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Regulation of Carbohydrate Transport Activities in *Salmonella typhimurium*: Use of the Phosphoglycerate Transport System to Energize Solute Uptake

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The phosphoglycerate transport system was employed to supply energy-depleted, lysozyme-treated *Salmonella typhimurium* cells with a continuous intracellular source of phosphoenolpyruvate. When the cells had been induced to high levels of the phosphoglycerate transport system, a low extracellular concentration of phosphoenolpyruvate (0.1 mM) half maximally stimulated uptake of methyl α-glucoside via the phosphoenolpyruvate:sugar phosphotransferase system. If the phosphoglycerate transport system was not induced before energy depletion, 100 times this concentration of phosphoenolpyruvate was required for half-maximal stimulation. Phosphoenolpyruvate could not be replaced by other energy sources if potassium fluoride (an inhibitor of enolase) was present. Inhibition of [^{14}C]-glycerol uptake into energy-depleted cells by methyl α-glucoside was demonstrated. A concentration of phosphoenolpyruvate which stimulated methyl α-glucoside accumulation counteracted the inhibitory effect of the glucoside. In the presence of potassium fluoride, phosphoenolpyruvate could not be replaced by other energy sources. Inhibition of glycerol uptake by methyl α-glucoside in intact untreated cells was also counteracted by phosphoenolpyruvate, but several energy sources were equally effective; potassium fluoride was without effect. These and other results were interpreted in terms of a mechanism in which the relative proportions of the phosphorylated and nonphosphorylated forms of a cell constituent influence the activity of the glycerol transport system.

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) catalyzes the translocation of several sugars into bacterial cells by a mechanism which couples transport with sugar phosphorylation (5, 6). Among the substrates of this enzyme system are a variety of hexoses, hexitols, and *N*-acetylhexosamines. Extensive studies, particularly in the laboratory of Roseman, have defined the enzymology of the system (3, 4, 6, 18). It consists of two "general" proteins, enzyme I and HPr, which are required for the translocation and phosphorylation of all sugar substrates of the PTS, and a family of sugar-specific enzyme II complexes (6). Each of the membrane-associated enzyme II complexes catalyzes the phosphorylation of only one or a few sugars.

Recently, we provided evidence for a complex mechanism whereby the PTS regulates the activities of several distinct membrane-associated enzyme systems. These include certain non-PTS carbohydrate permeases and adenylate cyclase (1, 7-15). To further characterize these regulatory interactions, we examined glycerol uptake and methyl α-glucoside uptake in energy-depleted, lysozyme-treated bacterial cells. The study of solute transport in closed membrane preparations usually requires a continuous supply of an appropriate energy source. With this problem in mind, we characterized a transport system in *Salmonella typhimurium* which allows phosphoenolpyruvate to cross cell membranes without prior hydrolysis (16). This system provides a means of supplying chemical energy to bacterial cells or membrane vesicles for the uptake of sugars via the PTS or for the continuous generation of ATP in the presence of pyruvate kinase and ADP. In the present communication, the conditions which maximize the activity of the phosphoglycerate transport system in energy-depleted cells are defined, and the system was employed to energize the uptake of methyl α-glucoside via the PTS. We further show that an adequate supply of intracellular phosphoenolpyruvate decreases the sensitivity of glycerol uptake to inhibition by a substrate of the PTS, such as methyl α-glucoside.

MATERIALS AND METHODS

Materials. Radioactive substrates were purchased from New England Nuclear Corp., with the exception of [^{14}C]methyl α-glucoside, which was from Amer-

sham/Searle, and [^{3}H]melibiitol, which was synthesized by reduction of melibiose with [^{3}H]$NaBH_4$ (12). The specific activities of the radioactive compounds used for the uptake studies were all 5 μCi/μmol. The enzymes used for the preparation of lysozyme-treated, energy-depleted cells and 2- and 3-phosphoglycerates (sodium salts) were purchased from Sigma Chemical Co. D-Lactate (lithium salt) was from Calbiochem. Other compounds were of the highest purity available commercially.

Bacterial strains. The strain used in most of the studies was *S. typhimurium* strain *glpT116*, a mutant isolated from strain LT-2 by R. Weppelman in the laboratory of B. N. Ames, Department of Biochemistry, University of California, Berkeley. This mutant, which lacked α-glycerophosphate transport activity, was used because glycerol uptake studies with membrane preparations derived from wild-type cells were complicated by the presence of the α-glycerophosphate transport system whenever glycerol phosphorylation was possible. The latter was very active in preparations of wild-type organisms after growth in glycerol-containing medium. Uptake of 3-phospho[^{14}C]glycerate or [^{14}C]methyl α-glucoside was not altered by the *glpT116* mutation. Other strains used were *S. typhimurium* strains SB1476 (*ptsI17*), a leaky enzyme I mutant, LJ68 (*glp-261*), a mutant in which glycerol uptake is resistant to PTS-mediated regulation, and the parent, LT-2 (11–13, 15).

Energy depletion. Cells were grown in minimal media as described in the table footnotes and figure legends. They were harvested in the exponential phase of growth because cells entering the stationary growth phase exhibited greatly depressed rates of phosphoglycerate uptake (16). Cells were treated essentially as described by Kaback for the preparation of membrane vesicles through the differential centrifugation step (2). Although uptake activities for 3-phospho[^{14}C]glycerate and [^{14}C]methyl α-glucoside were relatively stable in these preparations, that for [^{14}C]glycerol was unstable (most of the activity was lost within 24 h at 0°C). Because the procedure involved extended incubation of the cells at 37°C (in the presence of lysozyme), they were depleted of endogenous energy reserves (see Fig. 1 and Table 1), and the cell envelopes lost some of their native properties. However, *Salmonella* cells treated in this way retained their rod-shaped appearance by phase-contrast microscopy, and a substantial fraction of the cells in the preparation retained viability, as determined by a plating assay. Fresh preparations retained sufficient glycerokinase and ATP for accurate measurements of [^{14}C]glycerol uptake rates (17), but enolase, which was insensitive to KF inhibition in whole cells, was inhibited by fluoride in the treated preparations (see Table 1). *Escherichia coli* membrane vesicles could not be used in these studies, first, because they lacked sufficient glycerokinase and ATP to allow intravesicular trapping of [^{14}C]glycerol (which enters the cell by facilitated diffusion) and second, because *E. coli* lacks the genes which code for the phosphoglycerate transport system (16).

Uptake studies. Uptake studies were conducted at 28°C with aeration in 18-mm test tubes containing from 0.25 to 0.75 ml of a solution consisting of 0.08 M potassium phosphate (pH 6.6), 0.1 M potassium chloride, 0.02 M magnesium sulfate, 2 to 4 mg of protein per ml, and the inhibitors, energy sources, and radioactive substrates indicated below. The assay mixture (complete except for radioactive substrate) was preincubated at 28°C for 10 min before initiation of the uptake experiment. Samples (0.1 ml) were periodically removed, pipetted into 6 ml of 0.1 M LiCl at room temperature, filtered with membrane filters (type HA; pore size, 0.45 μm; Millipore Corp.), and washed with two 6-ml portions of 0.1 M LiCl. Dilution, filtration, and washing were conducted in less than 20 s. Subsequently, filters were removed from the suction apparatus and dried, and radioactivity was determined by scintillation counting (13).

RESULTS

Uptake of 3-phospho[^{14}C]glycerate into energy-depleted, lysozyme-treated cells. Under most conditions employed, rates of 3-phospho[^{14}C]glycerate uptake were nearly constant for the duration of the experiment (12 min), decreasing only slightly during the second half of this time period. The pH optimum was between 6 and 7, and comparable uptake rates were observed in potassium phosphate and sodium phosphate buffers at pH 6.6. However, omission of magnesium ions and inclusion of EDTA in the assay mixture strongly inhibited uptake. The effects of various energy sources and inhibitors on 3-phospho[^{14}C]glycerate uptake were studied (data not shown). In the absence of other compounds, 20 mM D-lactate stimulated 3-phosphoglycerate uptake, but the stimulatory effect of lactate was more pronounced in the presence of 40 mM KF, which depressed the endogenous activity. A variety of other potential energy sources stimulated poorly or did not stimulate 3-phoshoglycerate uptake appreciably. Inhibitors of energy metabolism counteracted the stimulatory effect of lactate, and high concentrations of any of the three nonradioactive substrates of the phosphoglycerate transport system abolished uptake of the radioactive compound. These results suggest that D-lactate can serve as an energy source for the uptake of substrates of the phosphoglycerate transport system.

Dependence of [^{14}C]methyl α-glucoside uptake on phosphoenolpyruvate. Time courses for the uptake of methyl α-glucoside into lysozyme-treated, energy-depleted cells grown in the presence of both glucose and 3-phosphoglycerate are shown in Fig. 1. Uptake of the glucoside was stimulated more than fivefold by 2 mM phosphoenolpyruvate. Figure 1 shows the dependence of methyl α-glucoside uptake on phosphoenolpyruvate concentration in treated cells which either had or had not been grown in the presence of 3-phosphoglycerate. Only the former

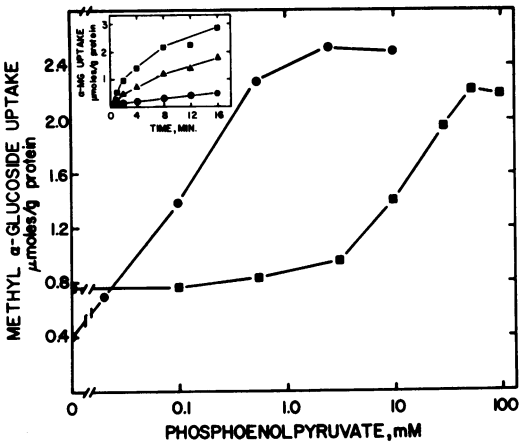

FIG. 1. *Uptake of methyl α-glucoside (α-MG) into lysozyme-treated energy-depleted cells as a function of phosphoenolpyruvate concentration. glpT116 cells were grown in medium 63 (13) containing 0.5% glycerol as the carbon source. At 2 h before the cells were harvested, 0.2% glucose and 0.1% 3-phosphoglycerate were added to the exponentially growing culture in order to induce synthesis of the glucose enzyme II complex (8) and the phosphoglycerate transport system (16) (●). Alernatively, cells were grown under identical conditions except that 3-phosphoglycerate was omitted (■). Cells were treated and transport experiments were conducted as described in the text in the presence of the phosphoenolpyruvate concentrations shown on the abscissa. The extracellular concentration of [^{14}C]methyl α-glucoside was 40 μM. Uptake values are expressed as micromoles of methyl α-glucoside accumulated in 10 min at 28°C per gram of protein. (Insert) Time course for the uptake of methyl α-glucoside. Uptake was followed in the absence of an exogenous energy source (●) or in the presence of 0.1 mM (▲) or 2 mM (■) phosphoenolpyruvate.*

cells showed the capacity to take up 3-phospho[^{14}C]glycerate. Low concentrations of phosphoenolpyruvate stimulated methyl α-glucoside uptake when the cells possessed high phosphoglycerate transport activity. Half-maximal stimulation occurred at about 0.1 mM phosphoenolpyruvate. Approximately 100 times this concentration was required to half maximally stimulate methyl α-glucoside uptake when the phosphoglycerate transport system was not induced (Fig. 1).

A variety of energy sources were tested for ability to stimulate methyl α-glucoside uptake (Table 1). ATP, D-lactate, glycerol, and several other compounds tested were without effect. In contrast, phosphoenolpyruvate, 3-phosphoglycerate, and 2-phosphoglycerate were nearly equally effective in stimulating uptake, even at low concentrations. Presumably the cells were capable of metabolizing 2- and 3-phosphoglycerates to phosphoenolpyruvate. This possibility was substantiated by using potassium fluoride to inhibit enolase activity. Table 1 shows that potassium fluoride reduced endogenous methyl α-glucoside uptake considerably, and in the presence of this salt neither 2- nor 3-phosphoglycerate stimulated methyl α-glucoside uptake appreciably. In contrast, low concentrations of phosphoenolpyruvate were almost as effective as in the absence of fluoride, and a 2 mM concentration of this phosphate donor stimulated maximally.

TABLE 1. *Dependence of [^{14}C]methyl α-glucoside uptake into energy-depleted Salmonella cells on exogenous energy sources*[a]

KF (40 mM)	Energy source	Methyl α-glucoside uptake (μmol/g of protein)
−		0.19
−	Na^+-ATP (10 mM)	0.17
−	Glycerol (20 mm)	0.22
−	Na^+-succinate (20 mM)	0.22
−	Li^+-D-lactate (20 mM)	0.24
−	Mannitol (10 mM)	0.23
−	Phosphoenolpyruvate (2 mM)	1.48
−	2-Phosphoglycerate (2 mM)	1.55
−	3-Phosphoglycerate (2 mM)	1.35
−	Phosphoenolpyruvate (0.1 mM)	0.91
−	2-Phosphoglycerate (0.1 mM)	0.87
−	3-Phosphoglycerate (0.1 mM)	0.66
+		0.07
+	Phosphoenolpyruvate (10 mM)	0.94
+	Phosphoenolpyruvate (0.1 mM)	0.51
+	2-Phosphoglycerate (10 mM)	0.14
+	3-Phosphoglycerate (10 mM)	0.09

[a] *glpT116* cells were grown as described in the legend to Fig. 1. Subsequently, cells were depleted of energy, and transport experiments were conducted in the presence of the compounds indicated, as described in the text. The extracellular concentration of methyl α-glucoside was 40 μM. Uptake values are expressed as micromoles of methyl α-glucoside accumulated in 10 min at 28°C per gram of protein.

The intracellular radioactive products which accumulated during the phosphoenolpyruvate-stimulated uptake of [^{14}C]methyl α-glucoside were analyzed. Samples were analyzed on Biorad AG1-X2 (Cl^-) anion-exchange columns (3) and by paper chromatography with butanol-pyridine-water (10:3:3) as solvent (10) before and after treatment with alkaline phosphatase. Most of the radioactive material (70%) adhered to the anion exchange columns. Phosphatase treatment converted this material to a neutral compound which cochromatographed with authentic methyl α-glucoside. These results suggest

that the accumulated radioactivity was a mixture of methyl α-glucoside phosphate and methyl α-glucoside.

A study of glycerol uptake in the presence and absence of methyl α-glucoside revealed that this glucoside strongly inhibited glycerol uptake (Fig. 2) and that little or no preincubation of the inhibitor with the vesicles was necessary for maximal inhibition. Rates of glycerol uptake were constant with time for the duration of the experiment under a variety of conditions. The energy sources listed in Table 1 were tested for their effects on glycerol uptake into energy-depleted *glpT116* cells. Slight stimulation (less than 50%) into some preparations was observed with phosphoenolpyruvate, D-lactate, and ATP, but uptake into most preparations was not appreciably altered by these energy sources (data not shown).

Although the energy sources showed little or no stimulatory effect on glycerol uptake, phosphoenolpyruvate was capable of partially overcoming the inhibitory effect of methyl α-glucoside. At low concentrations of the glucose analog, inhibition was completely overcome by phosphoenolpyruvate, but this phosphate donor was only partially effective when higher inhibitor concentrations were employed (Fig. 2). If the cells had been induced for the synthesis of the phosphoglycerate transport system, 2 mM phosphoenolpyruvate was maximally effective in counteracting methyl α-glucoside inhibition. A 0.1 mM concentration had an intermediate effect (Fig. 2). In contrast, if the phosphoglycerate transport system had not been induced, much higher concentrations of phosphoenolpyruvate were required to counteract inhibition (data not shown).

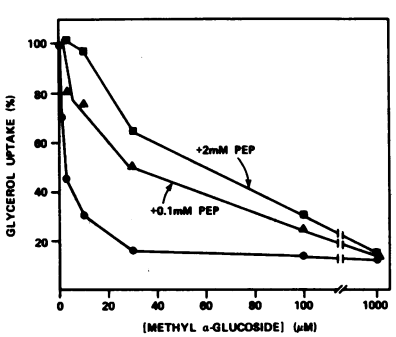

FIG. 2. *Effect of methyl α-glucoside and phosphoenolpyruvate (PEP) on the uptake of [^{14}C]glycerol into energy-depleted Salmonella cells. glpT116 cells were grown as described in the legend to Fig. 1 so that syntheses of the glycerol, glucose, and phosphoglycerate transport systems were induced. Cells were energy depleted, and uptake experiments were conducted by using standard conditions. Cells were preincubated with methyl α-glucoside and phosphoenolpyruvate for 10 min before initiation of the uptake experiment. Uptake of [^{14}C]glycerol (20 μM) was measured 5 and 10 min after the addition of the radioactive substrate. The uninhibited rate of [^{14}C]glycerol uptake in the absence of phosphoenolpyruvate was 36 nmol/min, and the uptake rates in the presence of 0.1 and 2 mM phosphoenolpyruvate were the same within 5%.*

A variety of energy sources were tested for ability to replace phosphoenolpyruvate in relieving methyl α-glucoside inhibition. In the absence of potassium fluoride, 2-phosphoglycerate and, to a lesser extent, 3-phosphoglycerate were effective in this regard (data not shown). The other energy sources tested (D-lactate, succinate, ATP) were without effect. The specificity for energy source was studied by using potassium fluoride (Table 2). In the presence of this salt, only phosphoenolpyruvate appreciably counteracted inhibition by methyl α-glucoside. Therefore, it appeared that phosphoenolpyruvate alone was effective and that the activity of the phosphoglycerates was due to their conversion to phosphoenolpyruvate.

To facilitate interpretation of the above results, inhibition of glycerol uptake was studied in strain LJ68, which carries a mutation which specifically renders glycerol uptake resistant to PTS-mediated regulation (15). Control experiments showed that this strain transported methyl α-glucoside and 3-phosphoglycerate at normal rates. The inhibition of glycerol uptake was studied by using energy-depleted cells of

TABLE 2. *Effects of various energy sources on inhibition of glycerol uptake by methyl α-glucoside in the presence of potassium fluoride*[a]

Addition	Glycerol uptake (nmol/min per g of protein)		% Inhibition
	Without methyl α-glucoside	With methyl α-glucoside	
None	101	47	53
2-Phosphoglycerate (2 mM)	96	51	47
3-Phosphoglycerate (2 mM)	94	48	49
Phosphoenolpyruvate (2 mM)	105	89	15
D-Lactate (20 mM)	120	61	49
ATP (10 mM)	112	51	54

[a] *glpT116* cells were grown and treated as described in the legend to Fig. 1. Uptake measurements were as described in the legend to Fig. 2, with the [^{14}C]glycerol and methyl α-glucoside concentrations at 40 μM and the potassium fluoride concentration at 20 mM.

strain LJ68. Uptake of glycerol in these cells was considerably less sensitive to inhibition by methyl α-glucoside as compared with the parental strain. Furthermore, phosphoenolpyruvate exerted only a marginal effect on this inhibition (data not shown). Therefore, it appears that inhibition of glycerol uptake in energy-depleted cells is controlled by the same factors which are responsible for these regulatory interactions in untreated cells (7, 9, 15).

Effects of exogenous energy on the regulation of permease function in untreated cells. Figure 3A shows the effect of phosphoenolpyruvate on inhibition of glycerol uptake by methyl α-glucoside in intact *glpT116* cells grown as described in the legend to Fig. 1. This phosphate donor partially relieved inhibition due to low concentrations of methyl α-glucoside. This behavior was similar to that demonstrated in energy-depleted, lysozyme-treated cells, except that, in contrast to the results obtained with the treated cells (Table 2), lactate, 3-phosphoglycerate, and other utilizable exogenous energy sources (in addition to phosphoenolpyruvate) effectively decreased sensitivity to inhibition and potassium fluoride (40 mM) had no effect. Moreover, the same energy sources enhanced methyl α-glucoside accumulation about twofold, showing that energy was a limiting factor for transport (data not shown). These results are in contrast to those obtained with the "leaky" enzyme I mutant *ptsI17* (13). This strain is more sensitive to the regulation of permease function by substrates of the PTS than is the parental strain (12). Phosphoenolpyruvate and other energy sources did not decrease sensitivity of glycerol uptake to inhibition by methyl α-glucoside in *ptsI17* cells.

These observations were extended to the melibiose and maltose transport systems (12). DL-Lactate, when added to wild-type or amylomaltase-negative cells grown in maltose plus glucose, decreased the sensitivity to maltose uptake to inhibition by methyl α-glucoside (Fig. 4A). Similar results were obtained when melibiose permease was studied (data not shown). Both wild-type and α-galactosidase-negative cells were less sensitive to inhibition of [^{3}H]melibiitol uptake by methyl α-glucoside in the presence of DL-lactate than in its absence, but this energy

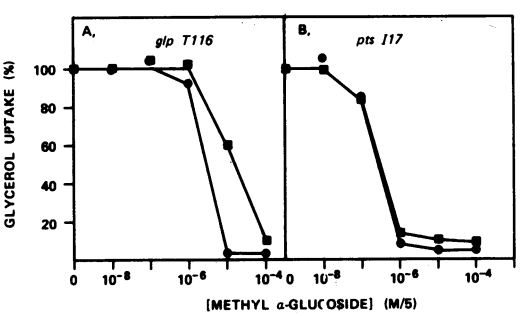

FIG. 3. *Energy dependence of the regulation of glycerol uptake in intact Salmonella cells. glpT116 cells (A) were grown as described in the legend to Fig. 1, and ptsI17 cells (B) were grown under the same conditions except that glucose was omitted from the growth medium. Cells were harvested during exponential growth, washed three times with medium 63, and resuspended in the same medium to a cell density of 0.2 mg (dry weight) per ml. Glycerol uptake rates were measured in the presence (■) or absence (●) of 4 mM phosphoenolpyruvate in the presence of the methyl α-glucoside concentrations indicated on the abscissa. Phosphoenolpyruvate did not stimulate glycerol uptake in either strain but enhanced methyl α-glucoside accumulation in glpT116 cells twofold. M/5 indicates one-fifth of the molar concentration indicated on the abscissa.*

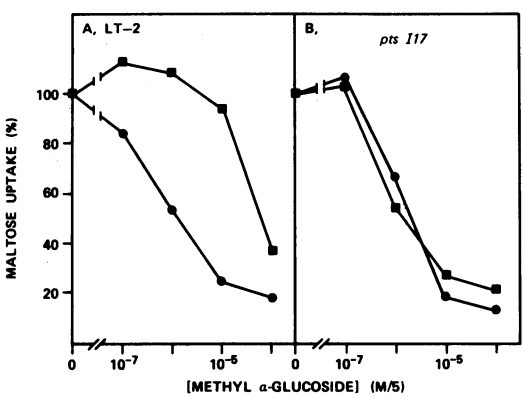

FIG. 4. *Effect of DL-lactate on inhibition of maltose uptake into wild-type and enzyme I-deficient cells of S. typhimurium. S. typhimurium strains LT2 (A) and ptsI17 (B) were grown at 37°C with aeration for 6 h in medium 63 containing 0.5% maltose as carbon and energy source before cells were harvested in the exponential phase of growth. At 2 h before harvesting, glucose (0.4%) was added to the LT-2 cell suspension in order to induce synthesis of glucose enzyme II. No addition was made to the ptsI17 cells because growth of this strain in the presence of glucose did not enhance sensitivity of maltose uptake to inhibition by methyl α-glucoside (8, 9). The harvested cells were washed three times with medium 63 and resuspended in the same salts medium (0.2 mg [dry weight] per ml). Uptake of [^{14}C]maltose was measured at various methyl α-glucoside concentrations (indicated on the abscissa) in the presence (■) or absence (●) of 0.5% DL-lactate. The uninhibited rates of [^{14}C]maltose uptake in the absence of lactate were 0.6 and 1.1 μmol/min per g (dry weight) for strains LT-2 and ptsI17, respectively. These rates were stimulated by lactate 42 and 26%, respectively. M/5 indicates a molar concentration which was one-fifth of that indicated on the abscissa.*

source did not appreciably affect regulation of melibiitol transport in strain *ptsI17*. These results suggest that the enzyme I deficiency in strain *ptsI17* changed the rate-limiting step for regulation of permease function.

DISCUSSION

In a previous report we showed that 3-phosphoglycerate, and presumably 2-phosphoglycerate and phosphoenolpyruvate, could be transported across the *Salmonella* membranes via an inducible transport system specific for these compounds (16). The process did not involve prior hydrolysis of the phosphate esters on the external side of the membranes. In the present study this transport system was used to supply energy-depleted cells with a continuous source of intracellular phosphoenolpyruvate. If synthesis of the phosphoglycerate transport system was induced to high levels, low concentrations of phosphoenolpyruvate markedly stimulated uptake of [^{14}C]methyl α-glucoside. In contrast, 100-fold-higher concentrations were required to stimulate methyl α-glucoside uptake to a comparable extent if the phosphoglycerate transport system was not induced. The results suggested that this effect was specific to phosphoenolpyruvate since other compounds were without effect when potassium fluoride was present to inhibit enolase. These observations confirm the work of Kaback (2) and provide evidence that only intracellular phosphoenolpyruvate can serve as an energy source for methyl α-glucoside accumulation. Since the phosphoglycerate transport system can be employed to energize uptake of sugar substrates of the PTS, it may also be of value for generating other sources of energy, such as ATP, in bacterial cells and membrane vesicles.

[^{14}C]glycerol uptake into *Salmonella* cells is subject to inhibition by methyl α-glucoside (7–9, 12). Since phosphoenolpyruvate stimulates uptake of the inhibiting sugar, it might be expected that this energy source would enhance sensitivity to inhibition. However, just the opposite was observed (Fig. 2). Intracellular phosphoenolpyruvate diminished the sensitivity of glycerol uptake to inhibition by methyl α-glucoside. Moreover, the same low concentration of phosphoenolpyruvate (10^{-4} M) which half maximally stimulated methyl α-glucoside uptake was effective in relieving inhibition.

Two interpretations of these results were considered. First, methyl α-glucoside might inhibit glycerol uptake by competing for the energy (ATP) required for the uptake and/or phosphorylation of glycerol. Second, phosphoenolpyruvate might serve as a phosphate donor for the phosphorylation of a cell constituent, presumably a protein, which is involved in regulating glycerol uptake (6–9). According to this second hypothesis, the phosphorylated form of the protein would be ineffective as an inhibitor, whereas the free form would be able to interact with the glycerol permease and regulate its activity. If this protein were phosphorylated in a reaction catalyzed by enzyme I and HPr, the observation that leaky enzyme I mutants are hypersensitive to regulation by sugar substrates of the PTS (1, 13) would be explained. In these mutants, phosphorylation of the protein would be a slow process (enzyme I is rate limiting), but dephosphorylation, which might result from transfer of the phosphoryl group from the protein to a sugar, should not depend on enzyme I activity and would be expected to occur rapidly. In view of the facts that (i) enzymes III of the PTS are phosphorylated (4, 18), (ii) these phosphoenzymes are of high energy and therefore presumably in equilibrium with one another (4, 7, 18), and (iii) the enzyme III^{glc} activity is apparently depressed in *crrA* mutants which are resistant to regulation (11), this possibility seems increasingly attractive.

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