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Control of phospho*enol*pyruvate-dependent phosphotransferase-mediated sugar transport in *Escherichia coli* by energization of the cell membrane

(protonmotive force/membrane vesicles/uncouplers/D-lactate oxidation)

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ABSTRACT **The phospho*enol*pyruvate-dependent phosphotransferase-mediated sugar transport in *Escherichia coli* is inhibited by the energized state of the membrane. This was shown in intact cells as well as in membrane vesicles. Relaxation of the proton gradient by uncouplers stimulated the uptake of sugars via the phosphotransferase system in aerobically cultured cells. No such effect was seen in anaerobic cells, apparently because the cell membrane of these cells is poorly energized. Energization by respiration of D-lactate or ascorbate inhibited the phosphotransferase uptake system in membrane vesicles. This inhibition was reversed by the addition of cyanide. Oxamate, a specific inhibitor of lactate dehydrogenase, prevented the inhibitory effect of D-lactate. Membrane vesicles prepared from a cytochrome-less mutant were not energized by D-lactate oxidation and the phosphotransferase uptake system was not inhibited.**

The intensive study of bacterial transport systems within the last decade has greatly enhanced our understanding of their molecular mechanism (for review see ref. 1). However, little effort has been devoted to the elucidation of the control of these transport systems. Many sugars are accumulated by the vectorial phosphotransferase system (PTS), which is genetically and biochemically well characterized (2). The uptake of PTS sugars simultaneously suppresses the uptake of non-PTS sugars either directly via inducer exclusion (3) or indirectly via catabolite repression (4). We understand the mechanisms that lead to the suppression of non-PTS sugar uptake; however, little is known about how the PTS itself is controlled.

Del Campo and coworkers (5) observed a correlation between oxygen consumption and the uptake of the PTS sugar methyl α-D-glucopyranoside (α-MeGlc) in wild-type and Ca^{2+},-Mg^{2+}-ATPase-deficient cells. They suggested that the energization of the cell membrane inhibits α-MeGlc accumulation. In intact cells the energized state of the membrane is coupled to many different reactions, such as various protonmotive force-driven processes. Therefore we elucidated the causal link between the energization of the membrane and the control of PTS, using membrane vesicles as a model system.

Our experiments demonstrate the control of PTS sugar uptake by the energized state of the membrane. PTS sugars are accumulated in membrane vesicles when exogenous phospho*enol*pyruvate (PEP) is added. Energization of the membrane via substrate oxidation suppresses this accumulation. Subsequent deenergization abolishes this suppression.

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MATERIALS AND METHODS

Materials. PEP, monosodium salt, was purchased from Boehringer Mannheim. The pH was adjusted to 6.6 with NaOH. D-(−)-Lactate, lithium salt, from Serva Heidelberg; L-(+)-ascorbic acid from Merck; and lysozyme (EC 3.2.1.17) from Sigma. Radioactive materials were obtained from Amersham.

Bacterial Strains. *Escherichia coli* B_{s-1} (6), strain BH 273 (*uncAB*$^-$) (7), and A 1004 c (8) were used. A 1004 c is a 5-aminolevulinic acid synthetase-deficient mutant of *E. coli* K-12 and was obtained from H. U. Schairer.

Growth Conditions. Cells were grown to mid-logarithmic phase (about $5 \cdot 10^8$ cells per ml) in M-9 minimal medium (42 mM Na_2HPO_4/22 mM KH_2PO_4/19 mM NH_4Cl/8.5 mM NaCl) supplemented with $MgSO_4$ (0.1 mM) and glucose (22 mM). A 1004 c cells were grown anaerobically in M-9 medium supplemented with $MgSO_4$ (0.1 mM), casamino acids (0.1%, wt/vol), methionine (20 μg/ml), isoleucine (20 μg/ml), and glucose (22 mM) as described (8). Cells were tested for their inability to grow in the absence of 5-aminolevulinic acid on agar plates containing succinate as the sole energy source.

Preparation of Vesicles. Vesicles were prepared by the method of Kaback (9); sodium was substituted for potassium; vesicles were stored at −70°C. The protein concentrations were determined by the method of Lowry *et al.* (10) with bovine serum albumin as standard.

Transport Experiments. *Sugar uptake.* Vesicles were suspended in a reaction medium such as to produce a final protein concentration of approximately 0.3 mg/ml. The final reaction mixture contained 50 mM sodium phosphate buffer at pH 6.6, 10 mM $MgSO_4$, 300 mM LiCl, and 100 mM PEP. NaF was added to produce a final concentration of 10 mM, where indicated, to abolish the endogenous transport activity (11). The reaction mixture was preincubated for 15 min at 37°C before the radioactively labeled sugar was added. Aliquots (100 μl) were taken at various times after the addition of the labeled compound, filtered through membrane filters (Sartorius, 0.45 μm pore diameter), and washed immediately with 5 ml of cold 0.5 M LiCl. The filters were dried and the radioactivity was determined. Total phosphorylation of α-MeGlc was controlled by the method described by Kaback (12).

Proline uptake. Because proline uptake is inhibited by sodium (11), vesicles were centrifuged, washed once with 50 mM

Abbreviations: PTS, phospho*enol*pyruvate-dependent hexose phosphotransferase system; PEP, phospho*enol*pyruvate; α-MeGlc, methyl α-D-glucopyranoside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, dinitrophenol; ATPase, Ca^{2+},Mg^{2+}-activated ATPase (EC 3.6.1.3).

potassium phosphate buffer, pH 6.6, containing 10 mM $MgSO_4$, and resuspended in this medium. The suspension was preincubated at 30°C for 15 min and D-lactate (20 mM) and radioactively labeled proline were added. All further procedures were performed as described above; filters were washed with 0.1 M LiCl.

Concentrations and specific activities of radioactive materials. L-[5-^{3}H]Proline (14 Ci/mmol), 50 μM (10 μl of 5 mM proline + 35.7 pmol of [^{3}H]proline per ml of suspension); methyl α-D-[*U*-^{14}C]glucopyranoside (79.0 mCi/mmol and 184 mCi/mmol, respectively), 2.75 μM; D-[*U*-^{14}C]glucose (248 mCi/mmol), 2.75 μM; D-[*U*-^{14}C]fructose (303 mCi/mmol), 2.75 μM; D-[*U*-^{14}C]mannose (3.0 mCi/mmol), 275 μM (this high concentration was necessary because of the low specific activity).

RESULTS

Uncouplers Enhance Uptake of α-MeGlc by *E. coli* Cells. α-MeGlc uptake was stimulated if the electrochemical proton gradient was abolished by uncouplers such as dinitrophenol (DNP) or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or reduced by poisoning the respiratory chain with cyanide (Fig. 1*A* and ref. 5). Little stimulation of α-MeGlc uptake by uncouplers was observed in anaerobic *E. coli* cells (Fig. 1*B*). The membranes do not appear to be optimally energized under anaerobic conditions. The anaerobic cells obtain energy only by glycolysis, and this inefficient mode of energy production apparently does not supply sufficient energy to the membrane. Low energization of the membrane was indicated by the fluorescence technique with 1-anilino-8-naphthalenesulfonate (data not shown). These anaerobic cells take up α-MeGlc at a high rate (38 pmol/min per mg of cells), which is comparable to the α-MeGlc uptake in CCCP-, DNP-, or cyanide-stimulated aerobic cells (36 pmol/min per mg of cells). Therefore, in anaerobic *E. coli*, α-MeGlc is taken up with close to maximal velocity and the rate cannot be stimulated by the addition of uncouplers. Further support for the involvement of membrane energy in the stimulation of α-MeGlc uptake is shown in Fig. 1*C* (and ref. 5). The *uncAB* mutant strain BH 273 (7), which was used in this experiment, was defective in the ATPase of oxidative phosphorylation. These cells charge their membranes with energy via the respiratory chain but they cannot utilize the membrane energy to synthesize ATP. Addition of the uncoupler CCCP stimulated the α-MeGlc uptake in these *uncAB*$^-$ cells as shown in Fig. 1*C*. Because the uncoupler interferes primarily with the membrane energy, its influence on α-MeGlc transport seems to be linked to the membrane energy.

D-Lactate and Cyanide Have Antagonistic Effects on α-MeGlc Transport in Membrane Vesicles. Membrane vesicles accumulated α-MeGlc for more than 60 min (Fig. 2). This α-MeGlc uptake was PEP dependent and no further energy source was required. The endogenous uptake activity was abolished by the addition of fluoride, which inhibits the endogenous PEP formation. The α-MeGlc accumulated by the vesicles was phosphorylated to about 90% (data not shown).

D-Lactate, a substrate for the respiratory chain, inhibited the PEP-dependent uptake of α-MeGlc (Fig. 2). Addition of cyanide, an inhibitor of respiration, had little or no effect on this accumulation (Fig. 2). However, cyanide counteracted the D-lactate-induced suppression of α-MeGlc uptake. Fig. 3 shows that D-lactate oxidation induces energization of the vesicle membrane under our experimental conditions. D-Lactate was utilized by the membrane vesicles to supply the PMF for the proline transport (13). When cyanide was present at the same time, proline was not accumulated. Thus, D-lactate energizes

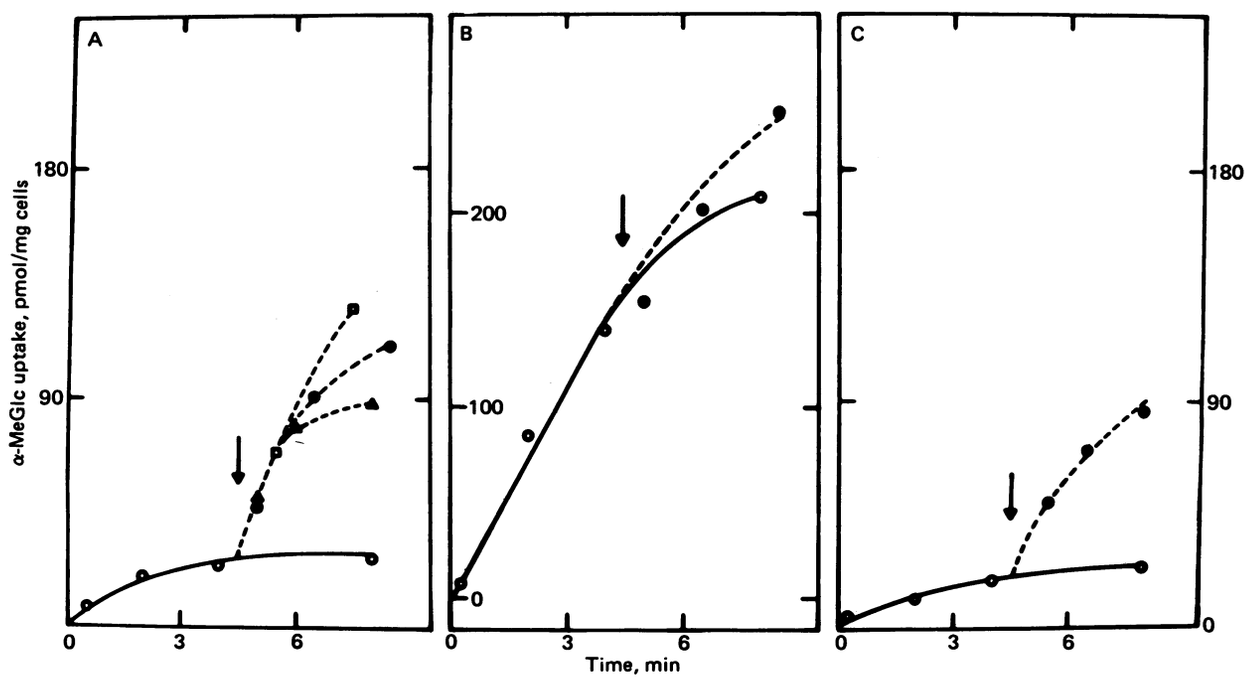

FIG. 1. Effect of uncouplers on α-MeGlc uptake in aerobic and anaerobic *E. coli* B_{s-1} and strain BH 273 *uncAB*$^-$ cells. Cells ($4\cdot10^8$ cells per ml) were grown in M-9 medium with 0.5% glycerol at 30°C either aerobically with shaking or anaerobically under nitrogen flow. At time zero α-Me[^{14}C]Glc (184 mCi/mmol; 1 μM) was added. After 4.5 min cells were treated with CCCP [20 μM (●)], DNP [1 mM (△)], or potassium cyanide [2 mM (□)]; ○, control without treatment. Uptake was determined in 0.5-ml aliquots, which were collected by filtration through Millipore filters (0.45 μm pore size). The filters were washed twice with 5.0 ml of potassium phosphate buffer (20 mM, pH 7.0) and dried, and radioactivity was determined. Note that $1\cdot10^9$ cells = 1 mg. (*A*) B_{s-1}, aerobic; (*B*) B_{s-1}, anaerobic; (*C*) strain BH 273, aerobic.

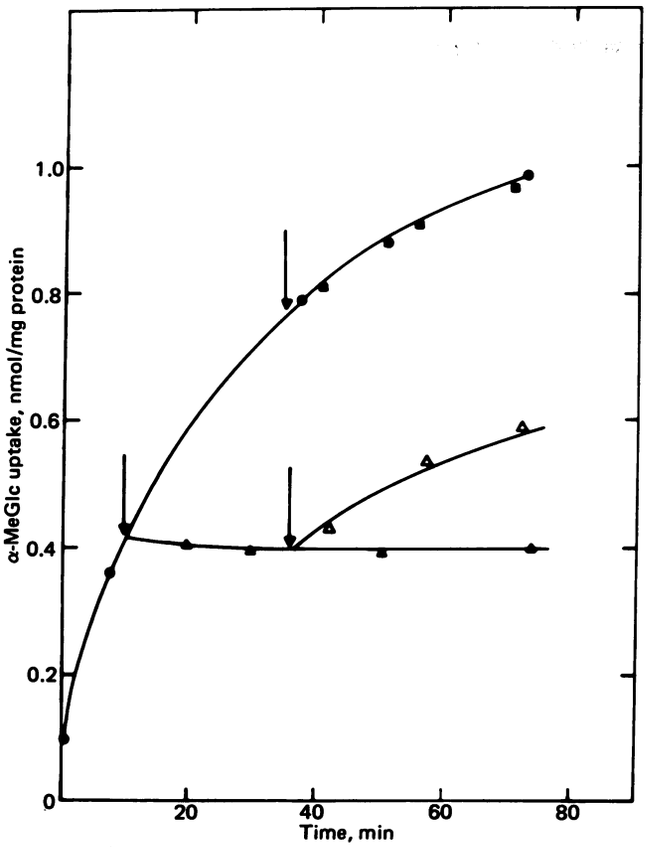

FIG. 2. Effect of D-lactate and cyanide on the accumulation of α-MeGlc in membrane vesicles of *E. coli* B_{s-1}. Vesicles were prepared and preincubated; the protein concentration was 0.15 mg/ml. α-Me[^{14}C]Glc (2.75 μM) was added at time zero and accumulation was assayed. At 10 min D-lactate (20 mM) was added. Uptake was followed either without further treatment (▲) or after addition of cyanide (3 mM) at 35 min (△). Control without addition of lactate (●) and in the presence of cyanide (■).

and cyanide deenergizes the vesicle membrane and parallel α-MeGlc uptake is reversibly inhibited. The inhibition of α-MeGlc uptake by D-lactate and the relief of this inhibition by cyanide indicate the regulatory effect of the energetic charge of the vesicle membrane.

Substrates of the Respiratory Chain Cause Inhibition of Accumulation of Various PTS Sugars. The oxidizable substrates D-lactate and ascorbate inhibited the PEP-dependent α-MeGlc uptake, whereas other substrates of intermediary metabolism such as pyruvate, acetate, or oxalacetate had no effect (data not shown). The specific suppression of the PTS was exerted only by substrates that delivered electrons to the respiratory chain. If the oxidation of D-lactate was prevented by the addition of oxamate, a specific inhibitor of D-lactate dehydrogenase (14), D-lactate did not affect the α-MeGlc uptake (Fig. 4).

The control of uptake by the membrane energy as elaborated for α-MeGlc uptake can be extended to various other PTS sugars (Fig. 5). Glucose, fructose, and mannose accumulations were also restricted by the supply of energy to the vesicle membrane by D-lactate.

An Intact Respiratory Chain Is Essential for Control of the PTS. Accumulation of α-MeGlc in a heme-deficient *E. coli* mutant that is unable to metabolize D-lactate due to the lack of cytochromes (8) did not respond to D-lactate (Fig. 6). Vesicles prepared from these cells accumulated α-MeGlc at a high rate. However, addition of D-lactate or ascorbate had little influence

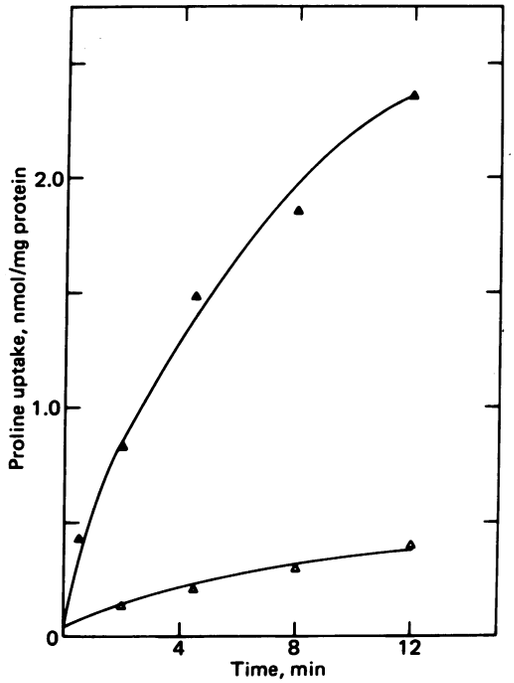

FIG. 3. Accumulation of proline in membrane vesicles prepared from *E. coli* B_{s-1}. The protein concentration was 0.42 mg/ml. The reaction was initiated by the addition of D-lactate (20 mM) and L-[^{3}H]proline (50 μM) at time zero and uptake was assayed (▲). △, Cyanide (10 mM) was added concomitant with lactate and proline.

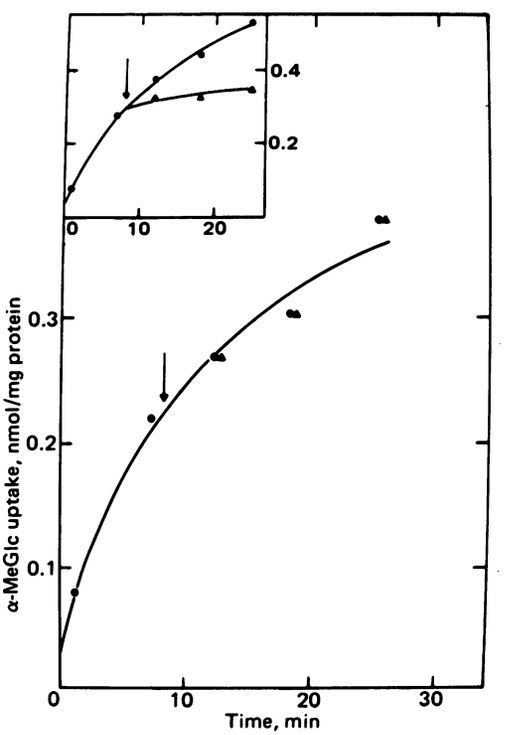

FIG. 4. Accumulation of α-MeGlc in the presence of oxamate and D-lactate in membrane vesicles of *E. coli* B_{s-1}. The experiment was performed as described in Fig. 2. The protein concentration was 0.50 mg/ml. At time zero α-Me[^{14}C]Glc (2.75 μM) and oxamate (30 mM) were added and at 8 min D-lactate (20 mM). The uptake was assayed as described. ●, Control; ▲, D-lactate added. (*Inset*) Control experiment without oxamate. By the addition of oxamate, α-MeGlc uptake is unspecifically inhibited by about 20%.

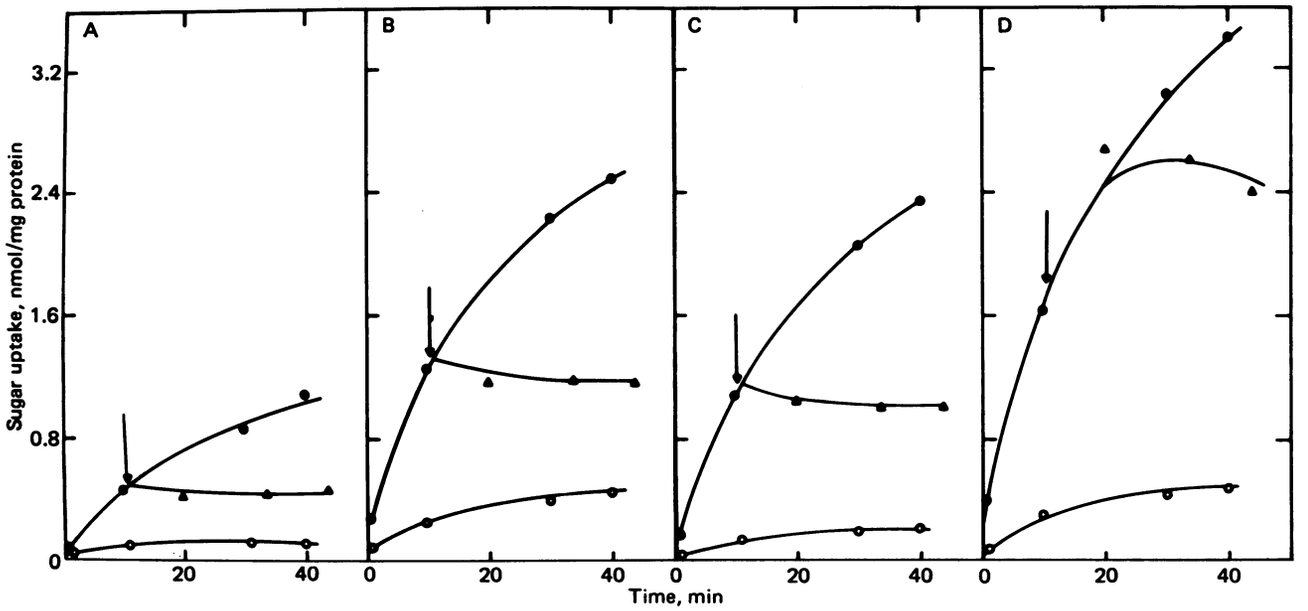

FIG. 5. Accumulation of various PTS sugars in membrane vesicles of *E. coli* B_{s-1}. The experiment was performed as described in Fig. 2. NaF (10 mM) was present throughout. The protein concentration was 0.24 mg/ml. The reaction was initiated by the addition of the radioactively labeled sugar and uptake was determined as described. ○, Control (− PEP); ●, + PEP; ▲, D-lactate was added at 15 min to the PEP-containing suspension. (*A*) α-Me[^{14}C]Glc (2.75 μM); (*B*) D-[^{14}C]glucose (2.75 μM); (*C*) D-[^{14}C]fructose (2.75 μM); (*D*) D-[^{14}C]mannose (275 μM). This high concentration was necessitated by the low specific activity of the commercially available [^{14}C]mannose. At this high concentration the change of the K_m due to energization is less apparent.

on the uptake. These membranes cannot be energized via the respiratory chain and therefore, as expected, PTS sugar uptake was not reduced.

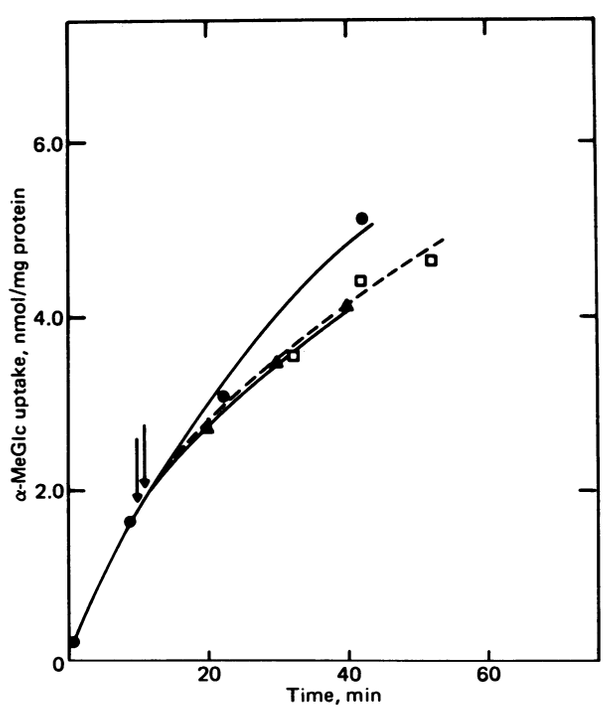

FIG. 6. Effect of D-lactate and ascorbate on the accumulation of α-MeGlc in membrane vesicles from a heme-deficient mutant (A 1004 c). α-MeGlc accumulation was measured as described in Fig. 2. The protein concentration was 0.21 mg/ml. D-Lactate (20 mM) was added at 10 min and ascorbate (20 mM) at 11 min. ▲, Uptake after lactate treatment; □, uptake after ascorbate treatment; ●, control.

DISCUSSION

In the present study we have shown that the energy content of *E. coli* cell membranes controls the uptake of PTS sugars. Treatment of *E. coli* cells with membrane-active colicins, e.g., Ia, enhances the accumulation of the PTS substrate α-MeGlc (15). In contrast, PMF- and ATP-driven transports are inhibited by these colicins (16). The molecular basis of the PTS stimulation in colicin-treated cells is not yet understood. A similar enhancement of α-MeGlc accumulation was observed in our laboratory after infection with phage T1 (17) or after addition of uncouplers of oxidative phosphorylation. Colicins (Ia, K, E1) (18) as well as T1 (19) and uncouplers relax the energized state of the membrane. These phenomena now can be understood easily on the basis of the PTS control by the energized state of the membrane. The membrane of intact cells is normally energized and therefore the PTS sugar accumulations are partially suppressed. The vesicle membrane is totally energy depleted due to the preparation procedure. These membrane vesicles accumulate PTS sugars at a high rate and are dependent upon added PEP. Uptake cannot be stimulated further by energy-dissipating agents. Supply of energy to the vesicle membrane via the respiratory chain inhibits PTS accumulation. However, this suppression is reversed by the addition of cyanide, which poisons the respiratory chain. Rapid PTS uptake is thereby restored.

The control of PTS sugar uptake by the energized state of the membrane is a clue to understanding the regulation of uptake of energy sources by the cell. A hierarchy in the uptake of various energy sources exists in microorganisms such as *E. coli*. The presence of glucose, for instance, suppresses the simultaneous accumulation of many other substrates that are sensitive to catabolite repression. This repression is mediated by the cyclic AMP levels, which are regulated by glucose or other PTS sugars (4). Thus, the problem of control of energy uptake in the presence of a PTS sugar is reduced to the regulation of the PTS sugar uptake itself. This uptake, however, is controlled by the energization of the membrane.

How does the membrane energy control the PTS uptake? We propose the following possibilities: The control can be exerted either directly by interaction with the components of the PTS or indirectly by regulation of the energy supply, the PEP. PEP is an anion that presumably crosses the vesicle membrane by passive diffusion. The generation of a membrane potential (inside negative) could tend to exclude the PEP from the cells or vesicles. In intact cells, however, PEP seems not to be limiting for the PTS (20). The direct control of the PTS can be exerted either at the soluble parts of the PTS [heat-stable protein (HPr), enzyme I] or at the membrane-bound part (enzyme II/III).

(*i*) Enzyme II, which is an integral membrane protein embedded in the phospholipid bilayer, is an essential constituent of the PTS. The physicochemical properties of this bilayer are altered by energization or deenergization of the membrane and thus the microenvironment of enzyme II is changed. The kinetic parameters of the enzyme could be altered by this change. Indeed, an alteration of the phospholipid bilayer by energization of the membrane can be visualized by fluorescence methods, NMR, or electron spin resonance (21). In addition, an interaction between enzyme II and the physicochemical state of the membrane is plausible. Thus, enzyme II is favored as the control element. This is supported by the decrease of the K_m for the α-MeGlc uptake as a consequence of deenergization of the membrane in intact cells (not shown here). The energization-dependent alteration of the K_m also explains the less pronounced effect of lactate on mannose uptake (Fig. 5*D*). For technical reasons, mannose was used at 100-fold higher concentration than the other sugars. A K_m alteration, of course, is less apparent if the substrate concentration is beyond the K_m.

(*ii*) An alternative model is based on a critical sensitivity of the PTS transport to changes in the internal cell milieu. The internal milieu is altered according to energization and deenergization of the membrane. Candidates to monitor the energized state of the membrane could be a discrete proton or ion gradient (e.g., potassium). For instance, the intracellular pH rises with energization and decreases after deenergization. Assuming a sharp pH sensitivity for the components of the PTS, the control via the energized state of the membrane is easily explained.

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