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Regulation of the Glucose Phosphotransferase System in *Brochothrix thermosphacta* by Membrane Energization

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Uptake of 2-deoxyglucose, α-methylglucopyranoside, and glucose into intact cells of *Brochothrix thermosphacta* (formerly *Microbacterium thermosphactum*, ATCC 11509) was stimulated by KCN or CCCP. The glucose analogs were recovered almost totally as the sugar phosphates. Membrane vesicles were isolated from protoplasts and shown to be right side out by freeze fracturing and by using ATPase as a marker for the cytoplasmic membrane surface. Uptake of glucose into vesicles was dependent on the presence of phosphoenolpyruvate. NADH oxidation, K^+-diffusion gradients, and externally directed lactate gradients (pH >7 initially) were used to generate transmembrane potentials across membrane vesicles. Above a threshold value of about −50 mV, uptake of glucose into membrane vesicles was reduced. Likewise, the maximum uptake of glucose and its two analogs into cells occurred when the protonmotive force was less than about −50 mV.

Brochothrix thermosphacta is a gram-positive, facultative anaerobe (34) which is associated with the spoilage of packaged meat products (12, 18). As part of a study of sugar metabolism in this organism, we prepared and characterized membrane vesicles and showed that glucose transport proceeds via a group translocation system that is represented by membrane energization.

Under anaerobic conditions, *B. thermosphacta* is comparable to the streptococci (11, 37) and carries out homolactic glucose fermentation. In *Streptococcus* spp., cytoplasmic P_i appears to play a major role in the regulation of glucose transport (17, 36, 38, 39). The high concentration of P_i in starved cells of *Streptococcus lactis* may inhibit pyruvate kinase activity (3, 4, 17, 39) and therefore provide high phosphoenolpyruvate (PEP) levels, which in turn keep the phosphotransferase system(s) in a poised or primed state.

Purified pyruvate kinase from *B. thermosphacta* (32) is also inhibited by P_i at physiological levels (<10 mM) even with saturating levels of fructose-1,6-biphosphate present. ^{31}P-nuclear magnetic resonance (NMR) analyses of intact cells during fermentation showed that in *Escherichia coli* cells with mixed acid fermentation (41) and in gram-positive organisms such as *Streptococcus faecalis* (30), *Staphylococcus aureus* (7), and *Streptococcus lactis* (39) during homolactic glucose metabolism, the level of cytoplasmic P_i is highly sensitive to adenylate charge and the intracellular accumulation of sugar phosphates.

Our preliminary studies (32) indicated that PEP levels in starved *B. thermosphacta* cultures are significantly higher than the values for cells isolated from logarithmic culture and also that a parallel relationship exists between cytoplasmic P_i and PEP concentrations. Thus, P_i may indirectly modulate sugar transport rates which are mediated by PEP-dependent group translocation. To date there are no reports on sugar transport in this organism, and therefore, to pursue the relationship between P_i and sugar metabolism, we set out to demonstrate that glucose uptake into cells and membrane vesicles is consistent with a group translocation system for which PEP can act as a phosphoryl donor.

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MATERIALS AND METHODS

Abbreviations. AcP, acetyl phosphate; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; CP, carbamyl phosphate; 2DG, 2-deoxyglucose; DMO, 5,5-dimethyl-2,4-oxazolidinedione; G6P, glucose-6-phosphate; αMeGlc, α-methylglucopyranoside; MES, 4-morpholineethanesulfonic acid; PEP, phosphoenolpyruvate; Δp, proton motive force; Ph_4P^+, tetraphenylphosphonium ion; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PTS, phosphotransferase system; Δψ, transmembrane electrical potential; ΔpH, transmembrane pH gradient.

Cell growth. *B. thermosphacta* ATCC 11509 was grown aerobically in batch culture in half-strength mineral salts medium (19) supplemented with 0.3% (wt/vol) Difco yeast extract–0.25% (wt/vol) glucose at 25°C.

Vesicle preparation. Washed cells (10 g [wet weight]) were suspended in 60 ml of 80 mM Tris-sulfate–10 mM $MgSO_4$ (pH 7) containing 300 mg of lysozyme and incubated at 30°C for 30 min. Saturated K_2SO_4 (15 ml) plus 150 ml of buffer containing 10 mg of both DNase and RNase were added, and the mixture was incubated with stirring for 20 min at 30°C. Potassium-EDTA was added to 4 mM, and the mixture was incubated for a further 10 min. $MgSO_4$ was added to 20 mM before centrifugation at 48,000 × *g* for 30 min at 4°C. The pellet was suspended in 50 ml of 40 mM Tris-sulfate (pH 7) and then centrifuged at 750 × *g* for 70 min at 4°C. The top two thirds of the supernatant was removed and recentrifuged at 48,000 × *g* for 30 min at 4°C. The pale yellow, vesicle pellet was resuspended in 40 mM Tris-sulfate–10 mM $MgSO_4$ (pH 7) to 10 to 20 mg of protein per ml, and 100-μl samples were frozen in liquid nitrogen or in Freon at liquid nitrogen temperature and then stored at −70°C.

ATPase assay. ATPase activity was measured by following the release of P_i in the presence of ATP. Membrane vesicles were diluted in 0.1 M Tris-sulfate–25 mM $MgSO_4$, 5 mM ATP (pH 7.5) at 20 to 100 μg of protein per ml. Samples were periodically removed, the reaction was terminated, and P_i was estimated colorimetrically as previously described (22). Triton was sometimes present in the reaction mix, as described in the text. "Stripped" vesicles were prepared by diluting the vesicles in 1 mM Tris-sulfate (pH 7) and washing by centrifugation at 48,000 × *g* for 10 min at 4°C. The

washing was repeated, and the pellet was suspended in the original volume with 1 mM Tris-sulfate buffer (pH 7). A unit of ATPase activity is equivalent to 1 μmol of P_i released per min.

Δψ estimation. Vesicle samples (10 μl) were diluted to 0.5 ml with 50 mM potassium phosphate–2 mM $MgSO_4$(pH 7.0) buffer. Glucose and glucose analog and in some cases CCCP or KCN were added as indicated in the text. Tetra[^{3}H]phenylphosphonium bromide (Ph_4P^+Br, 2.27 Ci/mM; 400 μM) was added to a final concentration of 20 μM. Samples (100 μl) were withdrawn, diluted with 2 ml of 0.5 M LiCl, collected on cellulose acetate filters and washed rapidly with 2 ml of 0.5 M LiCl. Dried filters were counted for radioactivity with Aquasol as the scintillant. Δψ was calculated by substitution of the extravesicular and intravesicular Ph_4P^+ concentrations into the Nernst equation. To estimate nonspecific Ph_4P^+ binding to membranes, vesicle samples (no additions) were preincubated (5 min) in cold Ph_4P^+ (0 to 50 μM), pelleted, and suspended in 20 μM Ph_4P^+ (2.27 Ci/mM). After 10 min the vesicles were recovered on filters and counted. The retained radioactivity decreased exponentially with increasing Ph_4P^+ concentrations in the preincubation and plateaued around 15 μM. The decrease in counts per minute due to preincubation with cold 20 μm Ph_4P^+ as compared to samples not preincubated, was subtracted from the observed counts in Δψ estimations. The confidence levels for Δψ values are ±8%. Δψ in intact cells was measured similarly, with the exception that 10 mM K-EDTA was included in the suspension buffer. The final cell density was 1 mg (dry weight) per ml. The intracellular volume was determined by the method of Rottenberg (27) and was 3.4 μl/mg of membrane protein. For intact cells, it was 1.6 μl/mg of cells (dry weight).

ΔpH estimation. The intracellular pH was calculated from the distribution of [2-^{14}C]DMO between the intracellular water space and the medium (27). The cell density was 1 mg (dry weight) per ml, and [2-^{14}C]DMO (11 Ci/mol) was present at 200 μM in the suspension buffer, 50 mM potassium phosphate–2 mM $MgSO_4$ (pH 7).

PEP-dependent sugar uptake into vesicles. Vesicles were thawed rapidly, adjusted to 200 μg of protein per ml in 300 mM LiCl–100 mM K-PEP–10 mM $MgSO_4$–50mM potassium phosphate (pH 7) and incubated at 23°C for 10 min. D-[^{14}C] glucose (50 Ci/mol), 2-deoxy-D-[1-^{3}H]glucose (25 Ci/mol), or α[U-^{14}C]MeGlc (50 Ci/mol) were added to final concentrations as specified in the text. CCCP or valinomycin were added in ethanolic solutions to 20 μM (final ethanol concentration <1%) and KCN when present was at 2 mM. Samples from the reaction mixture were rapidly filtered with cellulose mixed ester filters, and the filters were counted as previously described.

Sugar uptake into cells. Cells were suspended in 50 mM potassium phosphate–10 mM $MgSO_4$ (pH 7) containing 300 mM LiCl. D-[^{14}C] glucose (100 μM, 20 Ci/mol), α-[^{14}C]MeGlc (80 μM; 50 Ci/mol), or [^{3}H]2DG (50 μM, 25 Ci/mol) was added at zero time, and samples were filtered and counted as described above.

Recovery of sugar phosphates. Samples (500 μl) of the uptake reaction mixture were diluted with 10 ml of boiling water and incubated at 100°C for 5 min. Debris was removed by centrifugation, and the supernatant was loaded onto Dowex-1-Cl^-. Sugar phosphates were eluted with a solution of ammonium bicarbonate. Fractions were monitored for radioactivity.

Assay for glucose phosphorylation activity. Cells (1 mg/ml) were permeabilized by shaking vigorously in 0.1% toluene-acetone (0.01:0.09%) for 1 min at room temperature. G6P formation was measured by following NADPH absorbance at 340 nm in a reaction mixture that contained 6 U of G6P dehydrogenase, 1 mM NADP, 5 mM glucose, and 0.1 to 0.2 mg (dry weight) of decryptified cells in 50 mM potassium phosphate–5 mM $MgSO_4$ (pH 7). Potential phosphoryl donors were added as follows: 2 mM ATP, 5 mM CP, 5 mM AcP, or up to 10 mM PEP. In some cases 6 U of phosphoglucomutase was present, which enabled glucose-1-phosphate formation to be followed.

Lactate-efflux effects on Ph_4P^+ uptake and sugar transport into vesicles. Vesicles were thawed, concentrated with an Eppendorf centrifuge and suspended at 20 to 30 mg of protein per ml in 20 to 80 mM L-lactate–100 mM K-PEP–40 mM Tris–10 mM $MgSO_4$ (pH 7). This suspension was incubated for 30 min on ice before dilution of 2 μl of suspension in 200 μl of 40 mM Tris hydrochloride–10 mM $MgSO_4$ (pH 7) containing 6.8 μM [^{3}H]Ph_4P^+ (2.27 Ci/mmol) or 40 μM [^{14}C]glucose (250 Ci/mol). In control experiments this diluent buffer was supplemented with the same concentration of lactate as that used to load the vesicles. Uptake was arrested by rapid dilution of samples with 2 ml of 0.1 M LiCl, followed by filtration and counting.

Potassium efflux-induced inhibition of glucose uptake into vesicles. Concentrated vesicle suspensions (30 mg of protein per ml) in 40 mM Tris–10 mM $MgSO_4$ (pH 7) were incubated with 100 mM K-PEP and valinomycin (2 nmol/mg of protein) present on ice for 30 min. Samples (2 μl) of this suspension were diluted into 200 μl of either 100 mM potassium phosphate–10 mM $MgCl_2$ (pH 7) or 100 mM choline phosphate–10 mM $MgCl_2$ (pH 7) containing 40 μM [^{14}C]-glucose (250

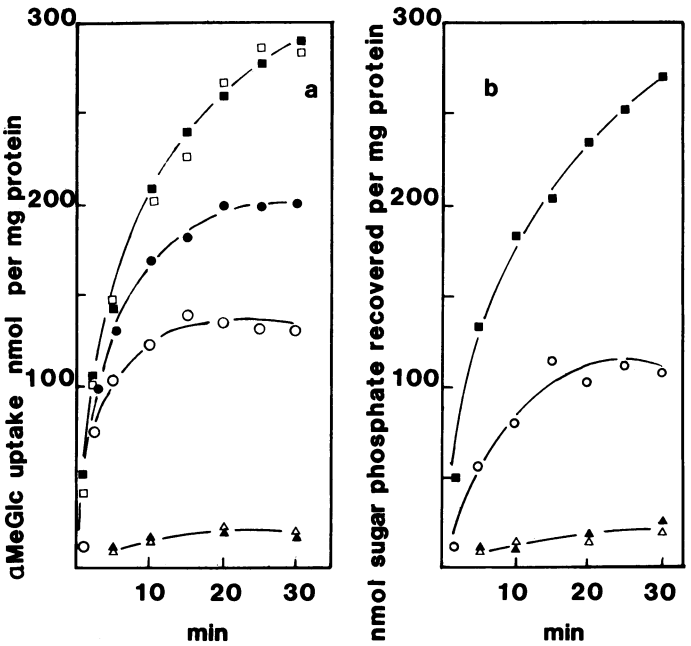

FIG. 1. Kinetics of αMeGlc uptake (a) and phosphorylation (b) by intact cells of *B. thermosphacta*. α-[^{14}C]-MeGlc (40 μM) was added to cells (1 mg [dry weight] per ml) at zero time (○); 10 mM KCN (■), 20 μM CCCP (●), 10 mM KCl plus 20 μM valinomycin (□), 0.2 mM chlorhexidine (▲) or 0.2 mM cyclohexidine plus 20 μM CCCP (△) additions were made at −5 min. Radioactivity was determined in cells retained by filtering and in the sugar phosphate fraction prepared by Dowex-1 fractionation of cell extracts.

Ci/mol) or 2 μM [^{3}H]Ph_4P^+ (2.27 Ci/mol). Uptake was stopped by rapid dilution of 30-μl samples with 2 ml of 0.1 M LiCl and assayed as described above.

Preparation of crude extracts of *B. thermosphacta*. An equal volume of packed cells and glass beads (0.5 mM diameter) in 2 volumes of 100 mM Tris sulfate (pH 7) were circulated in a Braun homogenizer at 0°C. A fraction from the supernatant obtained at 10,000 × *g* was prepared.

Electron microscopy. Cells and membrane vesicles were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 h at 4°C. Material for freeze fracturing was washed and suspended in 10% glycerol in cacodylate buffer overnight. Freeze fracturing was carried out with a type II device as previously described by Bullivant and Ames (2). Material for thin sectioning was washed, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Spurrs low viscosity embedding medium. Thin sections were stained with uranyl acetate and lead citrate and were viewed with a Philips EM 400 microscope.

Enzyme assays. Enolase (EC 4.1.2.13), L-lactate dehydrogenase (EC 1.1.1.27), and aldolase (EC 4.1.2.13) were assayed as described previously (20). NADH dehydrogenase (EC 1.6.99.3) was measured spectrophotometrically under aerobic conditions at 600 nm in 50 mM potassium phosphate–10 mM $MgSO_4$ (pH 6.6) with 0.1 mM dichlorophenolindophenol and 2 mM NADH present (ε dichlorophenolindophenol = 18.8 mM^{-1} cm^{-1}). D-lactate dehydrogenase (EC 1.1.1.78) was assayed similarly except that 10 mM D-lactate was the substrate. Oxidation of NADH (NADH-oxidase) was also measured aerobically with 1 mM NADH present in the above buffer. One unit of enzyme activity is equivalent to the loss of 1 μmol of substrate per min. Protein was measured by the method of Lowry with bovine serum albumin as standard.

Materials. Radiolabeled chemicals were obtained from Amersham Chemical Co. $Ph_4P^+Br^-$ was synthesized in our laboratory (42). CCCP and valinomycin were purchased from Boehringer and potassium phosphoenolpyruvate was from Sigma Chemical Co. Chlorhexidine was obtained from Abbot Laboratories. [1-^{13}C]glucose was obtained from Service des Molécules Marquées, France.

^{13}C-NMR of intact cells. Washed cells were suspended to 50 mg (dry weight) per ml in 100 mM PIPES–50 mM MES–10 mM potassium phosphate–5 mM $MgSO_4$–2 mM EDTA–200 mM KCl (pH 7.2). Spectra were obtained at 75.46 MHz on a Bruker CXP 300 spectrometer operating in the Fourier transform mode. Proton decoupled spectra of 2-ml samples in 10-mm diameter tubes were accumulated at 25°C in 2-min blocks using a 60° pulse and a 1-s repetition time. All peaks were referenced to tetramethylsilane using the α anomer of glucose as the internal reference at 92.9 ppm.

RESULTS

Transport of α-MeGlc and 2DG into intact cells. Assay of perchloric acid extracts of logarithmic-phase *B. thermosphacta* showed that the intracellular PEP concentration was about 1 mM (33). Uptake of labeled 2DG and α-MeGlc into intact washed cells was rapid (for example α-MeGlc; Fig. 1a). Specific uptake rates for these two sugars were comparable. The initial rate for glucose calculated from the uptake over a 2-min interval was slightly lower—about 75% of the 2DG value. The uptake of all sugars was markedly enhanced with CCCP (20 μM) or KCN (10 mM) present (Fig. 1a) but was significantly inhibited by 0.2 mM chlorhexidine (Fig. 1a). Chlorhexidine is an inhibitor of glucose specific PEP-phosphotransferase activity in some of the oral streptococci

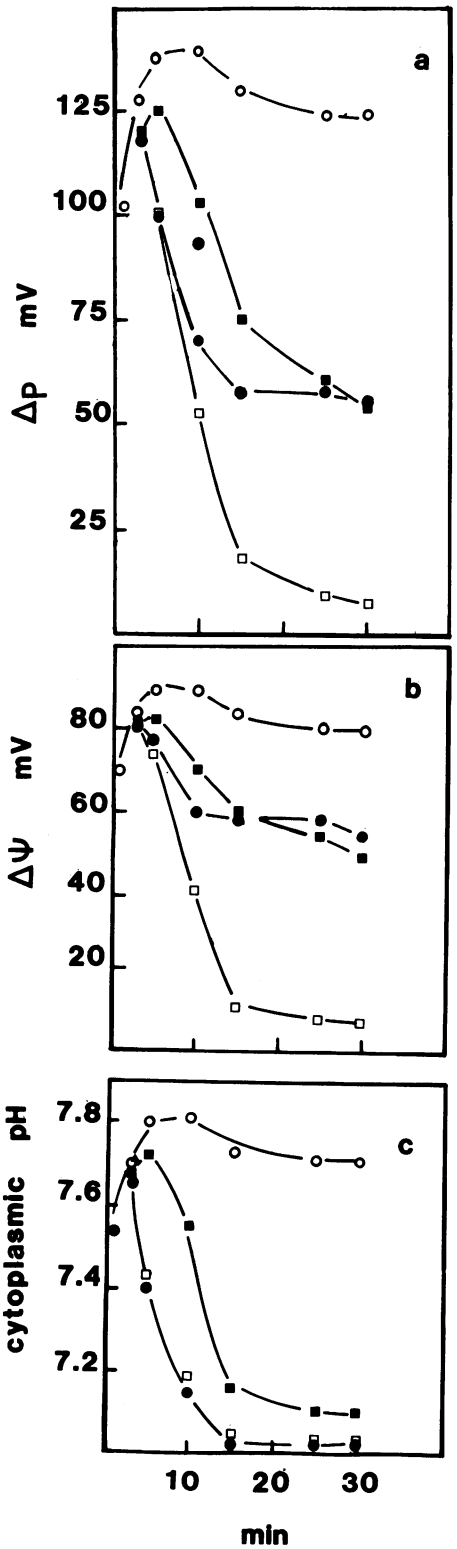

FIG. 2. Depolarization of the membrane potential of *B. thermosphacta* by ionophores and KCN. Logarithmic-phase cells were suspended in 50 mM potassium phosphate–10 mM $MgSO_4$–10 mM K-EDTA (pH 7) (1 mg [dry weight] per ml). [^{14}C]DMO (200 μM) and [^{3}H]Ph_4P^+ (20 μM) were added 5 min before the addition of 40 μM αMeGlc (zero time). At +2.5 min, KCN (2 mM) (■), CCCP (20 μM) (●) or valinomycin (20 μM) plus 10 mM KCN (□) were added. ○, Control with no additions.

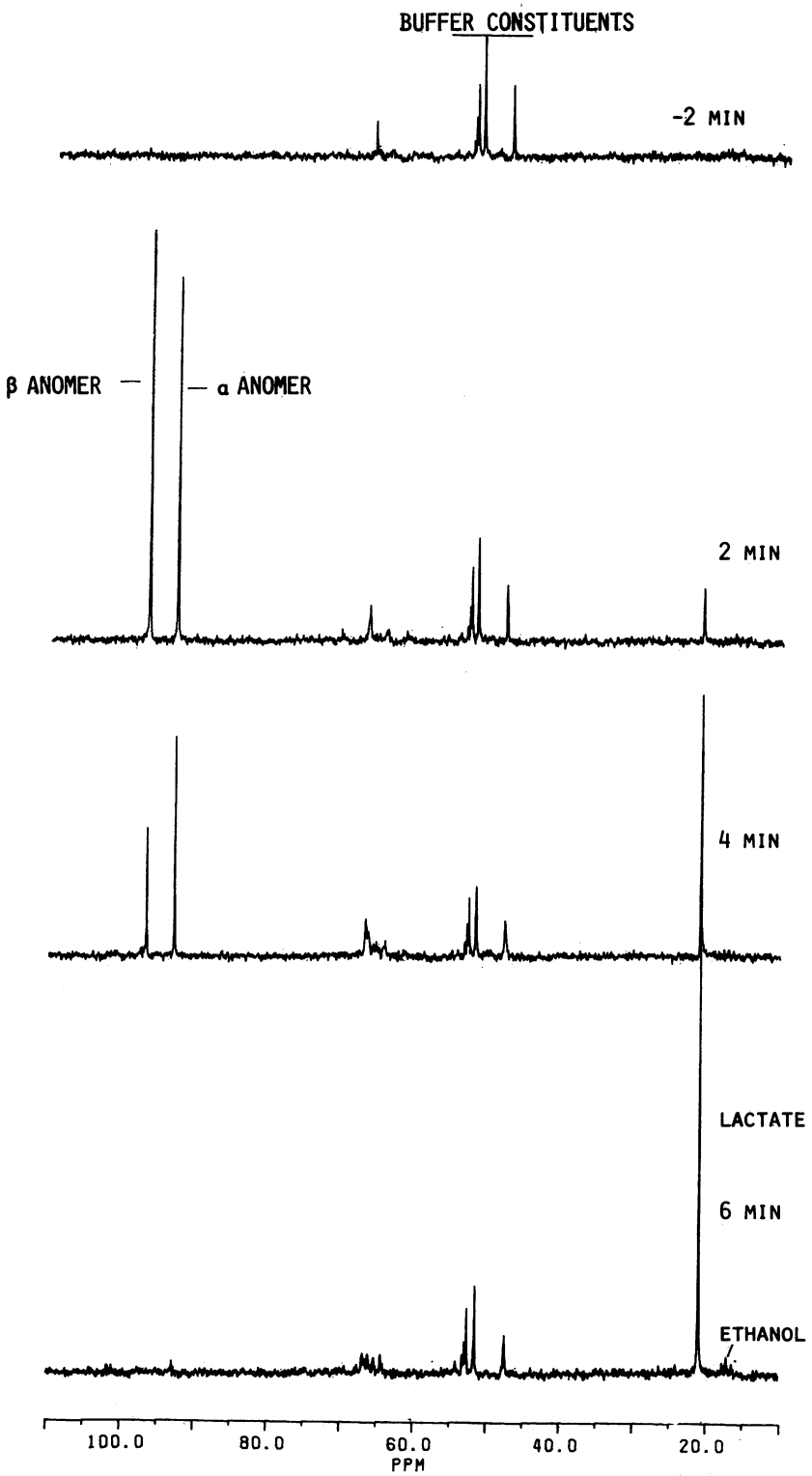

FIG. 3. ^{13}C-NMR of *B. thermosphacta* (50 mg [dry weight] per ml) before and after addition of 50 mM glucose at 25°C. Spectra were collected in 2-min blocks by using a 60° pulse and a 1-s repetition time. Times relative to glucose addition (0 min) are recorded on the spectra.

(9). No further reduction in uptake was observed when chlorhexidine and CCCP were both included. The majority of the incorporated label was recovered in the sugar phosphate fraction after Dowex-1 chromatography (Fig. 1b).

The stimulation of the glucose PTS in *E. coli* by membrane depolarization was first reported by Reider et al. (24). Therefore, we tested whether KCN and CCCP cause Δp to collapse in *B. thermosphacta* ($\Delta p = \Delta\psi - 2.3\ (RT/F)\ \Delta pH$; R, F, and T are the gas constant, Faraday's constant, and the temperature, respectively). The residual respiration rate of

washed suspensions of logarithmic-phase cells in the absence of added substrate was about 15 nmol of oxygen per min per mg protein, of which 90% was sensitive to 2 mM KCN inhibition. KCN addition collapsed ΔpH (Fig. 2) and decreased $\Delta\psi$ from −90 to −50 mV, within 30 min. Δp therefore decreased from −125 to −55 mV. Likewise, CCCP collapsed ΔpH and reduced $\Delta\psi$ to about −50 mV.

^{13}C-NMR. ^{13}C-NMR of intact cells metabolizing ^{13}C-enriched glucose (50 mM) showed that the β-anomer of glucose was metabolized faster than the α-form (Fig. 3). The preglucose spectrum showed signals that arise from the buffer constituents (Fig. 3). Addition of glucose resulted in resonances appearing at 20.8 nd 17.6 ppm, which have been assigned to lactate and ethanol, respectively. The 96.7 and 92.9 ppm signals are due to the β- and α-glucose anomers. The former signal is reduced more quickly than the α-anomer signal (Fig. 3). The small peak at 66.6 ppm was assigned to fructose-1,6-bisphosphate (40) and the peak at 64 ppm to mannitol-1-phosphate (assignments will be discussed elsewhere). In the presence of chlorhexidine, the same differential dismutation of the glucose anomers was observed, therefore showing that the substrate specificity of the carrier had not changed (spectra not shown). A similar preference for the β-anomer was reported for *E. coli* (40) and *Staphylococcus aureus* (7); we observed the same response with *Streptococcus lactis* and *Streptococcus faecalis* (unpublished data). In *E. coli* the PEP-phosphotransferase is the main if not the sole means of glucose transport (10, 23). Likewise, in *S. lactis* and *S. faecalis* there is no evidence of a second glucose carrier additional to the PTS (6, 17). However, in *CAC* mutants of *E. coli* which lack PTS activity (5), glucose uptake can occur when the galactose permease is induced. ^{13}C-NMR of these cells showed that α- and β-anomers are metabolized at equivalent rates (spectra not shown). In another series of NMR experiments, ^{13}C-enriched glucose was added to decryptified cells with either ATP (10 to 20 mM) or PEP (10 to 20 mM) present. With ATP, the decrease in the α- and β-anomer signals was equivalent; with PEP the β-anomer was preferentially metabolized in the same ratio as observed for untreated cells (spectra not presented). Thus, the preferential affinity for the β-form may be a marker for PTS activity. On this basis, glucose dismutation in *B. thermosphacta* is consistent with a PEP-phosphotransferase and the residual uptake of glucose in the presence of chlorhexidine is most likely due to incomplete inhibition of a PTS.

TABLE 1. Glucose phosphorylation activities in decryptified *B. thermosphacta*

Addition	Glucose phosphorylation activity (nmol of G6P formed per min/mg of cell protein)
PEP	54
PEP + ATP	95
PEP + ATP + 0.2 mM chlorhexidine	58
ATP	36
ATP + CP	35
ATP + CP + AcP	38
AcP	24
ATP + CP + AcP + 0.2 mM chlorhexidine	32
CP	8

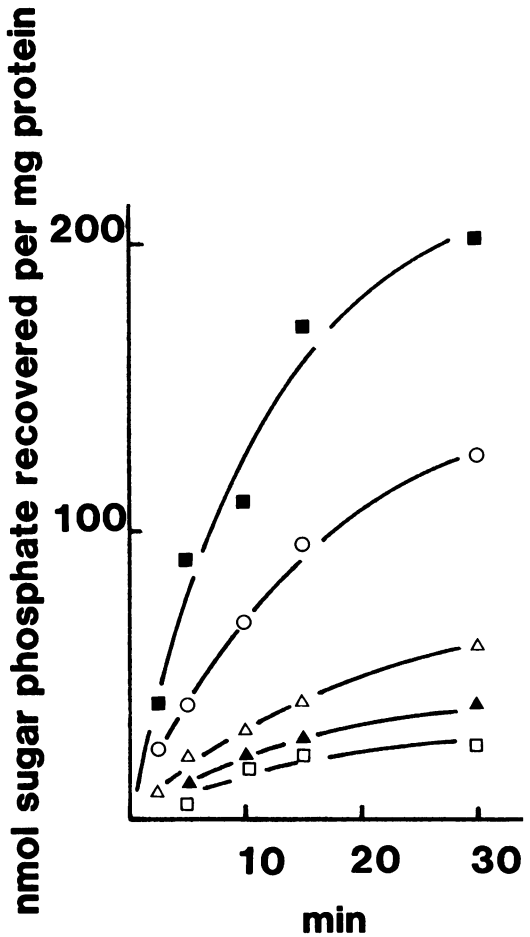

FIG. 4. Kinetics of phosphorylation of glucose by decryptified *B. thermosphacta*. The phosphoryl donors were added 5 min before addition of [^{14}C]glucose (100 μM) to permeabilized cells (1 mg [dry weight] per ml); 20 mM PEP (■), 10 mM ATP (○), 10 mM AcP (△), CP (▲), and no additions (□). Radioactivity was determined in the sugar-phosphate fraction after Dowex-1 chromatography of cell extracts.

Sugar transport into decryptified cells. The intracellular phosphorylation step was investigated by measuring the rate of phosphorylation of glucose in permeabilized cells in the presence of PEP, ATP, CP, and AcP. The rates were estimated by recovery of added glucose as sugar phosphate (Fig. 4) or by using a specific G6P dehydrogenase system (Table 1). By either method PEP was the best substrate, followed by ATP and AcP. There was also some phosphorylation observed when CP was added. The enzymatic assay monitored NADPH formation in the presence of added G6P dehydrogenase. There was no additional activity detected when phosphoglucomutase was added to the reaction mix, indicating that no glucose-1-phosphate was being formed. The PEP-dependent activity was also inhibited by chlorhexidine, whereas the phosphorylation with ATP or AcP present was not. The phosphorylation rates observed with ATP or PEP present were additive, showing that at least two enzyme systems are involved. However, the ATP-dependent rate was not enhanced with AcP present, probably because both are linked to the same enzyme. AcP could provide ATP for a glycokinase-mediated phosphory-

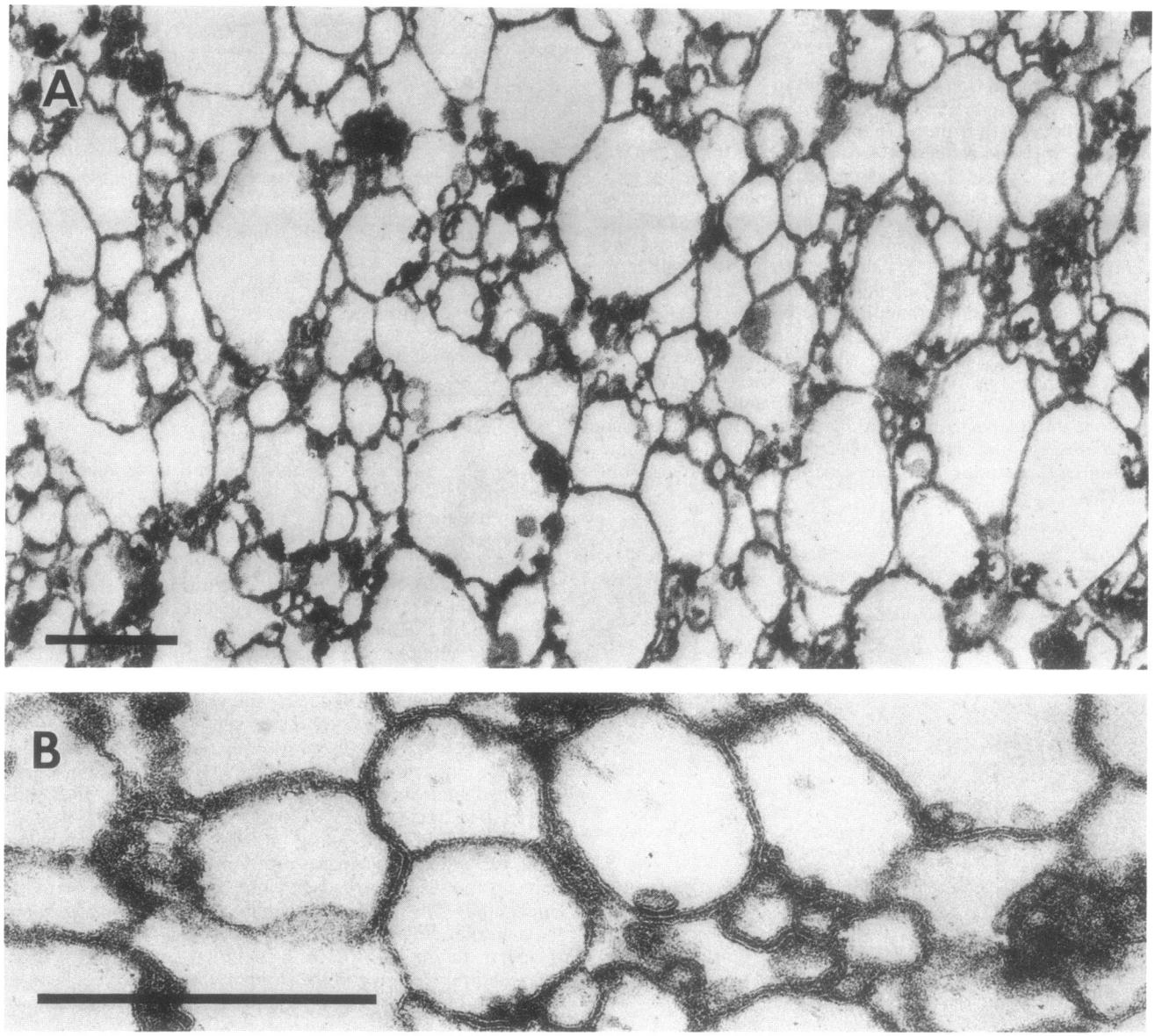

FIG. 5. Electron micrographs of thin sections of membrane vesicles of *B. thermosphacta*. Bars, 0.5 μm.

lation in view of the presence of acetate kinase in these cells. The *B. thermosphacta* enzyme will also accept CP as a phosphoryl donor (unpublished data). The K_m values for AcP and CP are comparable (0.3 mM), but the maximum rate with CP is only 10% of the velocity with saturating levels of AcP. The PEP-dependent phosphorylation rate by the enzymatic method was maximally 54 nmol/min per mg of protein, which is comparable to the initial velocity of αMeGlc or 2DG glucose uptake into intact cells (Fig. 1; 45 ± 10 nmol/min per mg of protein) and to the rate of phosphorylation of glucose in decryptified cells with PEP added (Fig. 4; 45 nmol/min per mg of protein).

Isolation of membrane vesicles. Membrane vesicles were prepared for transport studies by using a procedure which relies on a relatively high concentration of lysozyme (5 mg/ml) to produce protoplasts which are lysed by the addition of K_2SO_4 at high concentrations (20). About 100 mg of vesicle protein was obtained from 10 g (net weight) of packed cells. The internal volume of the vesicles was 3.4 μl/mg of membrane protein. Reduced-minus-oxidized difference spectra of membrane vesicles (dithionite reduced versus ferricyanide oxidized) indicated the presence of *b*-type and *a*-type cytochromes (data not shown). The membrane vesicles were almost devoid of cytoplasmic constituents as shown by the presence of less than 1% of the specific aldolase and endolase activity present in crude extracts (15 and 5 U/mg of protein, respectively).

The activities of the following enzymes were also determined: NADH dehydrogenase, L-lactate dehydrogenase, D-lactate dehydrogenase, fumarate reductase, succinate dehydrogenase, and malate dehydrogenase. Only the activities of NADH dehydrogenase (132 nmol of dichlorophenol-indophenol reduced per min/mg of protein) and D-lactate dehydrogenase (58 nmol of dichlorophenol-indophenol reduced per min/mg of protein) were significant, as the other enzyme activities were less than 4 nmol/min per mg of protein.

Electron microscopy of membrane vesicles. Electron micro-

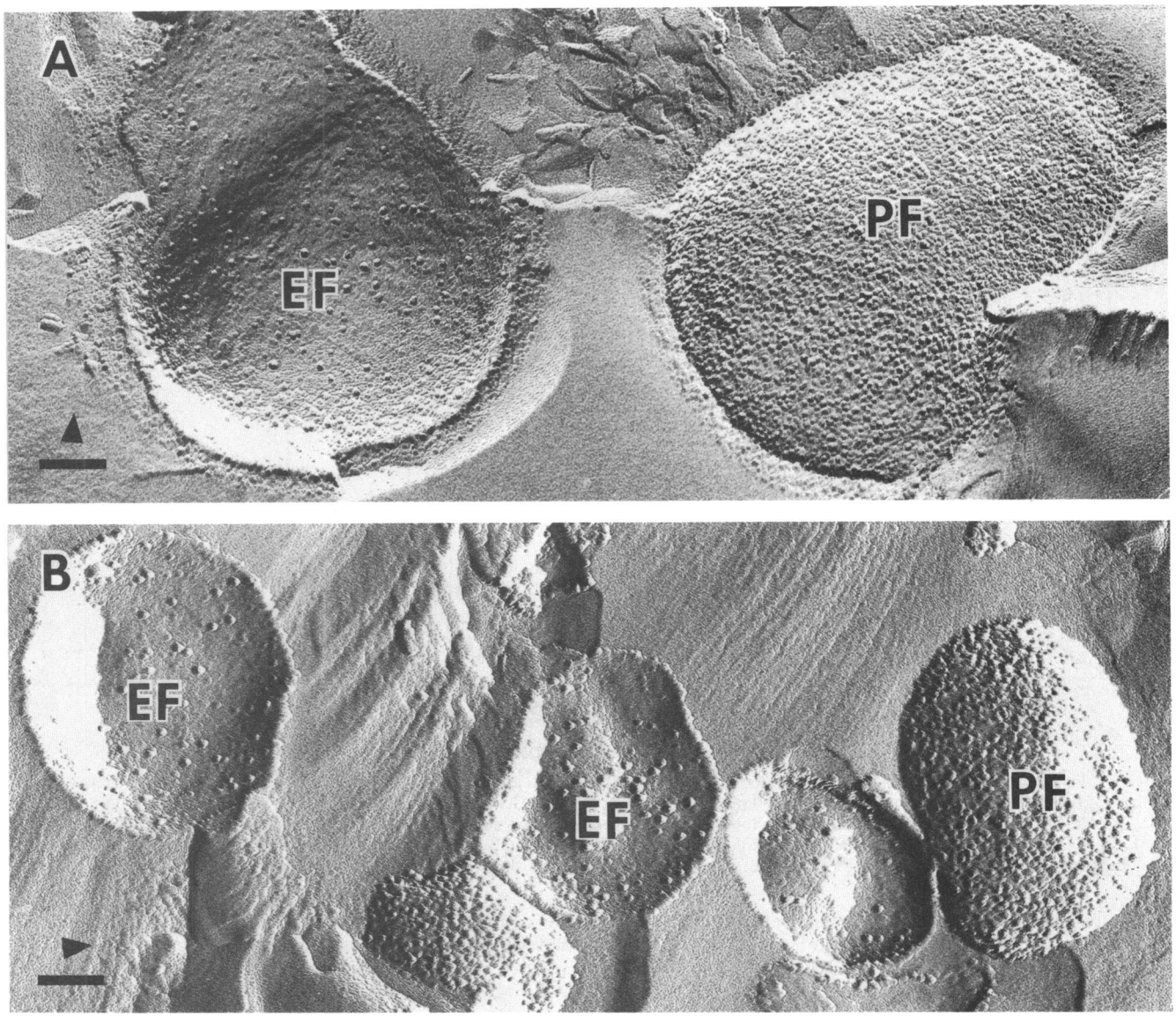

FIG. 6. Electron micrographs of freeze-fractures of (A) intact *B. thermosphacta* and (B) membrane vesicles. Both show protoplasmic fracture faces (PF) that are densely covered with particles and exoplasmic fracture faces (EF) that are sparsely covered. Bars, 0.1 μm. Arrows indicate the direction of shadowing.

graphs of thin sections of membrane vesicles showed closed sacs that possessed no recognizable internal structures (Fig. 5A and B). The maximum diameter of the vesicles was 0.65 μm, and the mean was 0.35 μm. Freeze fracturing gave an indication of the orientation of the membrane vesicles. Electron micrographs of replicas of freeze-fractured membrane vesicles and whole cells are compared in Fig. 6. As reported for other gram positive organisms (20), the convex protoplasmic fracture face (PF) of the outer membrane of cells is densely covered with particles (Fig. 6A) as compared to the sparsely covered concave exoplasmic fracture face (EF). A similar particle-density distribution was observed for membrane vesicles (Fig. 6B), which suggests that the orientation of the vesicle membranes is the same as that of intact cells.

Orientation of F_1F_0-ATPase in membrane vesicles. The orientation of the vesicle membranes was also established by using ATPase activity as a marker for the cytoplasmic surface. ATPase activity was measured as released P_i in the presence of ATP. Membrane vesicles maintained in 40 mM Tris-sulfate showed some activity (1.6 U/mg of protein). However Triton X-100 (0.2% [wt/vol]) enhanced activity by fivefold (8.2 U/mg of protein). When the membrane vesicles were stripped by washing in low-ionic-strength buffer (1 mM Tris-sulfate), the activity dropped from 1.6 to 0.5 U/mg of protein. The specific activity of stripped vesicles in the presence of Triton was lowered by almost the same amount (8.2 to 7.2 U/mg of protein). Therefore, we conclude that (i) the low-ionic wash releases ATPase from the external surface of the vesicles, (ii) there is no selective effect of the detergent on the ATPase as a consequence of orientation, and (iii) about 80% of the ATPase has the right-side-out orientation. These results support the freeze-fracture studies.

Sugar transport into membrane vesicles. The concentration of PEP in loaded vesicles (preincubation in 100 mM K-PEP

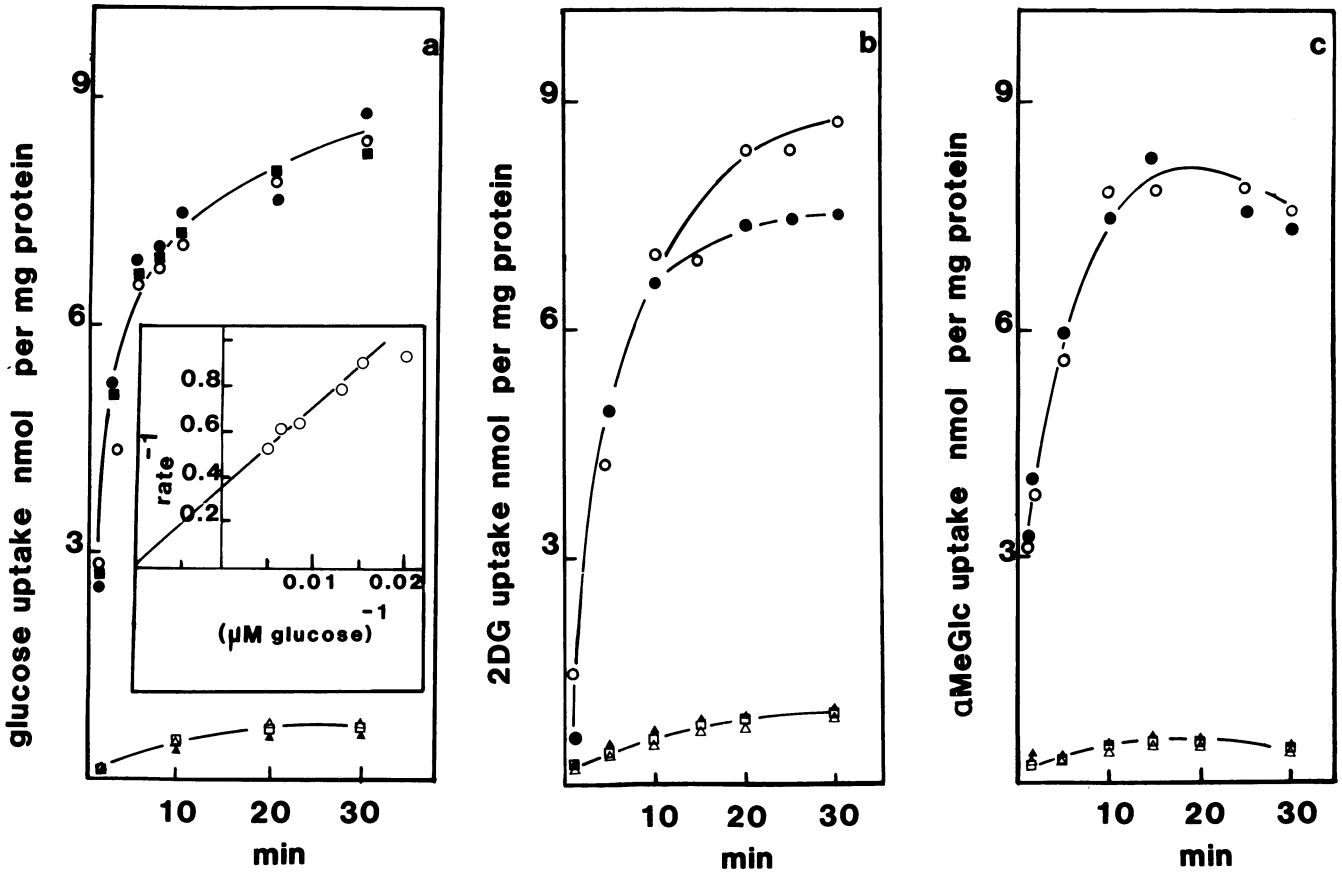

FIG. 7. Uptake of [^{14}C]glucose, [^{3}H]2 deoxyglucose, and α-[^{14}C]MeGlc (100 μM) into membrane vesicles from *B. thermosphacta*. Vesicles (200 μg of protein per ml) were preincubated in 300 mM LiCl, 10 mM $MgSO_4$, 50 mM potassium phosphate (pH 7) for 5 min at 0°C; PEP (100 mM) (○), 5 mM ATP (△), or 10 mM lithium-acetylphosphate (▲) were included during the incubation. The effects of 20 μM CCCP (●) and 2 mM KCN (■) on K-PEP-dependent glucose uptake are shown. CCCP or KCN in this instance were included in the preincubation. □, Uptake after preincubation without K-PEP present. The insert in panel a is a double reciprocal plot of uptake in the presence of K-PEP.

for 10 min) exceeded 50 mM. The membrane potential was less than −10 mV. These loaded vesicles supported active transport of glucose, αMeGlc and 2DG (Fig. 7). Exogenous ATP or AcP did not enhance uptake.

KCN (1 mM) and CCCP (20 μM) had no effect on the rate of uptake. The K_m and V_{max} for glucose transport were 100 μM and 3 nmol/min per mg of protein (Fig. 7a, insert). Phosphorylated sugar recovery accounted for 80 to 95% of the accumulated isotope.

Effect of membrane potential on glucose transport into vesicles. L-lactate, succinate, formate, or the artificial electron donor couple, ascorbate and phenazine methosulfate did not generate a membrane potential when added to vesicles under aerobic conditions. Both NADH and D-lactate produced a small potential which supported CCCP-sensitive uptake of labeled glycine (fivefold accumulation ratio; data not shown) but not glucose transport.

A larger membrane potential was formed by K^+-efflux from K^+-loaded vesicles (Fig. 8a). A transient $\Delta\psi$ of over −100 mV was produced by valinomycin-mediated efflux of K^+ (100 to 0.75 mM, outwardly directed gradient).

The potential did not support glucose uptake (Fig. 8b) but it did affect glucose transport into PEP-loaded vesicles (Fig. 8b). Vesicles were preincubated with 100 mM K-PEP with valinomycin present, before 100-fold dilution into buffer from which K^+ was omitted. Both the rate and absolute uptake of glucose into vesicles were decreased compared to the control in which the K^+ concentration in the diluent buffer matched the vesicle level (Fig. 8b).

When the diffusion potential was reduced to −60 mV by lowering the K^+ gradient (25 mM internal K^+-0 mM external), there was no significant effect on glucose transport (data not shown).

Lactate efflux-induced electrochemical gradient. Lactate efflux from membrane vesicles prepared from *E. coli* (35) and *Streptococcus cremoris* (20) creates an electrochemical gradient that will support active uptake of amino acids. In *Streptococcus faecalis*, lactate efflux can be coupled to ATP synthesis (31). Lactate-efflux from *B. thermosphacta* vesicles is also electrogenic at pH 7 and produces a significant membrane potential (Fig. 9a). CCCP addition prevented the formation of $\Delta\psi$. When concentrated suspensions of vesicles loaded with 75 mM potassium lactate and K-PEP were diluted into lactate-free medium, the rate of glucose uptake decreased as compared to the control (75 mM potassium lactate was present in the control diluent buffer). The activity of the glucose PTS is essentially constant from pH 6 to 7, but decreases on either side of this range (Fig. 10). Direct measurement of the external pH showed no significant change (<0.05 U) during lactate efflux as performed above. Inclusion of DMO (200 μM) to measure the intravesicular pH, showed that the internal value increased by less than 0.2 U. If the pH-activity profile is equally applicable to external and internal pH, a 20% decrease in PTS activity might be due

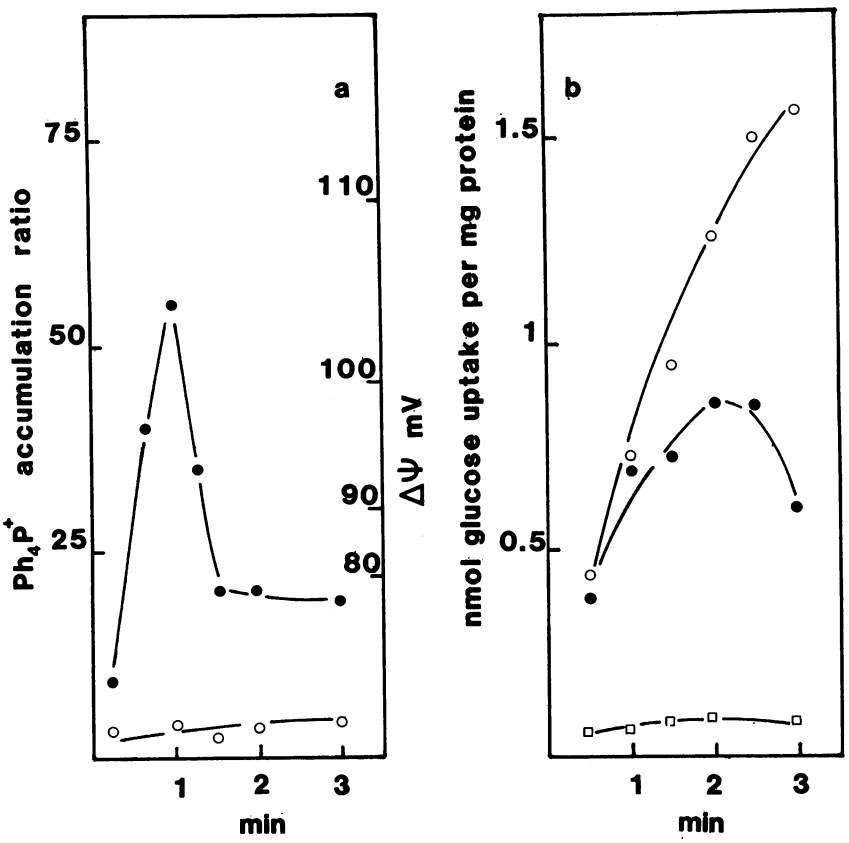

FIG. 8. (a) Time course of K^+ efflux-induced uptake of Ph_4P^+. Membrane vesicles (>30 mg of protein per ml) were incubated in 40 mM Tris–10 mM $MgSO_4$ (pH 7) for 30 min on ice with valinomycin (2 nmol/mg of protein) and 100 mM K-PEP present. The loaded vesicles were diluted 1:100 into either 100 mM choline phosphate–10 mM $MgSO_4$ (pH 7) (●) or 100 mM potassium phosphate–10 mM $MgSO_4$ buffer (pH 7) (○) in the presence of Ph_4P^+. (b) The effects of K^+ efflux-induced diffusion potential on glucose transport into membrane vesicles of *B. thermosphacta*. The K-PEP loaded vesicles were diluted into choline-phosphate buffer (●) or potassium phosphate buffer (○) with [^{14}C]glucose (40 μM) present; (□) vesicles incubated without K-PEP but with 100 mM potassium phosphate present were diluted into the choline-phosphate buffer with [^{14}C]glucose (40 μM) present.

to the increase in the internal pH. Both the decrease in the initial transport rate of glucose and the maximum accumulation rate during lactate efflux (Fig. 9) were greater than 20% (50 ± 15%, three experiments).

The data therefore support a causal relationship between membrane potential and inhibition of PEP-dependent glucose transport in *B. thermosphacta*.

DISCUSSION

Our aim was to show that glucose transport in *B. thermosphacta* can proceed via a PEP-phosphotransferase system. The presence of a PTS is supported by (i) the stimulation of the uptake of glucose analog into cells by membrane depolarization, (ii) the inhibition of this uptake by chlorhexidine, which specifically inactivates the glucose PTS in a number of related streptococci; (iii) the differential affinity of glucose uptake for the β-anomer of glucose; and (iv) the ability of PEP to act as phosphoryl donor for G6P formation in decryptified cells. In the latter, PEP was the most efficient substrate for phosphorylation of glucose, although ATP and AcP were also effective presumably because of the presence of glucokinase activity. A significant number of lactobacilli and streptococci have glucokinase activity (26) and although this may be suggestive of a second glucose uptake system, only in the case of *Streptococcus mutans* is there significant, albeit indirect evidence for a Δp-dependent glucose transport system (9, 14). In our studies, the residual glucose uptake in the presence of chlorhexidine was not reduced when ionophores were added and was not quantitatively different in terms of affinity for the β-glucose anomer as compared to control cells. ^{31}P-NMR and ^{13}C-NMR of *B. thermosphacta* have not detected significant changes in the glycolytic rate of cell suspensions in the presence of CCCP (20 μM) or N,N'-dicylohexylcarbodiimide (100 μM) (S. Singh and P. Rogers, Proceedings Australian Biochemistry Society Meeting, May 1985). We have no evidence for a second glucose transport system in these cells. We also note that *E. coli* which is dependent on phosphotransferase transport of glucose has multiple enzymes capable of phosphorylating glucose, including a constitutive glucokinase (5, 8).

Likewise, glucose uptake into membrane vesicles of *B. thermosphacta* was dependent on PEP, although the specific activity (3 nmol of glucose phosphorylated per min/mg of protein) is about 8% of the observed uptake rate in intact cells. Electron microscopy of thin sections of embedded vesicles showed largely empty sacs which were devoid of identifiable internal structure. The average size of the vesicles was 0.35 μm in diameter. ATPase activity (80%) was predominantly associated with the matrix site of the vesicles, and could only be accessed by disrupting the vesicles with detergent. Freeze fracturing also supports the conclu-

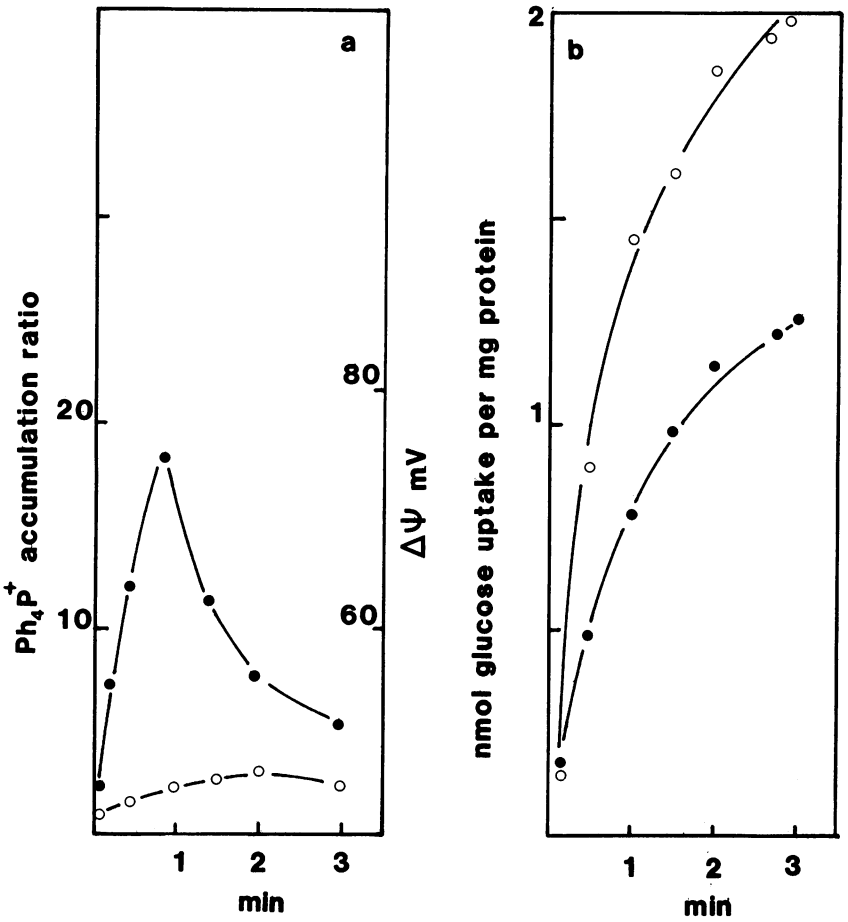

FIG. 9. (a) Lactate efflux-induced Ph_4P^+ uptake by membrane vesicles of *B. thermosphacta*. Membrane vesicles loaded with 75 mM potassium lactate, 100 mM K-PEP in 40 mM Tris–10 mM $MgSO_4$ (pH 7) were diluted into lactate-free medium (100 mM potassium phosphate, 10 mM $MgCl_2$ [pH 7]) containing Ph_4P^+ (●) or into the same buffer supplemented with 50 mM potassium lactate containing Ph_4P^+ (○). (b) The effects of lactate-efflux on the uptake of [^{14}C]glucose (80 μM) in the absence (○) and the presence (●) of an outwardly directed lactate gradient as in panel a above.

sion that the majority of the vesicles are right side out. NADH and D-lactate addition to vesicles produced a small membrane potential of about −30 mV, which supported limited uptake of labeled glycine (fivefold accumulation ratio). The presence of a redox-dependent H^+ pump was anticipated since KCN collapsed ΔpH and hence Δp by at least −80 mV in intact cells under aerobic conditions. The relatively low efficiency of the vesicle pump may be caused by limited coenzyme permeability or the NADH may only reach the respiratory sites at limited areas of membrane inversion. However, D-lactate which can be transported via the specific carrier also produced a Δψ of similar magnitude. The kinetics of Ph_4P^+ and glycine uptake were hyperbolic and Ph_4P^+ uptake during lactate efflux or K^+ exit from vesicles was comparable to the results of other workers using *E. coli* (35) and *Streptococcus cremoris* (20) vesicles. Therefore, although we cannot exclude the possibility that the low membrane potential generated with lactate and NADH is due to leaky membranes, we doubt that it is the major reason. Clearly, however, a low membrane potential did not affect glucose transport into vesicles. K^+-diffusion gradients were able to generate larger potentials. When Δψ was approximately −100 mV, there was a significant inhibition of glucose accumulation (Fig. 8) and a comparable effect on the initial rate of uptake. If the diffusion potential was reduced to approximately −50 mV, the effect on glucose uptake was very marginal. With intact cells, CCCP together with valinomycin effectively depolarized the membrane (residual Δp of −10 mV), and yet there was no significant difference in 2DG or αMeGlc uptake between these cells and cells with a Δψ of approximately −50 mV (plus KCN or CCCP). The data are consistent with a threshold potential above which inhibition of PTS activity is substantial. There have been proposals for a threshold potential to account for ATP synthesis in the streptococci by the reversal of the F_1F_0-ATPase due to the gating effect of K^+-diffusion potentials and ΔpH (15, 16). The sensitivity of the *E. coli* glucose PTS to membrane energization (24; for a review see reference 29) has been attributed to the effect of Δp on the equilibrium between vicinal dithiols and sulfydryl groups at the EII binding site, which then regulate the affinity of the carrier for the substrate (25).

We found that both K^+-diffusion potentials and H^+ efflux accompanying lactate exit from lactate-loaded vesicles were both effective in reducing PTS activity in vesicles. Electrogenic lactate-H^+ cotransport has been demonstrated in *E. coli* (35), *Streptococcus faecalis* (30, 31), and *Streptococcus cremoris* (20). The Ph_4P^+ accumulation driven by lactate efflux from vesicles of *B. thermosphacta* at pH 7 demonstrates that the lactate carrier in this organism can also be

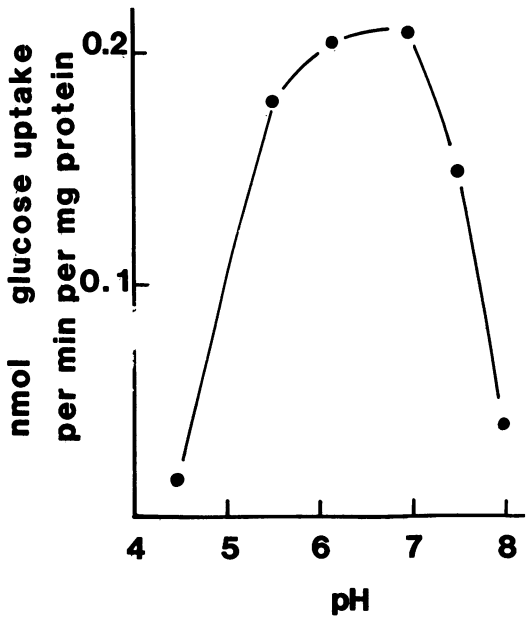

FIG. 10. The effect of pH on PEP-dependent uptake of glucose into membrane vesicles of *B. thermosphacta*. Conditions are the same as those described in the legend to Fig. 7 in the absence of KCN and CCCP.

electrogenic (28). The inhibition of PTS activity by lactate efflux from loaded vesicles may in part be attributable to a pH increase in the vesicle matrix, but the major effect appears to be due to $\Delta\psi$ formation.

We do not know whether the $\Delta\psi$-characteristics that we report for PTS activation are physiologically important. The washed cells we used were harvested from batch cultures after growth on rich medium, and contained a significant amount of ATP (3 mM, based on ^{31}P-NMR of cells). The membrane potential observed for these cells may not apply in other situations. Δp for example, can depend on aeration (13), growth rate (21), and pH (13); Δp will need to be quantified in *B. thermosphacta* under various growth conditions before this question is resolved.

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