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In Vivo Regulation of Glycolysis and Characterization of Sugar:Phosphotransferase Systems in *Streptococcus lactis*

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Two novel procedures have been used to regulate, in vivo, the formation of phosphoenolpyruvate (PEP) from glycolysis in *Streptococcus lactis* ML_3. In the first procedure, glucose metabolism was specifically inhibited by *p*-chloromercuribenzoate. Autoradiographic and enzymatic analyses showed that the cells contained glucose 6-phosphate, fructose 6-phosphate, fructose-1,6-diphosphate, and triose phosphates. Dithiothreitol reversed the *p*-chloromercuribenzoate inhibition, and these intermediates were rapidly and quantitatively transformed into 3- and 2-phosphoglycerates plus PEP. The three intermediates were not further metabolized and constituted the intracellular PEP potential. The second procedure simply involved starvation of the organisms. The starved cells were devoid of glucose 6-phosphate, fructose 6-phosphate, fructose-1,6-diphosphate, and triose phosphates but contained high levels of 3- and 2-phosphoglycerates and PEP (ca. 40 mM in total). The capacity to regulate PEP formation in vivo permitted the characterization of glucose and lactose phosphotransferase systems in physiologically intact cells. Evidence has been obtained for "feed forward" activation of pyruvate kinase in vivo by phosphorylated intermediates formed before the glyceraldehyde-3-phosphate dehydrogenase reaction in the glycolytic sequence. The data suggest that pyruvate kinase (an allosteric enzyme) plays a key role in the regulation of glycolysis and phosphotransferase system functions in *S. lactis* ML_3.

In 1964 Kundig et al. (23) reported the existence of a phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) in extracts prepared from *Escherichia coli*. The prime function of this system is the phosphorylative translocation of specific sugars across the cytoplasmic membrane (33). The system has since been detected in a wide range of anaerobic and facultative anaerobic bacteria (36), including *Streptococcus lactis* (26, 27).

The PTS is especially important to those species (e.g., *S. lactis*) whose energy requirements are derived from sugar fermentation via the Embden-Meyerhof pathway, since energy generated by glycolysis is conserved by the formation of a sugar phosphate during group translocation (38). The factors which regulate the activity of the glycolytic sequence are exceedingly complex (25, 32, 37), but it is clear that the rate of sugar uptake by the PTS in anaerobic bacteria must be dependent upon the availability and rate of PEP formation from glycolysis and vice versa. In this mutually self-regulating system, PEP must play a pivotal role in providing the essential link between sugar transport and glycolysis (33, 38).

The possible control of PTS activity by regulation of the intracellular concentration of PEP has been suggested, and attempts have been made to demonstrate such control in intact cells (33). However, the data obtained were difficult to evaluate because of the multitarget action of the inhibitors used and the fact that PEP levels had been determined in very few experiments. The lack of quantitative information in this area has been emphasized recently by Irani and Maitra (18). With the exception of a short communication by Kornberg and Reeves (20), no quantitative comparisons have been made between the rate of hexose uptake (and metabolism) and the experimentally determined activity of the PTS in the intact cells. The paucity of data reflects, in part, the difficulties inherent in regulating PEP formation by microorganisms which, in many instances, contain high levels of endogenous energy reserves or which may generate PEP from reactions other than glycolysis (e.g., PEP synthase). From this viewpoint, certain species of streptococci, including *S. lactis* (46) and *S. faecalis* (17), have proved to be particularly useful for studies concerned with the energetic aspects of solute transport in bac-

teria (16, 17). These organisms are essentially devoid of endogenous energy reserves and rely upon glycolysis for the generation of those compounds which serve directly (PEP), or indirectly (ATP), as energy donors for solute accumulation.

In this investigation, two novel methods have been used to regulate the in vivo formation of PEP from the glycolytic sequence in *S. lactis* ML_3. The intracellular PEP potential can be maintained at a high but finite concentration of ca. 40 mM, thereby permitting quantitative measurement of PTS activities to be correlated with (i) the rate of utilization of endogenous PEP and (ii) the rate of hexose metabolism by intact cells. The characteristics of the constitutive glucose PTS (glu-PTS) and the inducible lactose PTS (lac-PTS) have also been investigated. Intracellular concentrations of glycolytic intermediates have been determined in cells maintained under different physiological conditions. The in vivo data have confirmed the predictions from in vitro studies (5, 7, 44, 45) for the key role of pyruvate kinase in regulation of glycolysis in *S. lactis*.

MATERIALS AND METHODS

Strains. *S. lactis* ML_3 (group N) was obtained from the culture collection of the New Zealand Dairy Research Institute, Palmerston North, New Zealand.

Growth of cells. *S. lactis* ML_3 was grown in the chemically defined medium described previously (46) containing 0.5% (wt/vol) D-galactose as the fermentable energy source.

Preparation of starved cells. Cells from 200 ml of culture were harvested during midlogarithmic growth (optical density at 600 nm, 0.55 units; Spectronic 20, Bausch & Lomb), and the resultant cell pellet was washed twice by suspension in and centrifugation from 200 ml of 0.01 M $MgSO_4$ solution as described (46). A homogeneous suspension of washed (starved) cells containing 20 to 25 mg (dry weight) of cells per ml was prepared as described previously (46).

Transport studies. In the standard transport study procedure, 80 to 100 μl of thick cell suspension was added to 9.8 ml of 0.1 M tris-(hydroxymethyl)aminomethane-maleate buffer (pH 7.0) to obtain a final density of 200 μg (dry weight) of cells per ml. The appropriate sugars were added to this system (0.1 mM, specific activity of 0.2 to 0.5 μCi/μmol), and solute accumulation was followed by using previously described procedures (46). All transport experiments were performed at 30°C, and, unless stated otherwise, no exogenous energy sources were present in the transport medium. In kinetic studies, the initial rates of glucose and 2-deoxy-D-glucose (2-DG) accumulation (in micromoles per gram [dry weight] of cells per minute) were determined after 5 s of incubation.

Preparation of cells containing ^{14}C-labeled glycolytic intermediates. Washed cells were suspended in a 10-ml volume of 0.1 M tris(hydroxymethyl)aminomethane-maleate buffer (pH 7.0) containing 1.5 mM [*U*-^{14}C]glucose (0.2 μCi/μmol) at a density of 2 mg (dry weight) of cells per ml. From data obtained in a previous study (46), this concentration of glucose will support glycolysis for 6 to 7 min. To obtain radiolabeled starved cells (i.e., containing ^{14}C-labeled 3-phosphoglycerate [3-PG] and 2-PG and PEP) the incubation period was continued for 15 min to ensure exhaustion of [^{14}C]glucose from the medium. In other experiments, glycolysis was halted by the addition of iodoacetic acid (IAA) (10 mM) or *p*-chloromercuribenzoate (*p*-CMB) (1 mM) to the cell suspension after 2 min of incubation. Depending upon the experiment, radiolabeled cells were collected either (i) by vacuum filtration through 0.8-μm (pore diameter) membrane filters and subjection to trichloroacetic acid-diethyl ether extraction and thin-layer chromatography (see below) or (ii) by centrifugation (12,000 × *g*, 1 min) before suspension in the appropriate transport system.

Preparation of cell extracts. Cell extracts were routinely prepared by the trichloroacetic acid-ether extraction procedure of Collins and Thomas (5). Pyruvate, however, is readily extracted into the ether layer (30), with ca. 11 to 12% of the keto acid being lost at each of the five extraction stages (J. Thompson, unpublished data). When intracellular pyruvate levels were required, the filtered cells were extracted with 5 ml of 0.6 N $HClO_4$ at 0°C for 20 min, the suspension was adjusted to pH 7.4 by addition of triethanolamine-KOH solution as described by Maitra and Estabrook (28), and precipitated $KClO_4$ was removed by centrifugation (20,000 × *g*, 5 min). The supernatant fluid was transferred to a 10-ml volumetric flask. The $KClO_4$ pellet was rinsed with distilled water, and the wash liquid was transferred to the flask and made up to volume with distilled water. Pyruvate analyses were performed as soon as possible to avoid loss by polymerization. Recoveries of 90% were obtained by using 0.2 to 1 nmol of standard pyruvate.

Determination of glycolytic intermediates. Glycolytic intermediates were determined qualitatively and quantitatively by thin-layer autoradiography and enzymatic-fluorimetric analysis, respectively.

(i) Thin-layer autoradiography. Radiolabeled intermediates were separated by using a modification of the recently described method of Conyers et al. (6). Trichloroacetic acid-ether extracts (~4 ml) were frozen, freeze-dried, and then reconstituted with 150 to 200 μl of distilled water. No loss of radioactivity occurred during this process. Subsequently, 10 to 15 μl of solution (~25,000 cpm) was applied to precoated layers of polyethyleneimine-cellulose obtained from Macherey, Nagel and Co., Düren, West Germany. The chromatogram was developed in distilled water (15 cm, 3.25 h) to remove free sugar, neutral products, and impurities present in the layer. After drying for 24 h, the layer was developed (12 cm, 2 h) in a solution of 0.5 M LiCl-2 N formic acid (1:1) (34). Radioactive areas were visualized by autoradiography (Kodirex X-ray film, 5-day exposure) and identified by co-chromatography, using 0.05-μmol standard intermediates. The phosphorylated standards were identified upon

dipping the dried layer into a solution containing 0.1 g of $FeCl_3 \cdot 6H_2O$, 7 g of 5-sulfosalicyclic acid, 25 ml of water, and ethanol to a final volume of 100 ml. Phosphate compounds appeared as white fluorescent spots on a purple background, which faded rapidly (~10 min) but which reappeared to assume the original intensity after 24 to 48 h and were then stable for several weeks. R_f values (relative to the LiCl-formic acid front) obtained were: fructose-1,6-diphosphate (FDP), 0.11; PEP, 0.20; 2- and 3-PG, 0.22; dihydroxyacetone phosphate, 0.37; glucose 6-phosphate, 0.48; fructose 6-phosphate, 0.53; α-glycerophosphate, 0.55; and glyceraldehyde 3-phosphate, 0.77.

(ii) Enzymatic analyses for glycolytic intermediates. Glycolytic intermediates were assayed by standard fluorimetric techniques (9, 28, 47). Difficulty was encountered in the analysis of 3-PG and 2-PG in standard solution and cell extracts with the first of the two assays described by Czok and Eckert (9). In assay 1, pyruvate, PEP, 2-PG, and 3-PG were determined by sequential additions of lactate dehydrogenase, pyruvate kinase, enolase, and phosphoglycerate mutase to the previously described buffered system (47). Using a standard solution containing 1.5 nmol each of 2-PG and 3-PG, theoretical calculations predicted 18 units of fluorescence decrease for each isomer, assuming that the enolase and phosphoglycerate mutase reactions proceeded to completion. However, only ca. 55% of the anticipated decrease occurred upon addition of enolase (i.e., from 2-PG), and, more important, the addition of cofactor 2,3-diphosphoglycerate (in the absence of its enzyme, phosphoglycerate mutase) caused a large decrease in fluorescence (23 units), associated presumably with the assay of 3-PG. When repeated with 2,3-diphosphoglycerate present at the commencement of the assay, >92% (33 units) of fluorescence decrease occurred upon the addition of enolase, indicating the simultaneous assay of both phosphoglycerates, and no further decrease was observed on the further addition of phosphoglycerate mutase. These abnormalities suggested that one or more of the enzymes (lactate dehydrogenase, pyruvate kinase, or enolase) contained phosphoglycerate mutase as a contaminant, and, since cell extracts appeared to contain 2,3-diphosphoglycerate, this assay procedure resulted in the simultaneous assay of 2-PG and 3-PG derivatives upon addition of enolase alone. Since phosphoglycerate mutase addition caused no further change in fluorescence, the erroneous conclusion was reached in a previous study that starved cells contained only the 2-PG isomer (47).

The alternative assay, based on the sequential additions of glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and phosphoglycerate mutase, proved to be most satisfactory. In this procedure, 3-PG and 2-PG were determined by sequential additions of 75 μg (5.7 U) of glyceraldehyde-3-phosphate dehydrogenase, 7 μg (13 U) of phosphoglycerate kinase, and 8 μg (4 U) of phosphoglycerate mutase to a 2.5-ml system (including extracts or standards) containing 0.1 M triethanolamine-KOH (pH 7.6) buffer, 5 mM $MgSO_4$, 0.25 mM glutathione, 10 mM hydrazine, 0.5 mM ATP, and 2 μM reduced nicotinamide adenine dinucleotide. Theoretically predicted fluorescence decreases (18 units) were observed for 3-PG and 2-PG in standard mixtures after the addition of phosphoglycerate kinase and phosphoglycerate mutase, respectively, to the cuvette. Analyses of starved-cell extracts demonstrated conclusively that 3-PG was present at a ca. sixfold greater concentration than that of the 2-PG isomer. This contrasts with the results obtained by assay 1, which indicated the presence of only the 2-PG compound. All enzymatic analyses were performed at 25°C, using a Perkin-Elmer fluorescence spectrophotometer (MPF-2A) with excitation and emission wavelengths of 350 and 450 nm, respectively, and slit widths of 12 nm.

Identification of 2-DG derivatives formed during uptake and efflux. Starved cells were suspended at a density of 500 μg (dry weight)/ml in 10 ml of 0.1 M tris(hydroxymethyl)aminomethane-maleate (pH 7.0) buffer containing 0.1 mM 2-[^{3}H]DG (5 μCi/μmol). After exactly 20 s of incubation, 1-ml volumes of suspension were filtered through 0.45-μm membrane filters. One filter plus adhered cells was transferred to 2 ml of 0.01 M tris(hydroxymethyl)aminomethane-maleate (pH 7.0) buffer maintained at 90°C, and a second filter was transferred to 2 ml of the same buffer maintained at 30°C. After 30 min of incubation, the two suspensions, were clarified by centrifugation (20,000 × *g*, 5 min), and 0.5 ml of supernatant fluid was transferred to columns (4 by 0.6 cm) of Bio-Rad AG1-X4 anion-exchange resin (100 to 200 mesh) in the formate form. Free and phosphorylated compounds were eluted with distilled water and a solution of 0.5 M ammonium formate in 0.2 M formic acid, respectively (29). Eluted material was collected (0.5-ml fractions), 200 μl of each fraction was transferred to 10 ml of scintillation cocktail (containing 100 g of naphthalene and 5 g of 2,5-diphenyloxazole per liter of dioxane), and radioactivity was determined.

Glucose determination. Glucose was assayed by using Glucostat reagents (Worthington Biochemicals Corp., Freehold, N. J.).

Reagents. Chemicals and enzymes, unless stated otherwise, were obtained from the Sigma Chemical Co., St Louis, Mo. 5-Sulfosalicyclic acid and 6-DG were obtained, respectively, from BDH Chemicals Ltd., Poole, and Koch-Light Laboratories, Colnbrook, England. Radioactive materials including DL-[1-^{14}C] lactic acid (sodium salt), D-[*U*-^{14}C]glucose, 3-*O*-methyl-D-[*U*-^{14}C]glucose (3-*O*-[*U*-^{14}C]MG), 2-deoxy-D-[1-^{3}H]glucose (2-[1-^{3}H]DG), methyl-α-D-[*U*-^{14}C]glucopyranoside (α-[*U*-^{14}C]MG), [D-*glucose*-1-^{14}C]lactose, were purchased from the Radiochemical Centre, Amersham, England; β-D-[*methyl*-^{14}C]thiogalactopyranoside ([^{14}C]TMG) was obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Inhibition of glucose metabolism by sulfhydryl (—SH) reagents. The procedure which allows PEP potential (3-PG, 2-PG, and PEP) to be controlled in vivo by using *p*-CMB and dithiothreitol (DTT) was discovered during a study of the inhibitory effects of sulfhydryl-group reagents upon glucose metabolism by *S.*

lactis ML_3. Cells of *S. lactis* ML_3 grown previously on galactose as the sole fermentable sugar possess the capacity to metabolize glucose, galactose, and lactose by the probable pathways illustrated in Fig. 8. Nongrowing (resting) cells utilized glucose at a linear rate (ca. 110 μmol/g [dry weight] of cells per min), and ~85% of the hexose metabolized was recovered as L-lactic acid. The addition of *p*-CMB (1 mM) or IAA (10 mM) to glycolyzing cell suspensions caused immediate inhibition of sugar metabolism (data not shown), whereas *N*-ethylmaleimide (1 mM) had no significant inhibitory effect.

Glycolytic intermediates present in glycolyzing and —SH-inhibited cells. (i) Thin-layer chromatography and autoradiography. Cells of *S. lactis* ML_3 were incubated with [*U*-^{14}C]glucose, and, in duplicate systems, glycolysis was halted by either *p*-CMB or IAA addition. Cell extracts were prepared and assayed by thin-layer autoradiography as described in Materials and Methods. In the extract prepared from glycolyzing cells, three major bands were prominent (Fig. 1A). The first heavily labeled area (R_f, 0.85) was L-lactic acid, and the second (R_f, 0.24) corresponded to the mixture of 3-PG, 2-PG, and PEP derivatives, whereas the third major band (R_f, 0.12) corresponded to FDP. In the autoradiographic profiles of extracts prepared from *p*-CMB- and IAA-inhibited cells, the band corresponding to the PEP-potential intermediates was absent (Fig. 1C and D).

(ii) Quantitative analyses of glycolytic intermediates. Cell extracts were assayed for glycolytic intermediates by using standard fluorimetric procedures, and the data are shown in Table 1. The results of quantitative analysis correlated well with the autoradiographic profiles illustrated in Fig. 1. The following points were revealed by inspection of the data in Table 1: (i) the glycolyzing cells contained ca. 16 mM FDP and a total PEP potential of ca. 10 mM; (ii) —SH-inhibited cells contained ~20 mM FDP, similar to the amount in glycolyzing cells, but no PEP-potential intermediates were detectable; and finally (iii), the presence of those intermediates which precede glyceraldehyde 3-phosphate dehydrogenase in the glycolytic sequence (namely, glucose 6-phosphate, fructose 6-phosphate, FDP, and triose phosphates) and the depletion of those intermediates which follow (namely, 3-PG, 2-PG, and PEP) strongly suggested that glyceraldehyde-3-phosphate dehydrogenase was the intracellular target of the —SH reagents responsible for inhibition of glycolysis.

Regulation of PEP formation in vivo by use of *p*-CMB plus DTT. Enzymatic analyses showed that *p*-CMB-inhibited cells of *S. lactis* ML_3 contained high levels of FDP and triose phosphates, but no PEP-potential intermediates (Table 1). The addition of dithiothreitol (DTT) removed the block at glyceraldehyde-3-phosphate dehydrogenase caused by this inhibitor, and the preceding intermediates were immediately (<15 s) and quantitatively converted into 3-PG, 2-PG, and PEP (Table 2). The latter compounds were not further metabolized. The inhibitory effect of IAA upon glyceraldehyde-3-phosphate dehydrogenase could not be reversed by DTT (Table 3). By the judicious use of *p*-CMB plus DTT, it was possible to regulate the in vivo formation of PEP, thereby controlling PTS activities in intact cells (Fig. 2). In the experiment described, glycolysis was halted by *p*-CMB, and the inhibited cells were collected by centrifugation to remove extraneous glucose. Neither TMG nor 2-DG was significantly accumulated by the cells when they were resuspended in a buffered medium containing 1 mM *p*-CMB (Fig. 2). However, the addition of DTT to the appropriate system resulted in the immediate uptake of both sugar analogs by their respective PTS. Concomitant with sugar accumulation (e.g., 2-DG) the cells became depleted of intracellular PEP potential. No depletion occurred when the cells were presented with glu-

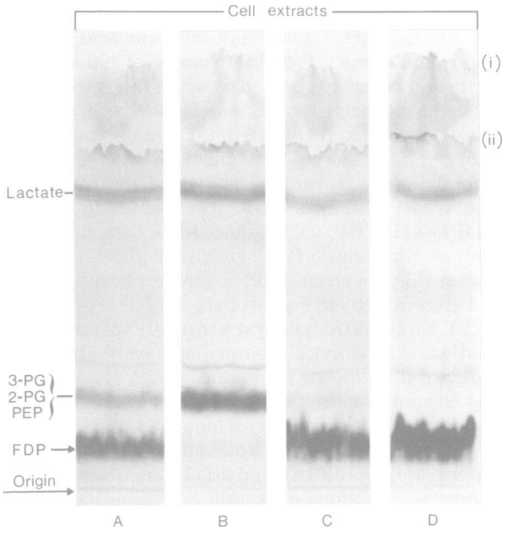

FIG. 1. *Qualitative autoradiographic analysis by thin-layer chromatography of* ^{14}*C-labeled glycolytic metabolites in cell extracts prepared from glycolyzing (A), starved (B), p-CMB-inhibited (C), and IAA-inhibited (D) cells of S. lactis* ML_3. *Experimental conditions were as described in the text. The solvent fronts designated (i) and (ii) are water and LiCl–formic acid fronts, respectively.*

TABLE 1. *Intracellular concentrations of glycolytic intermediates in glycolyzing, starved, and —SH-inhibited cells of S. lactis ML₃*

Glycolytic intermediate	Concn of intermediate (mM)[a] in:			
	Glycolyzing cells	Starved cells	1 mM *p*-CMB-inhibited cells	10 mM IAA-inhibited cells
Glucose 6-phosphate	1.64 ± 0.09	ND[b]	0.69 ± 0.17	0.95 ± 0.43
Fructose 6-phosphate	0.26 ± 0.09	ND	0.22 ± 0.05	0.35 ± 0.18
FDP[c]	15.76 ± 0.34	ND	20.45 ± 0.67	19.11 ± 1.68
Dihydroxyacetone phosphate / Glyceraldehyde 3-phosphate	6.70 ± 0.10	ND	8.04 ± 0.67	8.04 ± 0.10
3-PG[c]	6.24 ± 0.63	28.89 ± 0.32	ND	ND
2-PG[c]	1.24 ± 0.05	5.31 ± 0.94	ND	ND
PEP[c]	3.03 ± 0.28	11.29 ± 0.28	ND	ND
Pyruvate[d]	2.32 ± 0.09	1.12 ± 0.09	1.72 ± 0.17	1.64 ± 0.10

[a] Mean and mean deviations from two separate experiments.
[b] ND, Not detectable in cell extracts by fluorimetric-enzymatic analysis, indicating intracellular concentrations of <0.1 mM.
[c] Compounds comprising major bands as determined by autoradiography (Fig. 1).
[d] Intracellular pyruvate was determined by using the $HClO_4$ extraction procedure.

TABLE 2. *Reversible inhibition of glyceraldehyde-3-phosphate dehydrogenase in intact cells of S. lactis ML₃ by a combination of p-CMB plus DTT*

Glycolytic intermediate	Concn of intermediate (mM)[a] in cells inhibited with:	
	1 mM *p*-CMB	1 mM *p*-CMB followed by 10 mM DTT
Glucose 6-phosphate	0.11 ± 0.04	ND[b]
Fructose 6-phosphate	0.14 ± 0.04	ND
FDP	15.62 ± 1.31	0.51 ± 0.28
Dihydroxyacetone phosphate / Glyceraldehyde 3-phosphate	5.44 ± 1.22	0.55 ± 0.24
3-PG	1.21 ± 0.40	26.76 ± 1.07
2-PG	ND	2.01 ± 0.54
PEP	0.84 ± 0.19	9.87 ± 0.30
Approx total intracellular PEP potential	~38.9[c]	~39.7

[a] Mean and mean deviations from three experiments.
[b] ND, Not detectable in cell extracts by fluorimetric enzymatic analysis, indicating intracellular concentrations of <0.1 mM.
[c] Assuming complete transformation of FDP and triosephosphates upon reversal of *p*-CMB inhibition.

cose analogs which could not be transported via the glu-PTS (Table 4). The data revealed a close correlation between maximum uptake of 2-DG (ca. 32 mM [Fig. 2]) and the available PEP potential (ca. 39 mM [Table 2]), which indicated a stoichiometric ratio of sugar transported to PEP utilized of ~0.82.

Regulation of PEP potential by starvation. Autoradiography of extracts prepared from starved cells (i.e., after exhaustion of [^{14}C]glucose from the medium) (Fig. 1B) revealed only two major bands of radioactivity—the first corresponding to L-lactate and the other corresponding to the mixture of PEP-potential intermediates. These findings, which were confirmed by enzymatic analysis (Table 1), showed that the PEP potential in starved cells (ca. 45 mM) was approximately fourfold greater than that in glycolyzing organisms. Maximum accumulation of 2-DG by starved cells was attained within 20 s, and preincubation of the cells with either IAA or *p*-CMB had little inhibitory effect upon the activity of the glu-PTS (Fig. 3A). Starved cells accumulated TMG, via the lac-PTS, to a similar intracellular concentration to that of the glucose analog (~32 mM), though at a considerably slower rate (Fig. 3B). The lac-PTS was extremely sensitive to *p*-CMB inhibition, but, like the glu-PTS, the former system was also resist-

TABLE 3. *Irreversible inhibition of glyceraldehyde-3-phosphate dehydrogenase by IAA in intact cells of S. lactis ML₃*

Glycolytic intermediate	Concn of intermediate (mM)[a] in cells inhibited with:	
	10 mM IAA	10 mM IAA followed by 10 mM DTT
Glucose 6-phosphate	1.21 ± 0.04	1.00 ± 0.06
Fructose 6-phosphate	0.30 ± 0.04	0.24 ± 0.04
FDP	23.52 ± 1.19	19.94 ± 1.89
Dihydroxyacetone phosphate / Glyceraldehyde 3-phosphate	7.49 ± 1.20	7.49 ± 0.30
3-PG	ND[b]	ND
2-PG	ND	ND
PEP	ND	ND

[a] Mean and mean deviations from three experiments.
[b] ND, Not detectable in cell extracts by fluorimetric enzymatic analysis.

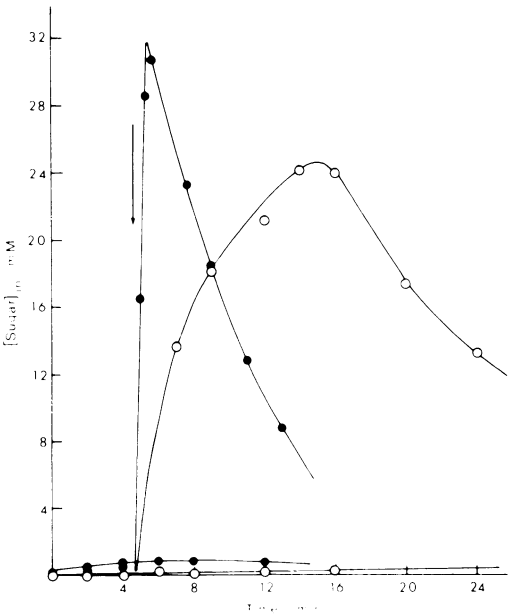

FIG. 2. *Accumulation of 2-DG and TMG by cells of S. lactis ML_3 previously treated with p-CMB (to halt glycolysis). Cells were suspended in buffered medium containing glucose, and glycolysis was halted by addition of p-CMB (1 mM). The cells were harvested and washed with tris(hydroxymethyl)aminomethane-maleate buffer containing p-CMB and finally resuspended (at 200 μg [dry weight]/ml) in the same solution, to which the following additions were made: (●) 0.1 mM 2-[^{3}H]DG (0.5 μCi/μmol) followed by 10 mM DTT at 5 min (○) 0.1 mM [^{14}C]TMG (0.2 μCi/μmol) followed by 10 mM DTT at 5 min.*

TABLE 4. *Depletion of intracellular PEP potential (3-PG, 2-PG, and PEP) during uptake of 2-DG by starved cells of S. lactis ML_3*

Endogenous glycolytic intermediate[a]	Concn of intermediate (mM)[b] in cells incubated with[c]:			
	Control (no glucose analog)	2-DG	3-*O*-MG	1-*O*-MG
3-PG	29.64 ± 0.36	ND[d]	27.24 ± 0.64	25.14 ± 1.50
2-PG	2.54 ± 0.45	ND	2.39 ± 0.59	2.99 ± 0.87
PEP	11.37 ± 0.05	ND	10.08 ± 0.86	10.36 ± 1.44

[a] No other glycolytic intermediates were detectable in starved cells by fluorimetric analysis (see Table 1).

[b] Intracellular concentrations represent means and mean deviations from four experiments.

[c] Concentration of glucose analog (when present) was 0.1 mM, and incubation period was 60 s. Filtration, extraction and analytical techniques were as described in the text.

[d] ND, Not detectable in cell extracts by fluorimetric enzymatic analysis.

ant to IAA and *N*-ethylmaleimide. The potent inhibitory effect of *p*-CMB upon the lac-PTS could be readily reversed by DTT (Fig. 4). Addition of *p*-CMB to the incubation system caused immediate inhibition of TMG accumulation by starved cells and a resultant efflux of accumulated material by a process exhibiting first-order kinetics ($t_{1/2}$ ~ 11 min). The subsequent introduction of DTT (to a duplicate system) removed the inhibition, and TMG was again transported until exhaustion of the intracellular PEP reserve (ca. 18 min).

Transport of glucose analogs by starved cells of *S. lactis* ML_3. Maximum uptake of 2-DG (as the product 2-DG-6-phosphate, see below) occurred within 20 to 30 s (Fig. 5). Thereafter, efflux of material took place by a process which exhibited first-order kinetics (k_{ex} = 0.25 min^{-1}), and the half-time of exit, $t_{1/2}$, was 2.8 min (Fig. 5, inset). Ion-exchange chromatography (Fig. 6) revealed that ca. 87% of the accumulated sugar existed in phosphorylated form, and ca. 13% existed as the free sugar. The material which appeared in the medium during efflux existed entirely as the free sugar. Starved cells were unable to accumulate α-MG, and 3-*O*-MG was transported very slowly by comparison with

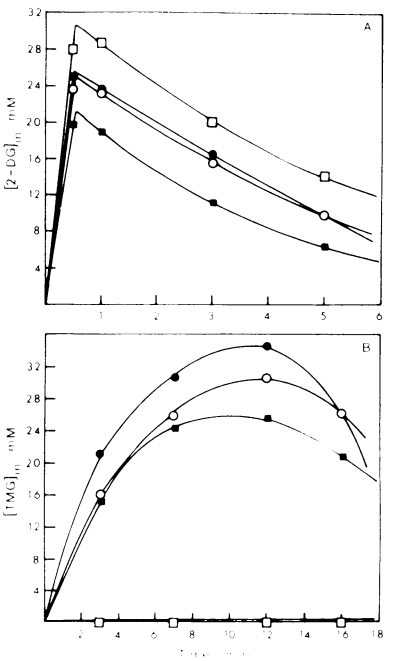

FIG. 3. *Accumulation of 2-DG (A) and TMG (B) by starved cells of S. lactis ML_3 in the presence and absence of —SH inhibitors. Cells were suspended at 200 μg (dry weight)/ml in 0.1 M tris(hydroxymethyl)aminomethane-maleate buffer (pH 7.0) containing the inhibitors at the concentrations specified: (●) control (no inhibitor), (○), 1 mM N-ethylmaleimide, (□) 1 mM p-CMB, and (■) 10 mM IAA. After 10 min of incubation, either 0.1 mM 2-[^{3}H]DG (specific activity, 0.5 μCi/μmol) or 0.1 mM [^{14}C]TMG (specific activity, 0.2 μCi/μmol) was added to the appropriate system.*

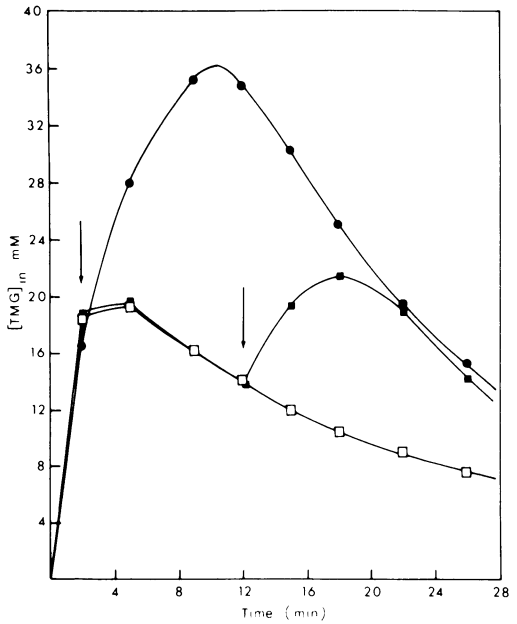

FIG. 4. *Reversible inhibition of TMG accumulation by starved cells of S. lactis ML₃ in the presence of p-CMB. The cells were suspended at a concentration of 200 μg (dry weight)/ml in 0.1 M tris(hydroxymethyl)aminomethane-maleate buffer (pH 7.0) containing 0.1 mM [¹⁴C]TMG (0.2 μCi/μmol), and the following additions were made: (●) control (no addition), (□), 1 mM p-CMB (at 2 min), and (■) 1 mM p-CMB (at 2 min) followed by 10 mM DTT (at 12 min). Arrows indicate times of additions of p-CMB and DTT to systems.*

the 2-DG analog.

Kinetic analysis of glucose and 2-DG accumulation by starved cells. The kinetic analysis of PTS-mediated transport of glucose could be meaningfully performed with IAA-inhibited, starved cells, since (i) the cells contained an endogenous PEP reserve, (ii) IAA did not inhibit glu-PTS activity, and (iii) IAA prevented the subsequent metabolism of accumulated sugar. The initial rates of uptake of both glucose and 2-DG by intact cells displayed high-affinity, Michaelis-Menten saturation characteristics (Fig. 7). Transformation of the initial rate data according to the method of Hofstee (Fig. 7, inset) yielded the following kinetic parameters: for glucose, V_{max} = 478 μmol/g (dry weight) of cells per min and K_m = 15.5 μM; for 2-DG, V_{max} = 390 μmol/g (dry weight) of cells per min and K_m = 80.6 μM. The data suggest that glycolytic activity (ca. 110 μmol of hexose metabolized per g [dry weight] of cells per min), rather than PTS activity (i.e., glucose transport), may be the rate-limiting factor for glucose utilization by nongrowing cells.

Specificity of the glu-PTS in *S. lactis* ML₃. The substrate specificity of the constitutive glu-PTS was investigated by monitoring [¹⁴C]glucose uptake by cells suspended in medium containing 10- and 100-fold greater concentrations of glucose analogs (Table 5) differing in a single substitution or positional change relative to D-glucopyranose. The results obtained showed that D-mannose, 2-DG, and D-glucosamine were effective inhibitors of glucose uptake, but no significant inhibition was found in the presence of α-MG, β-MG, 3-*O*-MG, galactose, xylose, or 6-DG. Inhibition by a structural analog does not necessarily prove that the analog and glucose bind to the same transport system, and this fact can be ascertained only by inhibition analysis. However, from the available data (Table 5 and Fig. 7) it is possible to calculate the expected rates of glucose transport in the presence and absence of 2-DG, an analog whose K_m (80.6 μM) is known. When the glucose concentration (S_G) is 20 μM, the theoretical rate of transport, V, can be calculated from: $V = (V_{max} \times S_G)/(K_m + S_G)$,

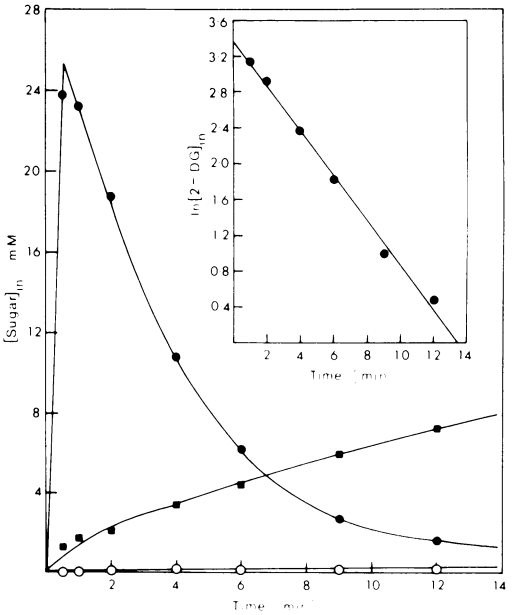

FIG. 5. *Uptake of non-metabolizable glucose analogs by starved cells of S. lactis ML₃. The cells were suspended at a concentration of 200 μg (dry weight)/ml in 0.1 M tris(hydroxymethyl)aminomethane-maleate buffer (pH 7.0). Glucose analogs were then introduced into the appropriate systems as follows: (●) 2-[³H]DG (0.5 μCi/μmol), (○) α-[¹⁴C]MG (0.2 μCi/μmol), and (■) 3-O-[¹⁴C]MG (0.2 μCi/μmol). Inset illustrates the first-order exit of accumulated materials from cells incubated with 2-[³H]DG. The rate constant of efflux, k_{ex}, was 0.25 min⁻¹, determined by linear regression analysis (correlation coefficient, 0.99) and half-time of exit, $t_{1/2}$ (ln 2/k_{ex}), was 2.76 min.*

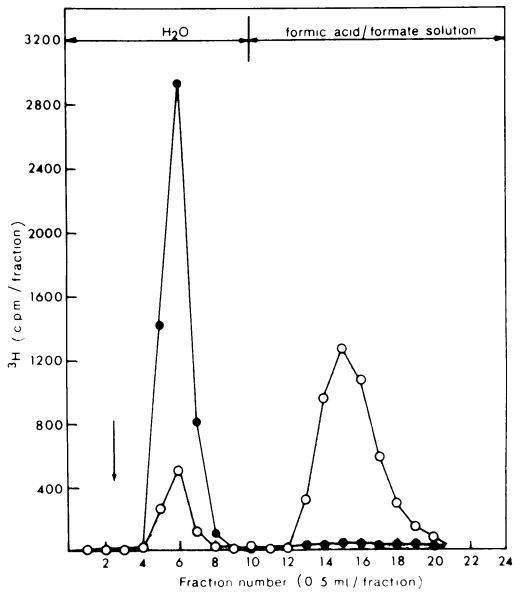

FIG. 6. *Identification by ion-exchange chromatography of 2-[^{3}H]DG derivatives formed during uptake and efflux from starved cells of S. lactis ML$_3$. Experimental procedure was as described in the text. Symbols: ○, material accumulated by cells within 30 s of incubation; ●, material appearing in medium during efflux. Arrow indicates application of 0.5 ml of cell extract to column.*

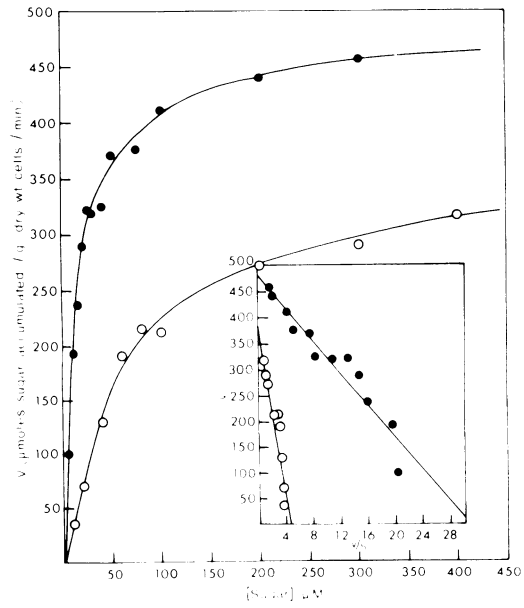

FIG. 7. *Kinetic analysis of (●) glucose and (○) 2-DG uptakes by the constitutive glu-PTS in S. lactis ML$_3$. Starved cells were preincubated for 10 min in buffered medium containing 10 mM IAA, and other experimental conditions were as described in the text.*

TABLE 5. *Inhibitory effect of glucose analogs upon the rate of [^{14}C]glucose uptake by starved, IAA-inhibited cells of S. lactis ML$_3$*

Sugar tested	Structural and positional changes relative to D-glucopyranose[a]	Analog concn (mM)	Initial rate of [^{14}C]glucose uptake (μmol/g [dry wt] of cells per min)[b]
D-Glucose[c]	None	0.2	27 (12)
		2	2 (1)
α-MG	C-1, 1-*O*-methyl (α-anomer)	0.2	201 (93)
		2	203 (94)
β-MG	C-1, 1-*O*-methyl (β-anomer)	0.2	232 (107)
		2	211 (97)
D-Mannose	C-2, axial —OH	0.2	91 (43)
		2	16 (7)
2-DG	C-2, deoxy	0.2	138 (64)
		2	43 (20)
D-Glucosamine	C-2, NH_2 substitution	0.2	174 (80)
		2	80 (37)
3-*O*-MG	C-3, -*O*-methyl	0.2	249 (114)
		2	201 (93)
D-Galactose	C-4, axial —OH	0.2	228 (105)
		2	213 (98)
D-Xylose	C-5, dehydroxymethyl	0.2	212 (98)
		2	215 (99)
6-DG	C-6, deoxy	0.2	211 (97)
		2	131 (60)

[a] All sugars considered as C1-chair conformers, with substituents as in D-glucopyranose (10, 11) except where indicated.

[b] The control rate of [^{14}C]glucose uptake was 217 μmol/g (dry weight) of cells per min (represented as 100%). The data shown are mean values obtained from two separate experiments, and values in parentheses represent observed [^{14}C]-glucose uptake as a percentage of the control rate. All systems contained 0.02 mM [^{14}C]glucose (0.2 μCi/μmol) and sugar analogs at either 0.2 or 2 mM. Other conditions were as described in the text.

[c] Unlabebed glucose.

where V_{max} = 478 μmol/g (dry weight) of cells per min, K_m = 15.5 μM, and S_G = 20 μM. The expected and observed rates of transport (see Table 5) were 270 and 217 μmol/g (dry weight) of cells per min, respectively. In the presence of a potential competitive inhibitor (i.e., 2-DG, 200 μM) the expected rate of glucose transport may be derived from: $V = (V_{max} \times S_G)/(\{K_m[1 + (i/K_i)]\} + S_G)$, where V_{max}, K_m, and S_G have the previous values and where i, the inhibitor concentration (2-DG), is 200 μM. If 2-DG is an alternate substrate of the glu-PTS, then K_i = $K_{m(2\text{-DG})}$ (for 2-DG transport alone) = 80.6 μM. The calculated and observed rates of glucose uptake in the presence of 200 μM 2-DG were 129 and 138 μmol/g (dry weight) of cells per min, respectively. The reasonably close agreement

Inset plots illustrate transformation of initial rate data according to Hofstee; the line of best fit was obtained by linear regression analysis, using a Hewlett-Packard (model HP 9830) desk top calculator.

between predicted and experimental data suggested that of the glucose analogs tested, 2-DG, at least, was transported via the glu-PTS.

DISCUSSION

The procedures described in this study permit intracellular PEP potential (3-PG, 2-PG, and PEP) to be controlled, and maintained at a high level, in intact cells of *S. lactis* ML_3. Experimentally, these techniques are particularly advantageous, since they eliminate the need for "permeabilizing" or "decryptifying" procedures so frequently used to introduce exogenous PEP into whole cells of other organisms (12, 13, 21, 41). The inherent risks of membrane disruption or enzyme inactivation associated with toluene treatment or freezing and thawing are thus avoided, and membrane functions, such as PTS activity, may be investigated in the physiologically intact cells. The capacity to regulate PEP formation in vivo has enabled several important interactions between glycolysis and PTS activity to be demonstrated. For example, quantitative studies revealed a direct correlation between PTS activities (e.g., TMG and 2-DG accumulation) and the rate of utilization of intracellular PEP, and a close stoichiometry was observed between PEP potential and maximum accumulation of PTS substrates. Furthermore, kinetic analysis of PEP-dependent uptake of glucose by intact cells of *S. lactis* ML_3 indicated that glycolytic activity, rather than the rate of hexose accumulation (PTS activity), was the rate-limiting factor for glucose metabolism in nongrowing cells.

Results obtained in a previous study (47) suggested that 2-PG was the predominant phosphoglycerate isomer in starved and glycolyzing cells of *S. lactis* ML_3. However, since the activity of phosphoglycerate mutase in vitro favors the formation of 3-PG, these findings indicated a significant departure from the expected equilibrium position for phosphoglycerate mutase (and enolase) in vivo (P. K. Maitra, personal communication). Using a more reliable assay, 3-PG (not 2-PG) has now been shown to be the predominant isomer. Furthermore, the close correlation between the calculated mass-action ratios and apparent equilibrium constants (Table 6) showed that in starved cells of *S. lactis* ML_3, both phosphoglycerate mutase and enolase reactions had attained equilibrium states. From thermodynamic considerations, neither phosphoglycerate mutase nor enolase reactions can be maintained at equilibrium during glycolysis (32), but the data (Table 6) indicate that these two sequential reactions must also be close to equilibrium under glycolyzing conditions.

Galactose-grown cells of *S. lactis* ML_3 have the capacity to metabolize glucose, galactose, and lactose by the probable pathways illustrated in Fig. 8, and it has thus been possible to characterize the constitutive glu-PTS and the inducible lac-PTS (26, 27) in the same cells. In *E. coli* and *Salmonella typhimurium*, there are two PTSs for glucose transport (for discussion and clarification, see references 1, 8, 19, and 33). The first is highly glucose specific and has been designated the GPTS (8) (also termed $III^{glu}/II\text{-}B^{glu}$, III/II-B', GPT-A, or GPT-G). The GPTS, which may be either constitutive or inducible, phosphorylates glucose and α-MG (K_m values of 10 and 6 μM, respectively [33]). The enzyme II-B' recognition protein is dependent upon an —SH function (3, 4, 14, 15, 22, 31). The second system, designated the mannose PTS (MPTS, [8]) is constitutive for glucose uptake (K_m, 15 μM [33]), but D-mannose, 2-DG (K_m ~ 200 μM [33]), and D-glucosamine (but not α-MG) are also transported by this system (also termed II-A/II-B, GPT-B, PTS-M, or PTS-X). The enzyme II-B recognition protein of the MPTS does not appear to be dependent upon —SH function (22).

In solution, D-glucopyranoside exists as a six-membered, cyclic chair of the C1 conformation (2, 10, 11), in which all the bulky substituents—hydroxyl and hydroxymethyl—project equatorially in the plane of the ring. Competition studies showed that 3-*O*-MG, α-MG, β-

TABLE 6. *Comparison of mass-action ratios* (Γ) *of phosphoglycerate mutase and enolase reactions determined in starved and glycolyzing cells of S. lactis ML_3 with equilibrium data obtained in vitro*

Enzyme	Reaction catalyzed	Apparent equilibrium constant (K')[a]	Γ[b] in:	
			Starved cells	Glycolyzing cells
Phosphoglycerate mutase	3-PG ⇌ 2-PG	$\frac{[2\text{-PG}]}{[3\text{-PG}]} = 0.1\text{–}0.2$	0.18	0.19
Enolase	2-PG ⇌ PEP	$\frac{[PEP]}{[2\text{-PG}]} = 2.8\text{–}4.6$	2.13	2.50

[a] Average K' values obtained from data in references 25, 32, and 37.
[b] Γ values calculated from data in Table 1.

MG, and galactose (single methylations at C3 or anomeric C1; and inversion of —OH to an axial position at C4, respectively) had no significant inhibitory effect on [^{14}C]glucose uptake (Table 5) by *S. lactis* ML_3. However, glucose (as expected) and the C2 analogs D-mannose, D-glucosamine, and 2-DG all produced marked inhibition of [^{14}C]glucose accumulation, suggesting a high affinity of these compounds for the constitutive glu-PTS in starved cells of *S. lactis* ML_3. The intact cells rapidly accumulated glucose (K_m, 15.5 μM) and 2-DG (K_m ~ 80 μM) but not α-MG, and 3-*O*-MG was only poorly transported. Sulfhydryl-group reagents IAA, *N*-ethylmaleimide, and *p*-CMB did not inhibit uptake of glucose or 2-DG. The glu-PTS in galactose-grown cells of *S. lactis* ML_3 (and also in *Streptococcus mutans* [40, 41]) exhibited the characteristics of the constitutive MPTS described previously in *E. coli* (8).

The accumulation of solutes by the glu-PTS (MPTS) in *S. lactis* ML_3 was insensitive to IAA and *p*-CMB (Fig. 3A), but these reagents produced immediate inhibition of glucose metabolism (glycolysis). The resistance of the MPTS to —SH reagents was not, therefore, simply due to failure of these compounds to reach or penetrate the cytoplasmic membrane (39). The lac-PTS-mediated accumulation of [^{14}C]lactose and TMG was also insensitive to IAA (and *N*-ethylmaleimide), but, surprisingly, this system was completely, but reversibly, inactivated by *p*-CMB (Fig. 4; J. Thompson, manuscript in preparation). This highly specific inhibition by the organic mercurial therefore permits the selective inactivation of the lac-PTS while allowing the glu-PTS (MPTS) to remain operational. The activities of the two systems, utilizing the PEP potential in starved cells, may be represented by:

$$(3\text{-PG} \rightarrow 2\text{-PG} \rightarrow \text{PEP} + \text{HPr} \xrightarrow{\text{enzyme 1, } Mg^{++}} \text{P-HPr} + \text{pyruvate} \quad (1)$$

$$\text{P-HPr} + \begin{cases} \text{glucose} \xrightarrow{\text{II-A/II-B}} \text{glucose 6-phosphate} + \text{HPr} & (2) \\ \text{lactose} \xrightarrow{\text{factor III}^{lac}/\text{II-B}^{lac}} \text{lactose phosphate} + \text{HPr} & (3) \end{cases}$$

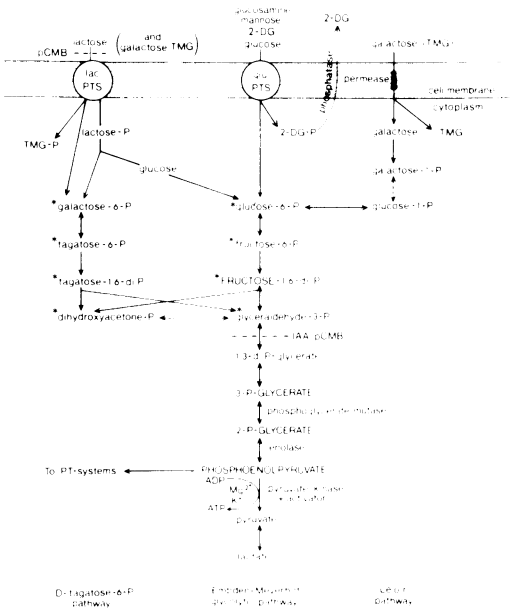

FIG. 8. *Probable pathways for transport and metabolism of sugars by galactose-grown cells of S. lactis ML_3 (modified after Thompson and Thomas [47]). Asterisks designate in vitro activators of pyruvate kinase, and dotted lines show points of inhibition by —SH reagents. Large type indicates metabolites of particular interest in this investigation, and PTS designates phosphoenolpyruvate:sugar phosphotransferase systems. The location of the phosphatase is not yet known.*

The point at which *p*-CMB inhibits the multicomponent lac-PTS (reaction 3) in *S. lactis* ML_3 is not yet known, but the resistance of the MPTS (reaction 2) to the inhibitor demonstrates that inactivation of the lac-PTS must occur at a stage subsequent to P-HPr formation.

In *S. lactis* ML_3, the accumulated hexose phosphates (glucose 6-phosphate and 2-DG-6-phosphate) were dephosphorylated before rapid efflux ($t_{1/2}$, ca. 2.8 min). It is of interest to note that hexose phosphatase activity has been found in several organisms (24) and also appears to be associated with the glu-PTS (factor III^{glu}) in *E. coli* (22). It should be stressed that the levels of hexose phosphates (30 to 40 mM) accumulated by starved cells of *S. lactis* ML_3 are considerably greater than the intracellular concentration of glucose 6-phosphate (2 to 6 mM) under normal growth conditions (48). During growth, the extent of phosphatase activity may therefore be insignificant, especially since the PTS sugar will rapidly enter the Embden-Meyerhof pathway. The physiological function of this phosphatase, whose substrate is the first metabolite of the glycolytic sequence, is not clear. High intracellular levels of hexose phosphates are known to have deleterious effects on cell metabolism, and the phosphatase in *S. lactis* ML_3 may play a detoxifying role to maintain the cellular hexose phosphate levels within physiologically acceptable limits. The mechanism of glucose (and 2-DG) exit subsequent to dephosphorylation is

also unknown. Further studies will be required to determine whether exit of free glucose is mediated via a non-PTS or whether the II-B protein of the MPTS can cause the facilitated diffusion of glucose and its analogs across the cytoplasmic membrane.

This investigation has provided in vivo evidence for the regulatory role of pyruvate kinase in glycolysis in *S. lactis*. By its location in the glycolytic pathway and from the importance of its substrate (PEP) in cellular energetics, pyruvate kinase would seem to be a likely target for metabolic control (32, 42). Modulation of pyruvate kinase activity, by controlling the intracellular PEP level, could determine the distribution of this key metabolite into two separate pathways—one leading to ATP generation and the other leading to membrane-located PTSs (Fig. 8). The enzyme has the potential, therefore, to synchronize the rate of energy (ATP) production with the rate of uptake of the energy source itself. A regulatory role for pyruvate kinase was suggested from in vitro studies with purified enzyme from *S. lactis* (5, 7, 45), which revealed the complex kinetics of a regulatory (allosteric) enzyme in that both PEP and ADP showed positive co-operativity. Pyruvate kinase was activated by FDP, and the sigmoid function between reaction velocity and PEP concentration was transformed into a typical Michaelis-Menten relationship in the presence of excess FDP (heterotropic effector). It was found that FDP decreased the K_m values for PEP and ADP, whereas in the absence of the activator, V_{max} was reduced by ca. 98%. In a more detailed study, all the phosphorylated metabolites designated by an asterisk in Fig. 8 were found to be effective activators of pyruvate kinase in vitro (45).

The development of theories of metabolic control (32, 35, 37) often requires extrapolation of in vitro characteristics of isolated enzymes to the unknown conditions of the intact cell. Ideally, to counteract criticism of such extrapolation (32, 43), the changes in those activators which are considered to regulate activity of pyruvate kinase should be demonstrated in vivo. Results obtained recently (47), and confirmed in this study, showed that starved cells of *S. lactis* ML_3 retained high levels of 3-PG, 2-PG, and PEP. These observations are consistent with the demonstrated absence of potential in vivo activators of pyruvate kinase (45, 47; see Fig. 8). The discovery of the selective and reversible inhibition of glyceraldehyde-3-phosphate dehydrogenase by *p*-CMB in this investigation has allowed two predictions of the pyruvate kinase activator hypothesis to be tested in vivo. First, inhibition of glycolysis by *p*-CMB should cause retention, or "trapping," of those metabolites (glucose 6-phosphate, fructose 6-phosphate, FDP and triose phosphates) which precede the blockage point (glyceraldehyde-3-phosphate dehydrogenase). However, since these intermediates are all potential activators of pyruvate kinase, the presence of these compounds (by ensuring the continued activity of this enzyme) should lead to depletion of those metabolites (3-PG, 2-PG, and PEP) occurring after the point of inhibition. Second, reversal of *p*-CMB inhibition by DTT should permit transformation of the trapped metabolites into 3-PG, 2-PG, and PEP. Such a transformation would result simultaneously in a depletion of pyruvate kinase activators, and the PEP potential intermediates would be expected to accumulate within the cell. The data obtained in this study have verified both predictions.

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