 mM phosphate, pH 6.5, buffer containing 10 mM MgCl₂ and 3 mM MSH (at 5°C) for 3 h. The sample was then dialyzed against the same buffer overnight at 5°C, 0.3 ml of this sample (protein concentration of 5 to 8 mg/ml) was then placed on a Sepharose 6B column (0.9 X 28 cm) equilibrated with 10 mM phosphate, pH 6.5, buffer containing 10 mM MgCl₂ and 3 mM MSH buffer at 5°C. The eluant was monitored for tryptophan fluorescence and peak fractions were monitored for pyruvate kinase activity. All samples with an activity of greater than 50 units/mg (1 unit is defined as the amount of enzyme which can convert 1 pmol of P-enolpyruvate to pyruvate in 1 min at 30°C) were diluted to 50% (v/v) with glycerol and stored at -20°C for up to a week with no measurable loss of activity.

Throughout the purification procedure protein was assayed for by the method of Lowry as modified by Bailey (20). Pyruvate kinase activity was routinely assayed for by monitoring the decrease in NADH absorption at 366 nm on a Cary 14 recording spectrometer at 30°C in a 1-cm light path cuvette containing (in a final volume of 2.5 ml) 50 mM (EtOH),N-HCl, pH 7.5, 8 mM MgCl₂, 80 mM KCl, 130 mM NADH, 10 μg of lactate dehydrogenase (10 μl in 50% glycerol), 2 mM Fru-1,6-P₂, 2 mM P-enolpyruvate, 2 mM ADP, and the enzyme sample. A 48-fold purification was accomplished with a 3.6% yield. The purified enzyme had a specific activity of 98 units/mg.

RESULTS

Starved State—Energy-starved cells are characterized by a high level of inorganic phosphate and a large P-enolpyruvate pool. Other glycolytic intermediates are absent (Table I). Since the P-enolpyruvate pool in starved cells remains high for long periods of time while the pyruvate and ATP concentrations are low, it is clear that pyruvate kinase must be inactive in the starved cells.

Cell Activation—Following the addition of glucose (time = 0 in Fig. 2) to starved cells substantial changes take place.

### Table I

Concentrations of glycolytic intermediates in starved state and 1½ min after glucose addition

<table>
<thead>
<tr>
<th>Glycolytic intermediate</th>
<th>Starved state</th>
<th>1½ min after glucose addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₂</td>
<td>47 ± 0.75</td>
<td>4 ± 0.75</td>
</tr>
<tr>
<td>P-enolpyruvate pool (PEP + 2PG + 3PG)</td>
<td>26 ± 0.5</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>ND</td>
<td>2.3 ± 1</td>
</tr>
<tr>
<td>Fru-1,6-P₂</td>
<td>ND</td>
<td>21 ± 0.5</td>
</tr>
<tr>
<td>Dihydroxyacetone-P</td>
<td>ND</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>Glyceraldehyde-3-P</td>
<td>ND</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Glc-6-P</td>
<td>ND</td>
<td>1.5 ± 0.75</td>
</tr>
<tr>
<td>Lactate</td>
<td>ND</td>
<td>70 ± 2</td>
</tr>
</tbody>
</table>

* Not detectable, intracellular concentrations determined were less than experimental error.

* 2PG, 2-P-glycerate; 3PG, 3-P-glycerate.

* Lactate measured includes intracellular and extracellular lactate, but amount measured is reported in terms of intracellular volume, that is, as if all lactate produced remained within the cells.

![Fig. 2. Glycolytic intermediates in cells of S. lactis metabolizing glucose at 15°C. Cells were suspended as described under "Materials and Methods" and samples were taken at the specified times. 1.07 pmol of glucose/dry weight of cells was added at time equal zero. The concentrations of the intermediates are expressed in terms of their intracellular concentration (millimolar).](image)
in cellular metabolite concentrations. In the first ½ min, inorganic phosphate and the P-enolpyruvate pool disappear while the combined Glc-6-P and the Fru-1,6-P2 pools rise from zero and plateau near 25 mM (Fig. 2). There is a rapid uptake of [14C]glucose (not shown), rapid production of lactate, and appearance of cellular ATP during the 1st min (Fig. 2). But after these initial changes have taken place, lactate production and glucose uptake cease for several minutes and the ATP concentration drops to near zero. The period of glycolytic inactivity lasts for about 3 min even though plenty of glucose is available to the cells and the Fru-1,6-P2 pool remains remarkably high during this period (indicating that the cells maintain the capacity for synthesizing ATP).

Steady State—About 4 min after glucose addition, the transient period of glycolytic inactivity ends. Glucose uptake and lactate extraction resume (Fig. 2). The ATP concentration increases and the cells move into a steady state condition in which metabolite concentrations (except lactate) remain constant even though glucose is being consumed at a rapid rate. In the steady state cells, the Fru-1,6-P2 pool is large and the P-enolpyruvate pool and Pi, level are very low.

Cell Shutoff—When the glucose in the suspension buffer becomes depleted, metabolite pools return to starved state levels. Thus, the levels of the P-enolpyruvate pools reverse and the Pi concentration moves from a very low value to greater than 30 mM (Fig. 4).

Pyruvate Kinase Kinetics—Collins and Thomas (8) and Thompson and Thomaar (9) have suggested that the absence of early glycolytic metabolites (which are pyruvate kinase activators) from energy-starved cells accounts for the observed pyruvate kinase inactivity, but kinetic studies of the purified enzyme carried out by Collins and Thomas (see Fig. 2a of Ref. 8) indicate that pyruvate kinase is active in the absence of all these activators. We have also examined in vitro pyruvate kinase activity and in Fig. 3 it can be seen that this enzyme indeed catalyzes the conversion of P-enolpyruvate to pyruvate in the absence of all glycolytic metabolite activators. Under the conditions of our assay, the reaction is only 0.1 time slower than the maximal rate found, too high to account for the maintenance of the P-enolpyruvate pool in the starved state. What is also clear from Fig. 3 is the strong inhibition of pyruvate kinase by less than 10 mM Pi (also found by Collins and Thomas (8) and Crow and Pritchard (10). Furthermore, complete inhibition of pyruvate kinase was observed at a Pi concentration of 50 mM, even in the presence of higher concentrations of early glycolytic activators (Fru-1,6-P2 and Glc-6-P) than we found in vivo (Figs. 3 and Fig. 2).

**DISCUSSION**

**Starved Cells**—Energy-starved _S. lactis_ maintain a large P-enolpyruvate pool for many hours even though it is thermodynamically unfavorable for them to do so (9). The resting level of P-enolpyruvate is useful because it allows the organism to quickly and actively scavenge glucose when it appears in the medium. We propose that the large concentration of Pi, (40 mM) in starved _S. lactis_ cells is primarily responsible for this organism. Even high concentrations of early glycolytic intermediates, which are known to be activators of pyruvate kinase do not overcome the strong inhibition at high Pi concentrations. Furthermore, the absence of pyruvate kinase activators cannot account for pyruvate kinase inactivity in starved cells because pyruvate kinase has substantial activity even in the absence of activators.

**Cell Activation**—In bacteria that obtain glucose from the medium solely via the phosphotransferase system and are carrying out glycolysis at a steady state rate, it is expected that the fraction of P-enolpyruvate passing through the phosphotransferase system to bring in a glucose molecule will be 0.5. The other half of the P-enolpyruvate will pass through pyruvate kinase to synthesize additional ATP. This scheme is illustrated in Fig. 1a. Under these steady state conditions, the cells produce 2 ATP and 2 lactate/glucose molecule consumed. On the other hand, immediately after energy-starved cells have been exposed to glucose, P-enolpyruvate will be...
rapidly consumed at the phosphotransferase system, but cannot be used at pyruvate kinase since the P_1 concentration is still high enough to inhibit the latter enzyme. Furthermore, as long as pyruvate kinase remains inhibited, all P-enolpyruvate produced by incoming glucose will be cycled through the phosphotransferase system in a process we define as "P-enolpyruvate-phosphotransferase system cycling (PEP-PTS cycling, for short). Pure PEP-PTS cycling is illustrated in Fig. 15.1.

It is expected that between the time of glucose addition to the starved cells and arrival of steady state glycolysis, the fraction of P-enolpyruvate moving through the phosphotransferase system must drop from 1.0 to 0.5 while the fraction of P-enolpyruvate moving through pyruvate kinase must rise from 0 to 0.5. From data in Fig. 2 it is possible to calculate these fractions at various times after addition of glucose to the starved bacteria. The results are shown in Fig. 5. Within 1 min of glucose addition, the fraction of P-enolpyruvate moving through the phosphotransferase system has dropped to 0.6 indicating that pyruvate kinase has become active during this time.

There is additional evidence pointing to the activation of pyruvate kinase shortly after glucose addition to the cells. Fig. 2c shows that there is a transient but significant increase in the ATP concentration at the 1-min mark. This amount of ATP certainly cannot arise at phosphoglucokinase during P-enolpyruvate-phosphotransferase system cycling because stoichiometric arguments can show that not enough ATP can be produced at this site during pure P-enolpyruvate-phosphotransferase system cycling to account for the metabolite changes that we observed in the cells during the first 1/2 min.

From Fig. 2 it can be seen that the activation of pyruvate kinase is coincident with the striking decrease in cellular P1, as the latter is incorporated into sugar phosphates. Also coincident with pyruvate kinase activation is the increase in Glc-6-P, Fru-6-P, Fru-1,6-P_2, Dihydroxyacetone P, and Glyceraldehyde-3-P, all of which are known to be activators of pyruvate kinase (8, 10, 21). Undoubtedly, the activation of pyruvate kinase results from changes in concentrations of all these modulators, with P_1 as the more important factor.

The transient, 3-min period of glycolytic inactivity that begins about a minute after glucose addition to the starved cells probably has little physiological significance in normal cell behavior. Its appearance is surprising, however, because at this time glucose is present in the medium, the Glc-6-P and Fru-1,6-P_2 pools are large and the P_1 concentration is low enough to permit pyruvate kinase activity. We think that the 3-min inactivation phenomenon can be explained in terms of an extreme depletion of cellular P_1 during this period that stops glycolysis at the glyeraldehyde-3-P dehydrogenase reaction, which requires P_1 as a substrate. Apparently ATP hydrolysis reactions which are normally able to replenish P_1 are not able to compete with phosphorylation reactions that consume ATP produced during the entire period of cell activation. Probably the major ATP sink during the inactivation period is the glycolytic priming reaction at phosphofructokinase which is being driven by Glc-6-P still present in the cells.

If this is true, then the cells can enter steady state glycolysis after the Glc-6-P concentration is reduced.

Steady State—The rate of glycolysis in steady state was found to be highly sensitive to the rate of ATP hydrolysis at the membrane-associated H^+-pump. Addition of ionophores that make the membrane leaky decrease the "load" on the ATPase and steady state glycolysis can be stimulated 5-fold over the rate in the absence of ionophores. On the other hand, addition of dicyclohexylcarbodiimide (data not shown) results in a 4-fold reduction in the rate of steady state glycolysis. Clearly, the rate of glycolysis is regulated by demand for ATP at the membrane bound H^+-pump (and presumably elsewhere in the cell as well). We will publish separately the results of our studies on the regulation of S. lactis glycolysis by cellular energy demand.

Shutdown—When glucose disappears from the medium, the cells make a transition from steady state metabolism, in which the Fru-1,6-P_2 pool is large and the concentrations of P_1 and the P-enolpyruvate pool are small, to the starved state, in which the Fru-1,6-P_2 pool is small and the concentrations of P_1 and the P-enolpyruvate pool are large. We propose the following mechanism to account for these spectacular shifts.

When the glucose in the medium is exhausted, all P-enolpyruvate synthesized in the cell must be converted to pyruvate by pyruvate kinase because the phosphotransferase system is substrate-limited (Fig. 1c). Thus, for every P-enolpyruvate metabolized, more ATP will be synthesized than was possible under the steady state conditions. Furthermore, no more Glc-6-P and Fru-6-P will be synthesized so the cells will quickly lose their requirement for ATP in the phosphorylation reaction at phosphofructokinase. Thus, the fraction of ATP used for phosphorylation decreases and the fraction of ATP used in hydrolysis reactions increases. As a result of these two effects, the intracellular P_1 concentration would be expected to increase, consistent with the data in Fig. 4. On the basis of this data, we propose that the sudden release of P_1 coupled with the decrease in the Glc-6-P and Fru-1,6-P_2 pools (which contain pyruvate kinase activators) leads to inhibition of pyruvate kinase. It is clear that a substantial inactivation of pyruvate kinase must precede full loss of the Fru-1,6-P_2 pool because the P-enolpyruvate pool reaches 12 mM by the time cell shutdown is completed. Thus, it is undoubtedly the increase in P_1 concentration following glucose depletion that leads to pyruvate kinase inactivation and production of the P-enolpyruvate pool that is found in the starved cells. Reversal

Although our experimental value for the P_1 concentration is 1 to 2 mM during this period, we think that this P_1 is not free within the cell and is therefore not available to glyeraldehyde-3-P dehydrogenase. Perhaps this phosphate is tightly bound to proteins or is released from phosphate esters during the assay procedure. Phosphate NMR experiments (23, 24) may allow resolution of this important question.
of the P-enolpyruvate and Fru-1,6-P₂ pools removes the cells from the optimal metabolic conditions for steady state glucose metabolism and places them in the metabolically inactive condition optimal for PTS-sugar uptake.

In 1905 Harden and Young (31), made the fundamental discovery that sugar fermentation depends on the presence of inorganic phosphate. We have shown that Pᵢ, by virtue of its strong inhibition of pyruvate kinase plays a major role in glycolytic metabolism of S. lactis. A particularly important result is regulation of the size of the P-enolpyruvate pool in starved versus metabolizing cells. Earlier it had been suggested that regulation of the P-enolpyruvate pool is effected by changing levels of phosphorylated glycolytic intermediates that are potential activators of pyruvate kinase. While we agree that the levels of phosphorylated metabolites contribute to the control of cellular P-enolpyruvate, we feel that their major function is to be found in cells during steady state metabolism. Under steady state conditions, the phosphorylated sugars modulate pyruvate kinase activity in order to direct more P-enolpyruvate on a path to the phosphotransferase system, so that more glucose can be brought in, or direct more P-enolpyruvate on the path through pyruvate kinase, so that more ATP can be synthesized. For example, if the levels of Fru-6-P, Fru-1,6-P₂, etc., become low in the glycolyzing cells, pyruvate kinase becomes less active so that a greater fraction of the P-enolpyruvate moves to the phosphotransferase system and the pools of phosphorylated early glycolytic intermediates are replenished.

The kinetics of the metabolite changes before and after glucose addition is very similar when the cells are suspended in phosphate-containing or phosphate-free buffer. In the energy-starved state, the cells are able to maintain high intracellular Pᵢ and therefore high P-enolpyruvate in spite of a very large transmembrane Pᵢ concentration gradient. At the other extreme, during steady state metabolism when intracellular Pᵢ is very low, cells suspended in 100 mM phosphate buffer do not show especially large increases in the sugar phosphate pools. These observations demonstrate the importance of the mechanisms controlling phosphate transport in S. lactis. These mechanisms have been discussed by Harold and Spitz (26) and Harold et al. (27).

While Romano and others (29, 30) have demonstrated the presence of the phosphotransferase system in numerous bacterial systems, there have been no data published on the intracellular concentrations of Pᵢ and P-enolpyruvate in these bacteria. It is possible that high Pᵢ and P-enolpyruvate levels may only be found in phosphotransferase system-containing bacteria that have no endogenous carbohydrate stores.

The time dependence of ATP concentration changes shown in Fig. 2c accounts for the previously unexplained fluorescence changes of a membrane potential dye (diS-C₂(5)) observed by Carbone and Waggoner (28). The cell membrane potential is directly related to the cellular ATP concentration and the fluorescence of the diS-C₂(5) decreases with membrane potential. Thus, it is not surprising that the kinetics of diS-C₂(5) fluorescence changes is similar in time course, but in an inverse direction from the ATP concentration changes.

The glycolytic control mechanisms found in S. lactis should be amenable to mathematical modeling because the metabolic pathways in this organism are simple, the cells have no endogenous carbon source, and because the metabolic rate of the organism can be substantially slowed by cooling, thereby facilitating sampling and determination of accurate kinetics for each metabolite.

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