Glucose 6-Phosphate Transport in Membrane Vesicles Isolated from *Escherichia coli*: Effect of Imposed Electrical Potential and pH Gradient†

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ABSTRACT: Imposition of a membrane potential ($\Delta\Psi$, interior negative) or a pH gradient (ΔpH, interior alkaline) across the membrane of *Escherichia coli* DF2000 leads to a marked, transient increase in glucose 6-phosphate transport that varies systematically with pH. Outwardly directed potassium diffusion gradients in the presence of valinomycin (i.e., generation of $\Delta\Psi$, interior negative) drive glucose 6-phosphate transport at pH 7.5 but much less effectively at pH 5.5, although the magnitudes of the transient $\Delta\Psi$ generated are comparable at both pH values. Similarly, imposition of $\Delta\Psi$ (interior negative) retards the rate of passive, carrier-mediated glucose 6-phosphate efflux down a concentration gradient at pH 7.5 but not at pH 5.5. In contrast, imposition of ΔpH (interior alkaline) by means of outwardly directed acetate diffusion gradients drives glucose 6-phosphate accumulation at pH 5.5 but is relatively ineffective at pH 7.5. The results are independent of the p*K* of glucose 6-phosphate and provide strong support for the argument that the glucose 6-phosphate porter catalyzes an electrically neutral reaction at acid pH and an electrogenic reaction at alkaline pH. In addition, they are entirely consistent with the hypothesis that the proton/glucose 6-phosphate stoichiometry increases at alkaline pH [Rottenberg, H. (1976) *FEBS Lett. 66*, 159; Ramos, S., & Kaback, H. R. (1977) *Biochemistry 16*, 854, 4271].

Cytoplasmic membrane vesicles isolated from *Escherichia coli* retain the same configuration as the membrane in the intact cell (Kaback, 1974a; Owen & Kaback, 1978, 1979a,b) as well as the capacity to catalyze active transport by a respiration-dependent mechanism that does not involve the generation of ATP or other high-energy phosphate intermediates (Kaback, 1974a). Moreover, it has become apparent over the past few years that chemiosmotic phenomena, as postulated by Mitchell (1961, 1966, 1968, 1973, 1979), play a central, obligatory role in the energetics and mechanism of active transport in this experimental system (Harold, 1976; Kaback, 1976; Konings & Boonstra, 1977; Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Padan et al., 1979; Reenstra et al., 1980).

In vesicles prepared from *E. coli* (Ramos et al., 1976; Ramos & Kaback, 1977a) and *Salmonella typhimurium* (Tokuda & Kaback, 1977), oxidation of D-lactate and reduced phenazine methosulfate (PMS)[1] leads to the generation of a proton electrochemical gradient across the membrane ($\Delta\bar{\mu}_{H^+}$, interior negative and akaline) that is composed of interconvertible electrical and chemical parameters according to the relationship

$$\Delta\bar{\mu}_{H^+} = \Delta\Psi - \frac{2.3RT}{F}\Delta\text{pH} \qquad (1)$$

where $\Delta\Psi$ represents the electrical potential across the membrane and ΔpH is the chemical difference in proton concentrations across the membrane ($2.3RT/F$ is equal to 58.8 mV at room temperature).

Transport of substrates such as lactose or glucose-6-P which are accumulated in relatively large amounts by the appropriate vesicles causes partial collapse of $\Delta\Psi$ (Schuldiner & Kaback, 1975) and/or ΔpH (Ramos & Kaback, 1977b), providing direct support for the argument that $\Delta\bar{\mu}_{H^+}$ drives solute accumulation via coupled movements with protons. Titration studies with the ionophores valinomycin and nigericin demonstrate that $\Delta\Psi$ and ΔpH can be varied reciprocally with little or no change in $\Delta\bar{\mu}_{H^+}$ (Ramos et al., 1976; Ramos & Kaback, 1977a,b; Tokuda & Kaback, 1977). Analogous studies with various solutes indicate that at pH 5.5 there are two classes of transport systems (Ramos & Kaback, 1977b): those driven primarily by $\Delta\bar{\mu}_{H^+}$ (lactose, proline, serine, glycine, tyrosine, glutamate, leucine, lysine, cysteine, and succinate) and those driven primarily by ΔpH (glucose-6-P, lactate, glucuronate, gluconate, inorganic phosphate,[2] and in *S. typhimurium* vesicles, citrate[2]). Strikingly, however, at pH 7.5 and above, all of these transport systems are driven by $\Delta\Psi$, which comprises the only component of $\Delta\bar{\mu}_{H^+}$. In addition, when the steady-state level of accumulation of transport substrates is examined as a function of pH, none of the profiles correspond to those described for $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\Psi$. Furthermore, at external pH values exceeding 5.5–6.0, $\Delta\bar{\mu}_{H^+}$ is insufficient apparently to account thermodynamically for the concentration gradients observed for most of the substrates if the stoichiometry between protons and substrates is 1:1 (Ramos & Kaback, 1977b). This finding and the observation that the accumulation of some organic acids and inorganic phosphate[2] is in equilibrium with $\Delta\Psi$ at alkaline pH, where ΔpH is absent, led to the suggestion that the stoichiometry between protons and transport substrates may vary as a function of external pH, and more direct evidence supporting this hypothesis was presented (Ramos & Kaback, 1977c).

Recent studies with intact cells (Zilberstein et al., 1979; Booth et al., 1979; Porter et al., 1979; Felle et al., 1980), however, have cast doubt on the contention that there is a discrepancy between the steady-state level of lactose accumulation and $\Delta\bar{\mu}_{H^+}$ at alkaline pH. Specifically, it has been demonstrated that in intact cells, as opposed to membrane vesicles, $\Delta\Psi$ increases markedly with pH in such a manner as to compensate for the decrease in ΔpH. Thus, $\Delta\bar{\mu}_{H^+}$ in intact cells does not decrease as drastically with increasing pH as observed in vesicles and the steady-state level of lactose accumulation at high pH can be accommodated without a

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[1] Abbreviations used: PMS, phenazine methosulfate; $\Delta\bar{\mu}_{H^+}$, electrochemical proton gradient; $\Delta\Psi$, electrical potential; ΔpH, pH gradient; P, phosphate; Dns^6-Gal, 6′-*N*-dansylaminohexyl 1-thio-β-D-galactopyranoside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPP^+, tetraphenylphosphonium.

[2] L. Patel and H. R. Kaback, unpublished information.

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change in proton/lactose stoichiometry. In addition, more direct studies of proton–lactose symport in deenergized cells are not indicative of a change in stoichiometry at high pH (Zilberstein et al., 1979; Booth et al., 1979).

On the other hand, numerous studies with both intact cells (Padan et al., 1976; Ogawa et al., 1978; Navon et al., 1977; Zilberstein et al., 1979; Felle et al., 1980) and isolated membrane vehicles (Ramos et al., 1976; Ramos & Kaback, 1977a,b; Tokuda & Kaback, 1977) demonstrate that ΔpH is absent at pH 7.5 and above. Thus, it is difficult to explain how the transport of certain organic acids and inorganic phosphate[2] can be coupled to ΔpH at acid pH and to $\Delta\Psi$ at alkaline pH without invoking a pH-dependent increase in proton/substrate stoichiometry (Rottenberg, 1976; Ramos & Kaback, 1977b,c), and direct measurements in intact cells supporting this notion have been presented recently (Taylor & Essenberg, 1979).

The experiments presented here describe the effects of artificially imposed electrical potentials and pH gradients on the transport of glucose-6-P as a function of pH and provide additional evidence for an increase in proton/glucose-6-P stoichiometry at alkaline pH.

Experimental Section

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* DF2000 (pgi^-ZWf^-), obtained from the *E. coli* Genetic Stock Center (No. CGSC 4873), is a mutant of *E. coli* K-12 that lacks phosphoglucose isomerase (pgi^-) and Zwishenferment (ZWf^-) activities (Fraenkel, 1968). Cells were grown on medium 63 (Cohen & Rickenberg, 1956) supplemented with thiamine, casamino acids, and disodium succinate (hexahydrate) at final concentrations of 2 mg/L, 0.35%, and 1.0%, respectively. For induction of the glucose-6-P transport system, disodium glucose-6-P was added to the cultures at a final concentration of 1.0 mM 90 min prior to harvesting. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was grown on minimal medium A (Davis & Mingioli, 1959) containing 1.0% disodium succinate (hexahydrate) or sodium glucuronate, as indicated. Membrane vesicles were prepared from *E. coli* DF2000 as described previously for *E. coli* GN-2 (Ramos & Kaback, 1977b) by using lysozyme and sucrose at final concentrations of 25 μg/mL and 30%, respectively, for the preparation of spheroplasts. Vesicles were prepared from *E. coli* ML 308-225 as described (Kaback, 1971; Short et al., 1975).

Transport Assays. Transport of glucose-6-P by membrane vesicles in the presence of ascorbate and PMS was determined as described (Kaback, 1974b). Other transport experiments were performed with concentrated vesicle suspensions equilibrated with buffered solutions of given composition and pH prepared according to Kaczorowski & Kaback (1979) and Kaczorowski et al. (1979). Thus, vesicles prepared in 100 mM potassium phosphate (pH 6.6) and stored in liquid nitrogen were thawed rapidly at 46 °C, diluted 30-fold with medium of desired composition, and equilibrated at room temperature for 30 min before centrifugation (45000*g* for 30 min). After the equilibration step was repeated twice, the final membrane pellet was resuspended in a minimal volume to give as concentrated a suspension as possible (usually 35–40 mg of protein per mL) and valinomycin was added to a final concentration of 2 nmol/mg of membrane protein.

Solute uptake in response to an artificially imposed $\Delta\Psi$ or ΔpH was measured as follows. An aliquot (2 μL) of vesicles that had been concentrated and equilibrated under a specified set of conditions was drawn into a 10-μL syringe (Hamilton No. 801) and then diluted rapidly 200-fold into a solution of given composition containing a radioactive solute that had been equilibrated previously to 25 °C. The suspension was quickly agitated and placed at 25 °C, and at given times the reaction was terminated by rapidly adding 2.0 mL of a solution containing 100 mM sodium or potassium phosphate (pH 5.5) and 100 mM lithium chloride and filtering the sample. The filter was washed once with the same salt solution and immediately removed from the filtration apparatus. Cellulose acetate filters (0.45 μm; Millipore Filter Corp.) were used for assays of [^{3}H]tetraphenylphosphonium ([^{3}H]TPP^+) uptake, while nitrocellulose filters (0.45 μm; Amincon Corp.) were employed for all other assays. Radioactivity was determined by liquid scintillation spectrometry using Bray's solution (National Diagnostics) with typical efficiencies of 80–90% for ^{14}C and 30–40% for ^{3}H.

For measurements of glucose-6-P efflux, a small aliquot of [U-^{14}C]glucose-6-P was added to a concentrated vesicle suspension to yield a final concentration of 20 mM and the sample was incubated at room temperature for 3 h to allow glucose-6-P to equilibrate with the intravesicular space. Aliquots (2 μL) of this suspension were then diluted 400-fold into media of given composition at 25 °C and assayed at specified times as described above.

Fluorescence Measurements. Fluorescence of 6′-*N*-dansylaminohexyl 1-thio-β-D-galactopyranoside (Dns^6-Gal) was observed at 90 °C from the excitation beam with a Perkin-Elmer MPF4 spectrophotofluorometer using 1 × 1 cm quartz cuvettes (Beckman) as described (Reeves et al., 1973; Schuldiner et al., 1975). The sample chamber was maintained at 25 °C with a Lauda Model K-2/R circulating water bath (Brinkman). Emission and excitation slits were adjusted to 6 nm. Additions were made to the cuvettes with Hamilton microsyringes or with a Gilson Pipetman microliter pipet, and mixing was accomplished within 5 s by using a plastic stick (Calbiochem).

Protein. Protein was measured according to Lowry et al. (1951) by using bovine serum albumin as standard.

Calculations. The internal concentration of solutes accumulated by the vesicles was calculated by using a value of 2.2 μL of intravesicular fluid per mg of membrane protein (Kaback & Barnes, 1971), and concentration gradients were determined from the ratio of internal and external concentrations. The membrane potential was calculated from the concentration gradient of [^{3}H]TPP^+ established under a given set of conditions by using the Nernst equation ($\Delta\Psi = -59 \log [TPP^+]_{in}/[TPP^+]_{out}$).

Materials

[U-^{14}C]Glucose-6-P was purchased from Amersham/Searle and [6-^{14}C]glucuronate from New England Nuclear. [^{3}H]-TPP^+ (bromide salt) was synthesized by the Isotope Synthesis Group at Hoffmann-La Roche under the direction of Dr. Arnold Liebman. Dns^6-Gal was synthesized as described (Schuldiner et al., 1975). Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Calbiochem. Nigericin was generously provided by Dr. John Wesley, Hoffman-La Roche, Inc. All other materials were reagent grade obtained from commerical sources.

Results

Effect of pH on $\Delta\Psi$-Driven Glucose-6-P Transport. It is well established that imposition of a potassium diffusion gradient across the *E. coli* membrane ($K^+_{in} \rightarrow K^+_{out}$) in the presence of the ionophore valinomycin leads to the transient generation of a $\Delta\Psi$ (interior negative) and that a $\Delta\Psi$ generated in this fashion drives the transport of various substrates

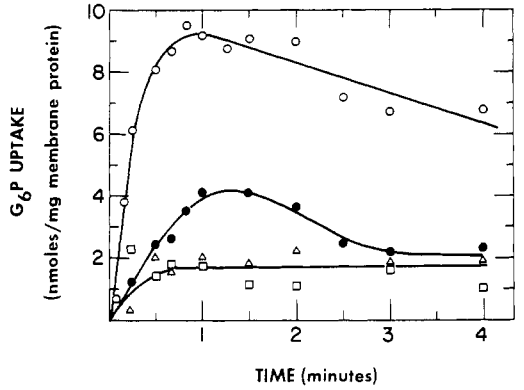

FIGURE 1: $\Delta\Psi$-driven glucose-6-P transport at pH 7.5 and 5.5. Membrane vesicles prepared from *E. coli* DF2000 induced for the glucose-6-P transport system as described under Methods were equilibrated with buffered solutions of given composition and pH (cf. below) and concentrated to 35–40 mg of protein per mL, and valinomycin was added to a final concentration of 2 nmol/mg of membrane protein. An aliquot (2 μL) of the suspension was then diluted rapidly 200-fold into a solution of given composition (cf. below) containing 0.6 mM [U-^{14}C]glucose-6-P (3.3 mCi/mmol) that had been equilibrated previously to 25 °C. The samples were incubated at 25 °C, and at the times indicated the reactions were terminated and assayed as described under Methods. (○) Vesicles equilibrated with 100 mM potassium phosphate (pH 7.5) and 10 mM magnesium sulfate diluted into 100 mM sodium phosphate (pH 7.5) containing 10 mM magnesium sulfate and 2×10^{-6} M valinomycin; (●) vesicles equilibrated with 100 mM potassium phosphate (pH 5.5) and 10 mM magnesium sulfate diluted into 100 mM sodium phosphate (pH 5.5) containing 10 mM magnesium sulfate and 2×10^{-6} M valinomycin; (△) vesicles equilibrated with 100 mM potassium phosphate (pH 7.5 or 5.5) and 10 mM magnesium sulfate diluted into 100 mM potassium phosphate (pH 7.5 or 5.5, respectively) containing 10 mM magnesium sulfate and 2×10^{-6} M valinomycin; (□) vesicles equilibrated with 100 mM potassium phosphate (pH 7.5 or 5.5), 10 mM magnesium sulfate, and CCCP at a final concentration of 4 nmol/mg of membrane protein diluted into 100 mM sodium phosphate (pH 7.5 or 5.5, respectively) containing 10 mM magnesium sulfate, 2×10^{-6} M valinomycin, and 5×10^{-6} M CCCP.

(Hirata et a., 1973, 1974; Lombardi et al., 1974; Schuldiner & Kaback, 1975). The data presented in Figure 1 illustrate time courses of glucose-6-P accumulation by *E. coli* DF2000 membrane vesicles in response to such a transient $\Delta\Psi$ under various conditions. When the vesicles are equilibrated with 100 mM potassium phosphate at pH 7.5, treated with valinomycin, and then diluted 200-fold into medium containing equimolar concentrations of sodium phosphate at pH 7.5, glucose-6-P is taken up rapidly for 15–30 s and the vesicles achieve a maximum level of accumulation of ~9.5 nmol/mg of membrane protein in 1 min, which corresponds to a concentration gradient of about sevenfold. Subsequently, glucose-6-P is lost from the vesicles and by ~8 min (not shown) the level returns to that of the control samples. During the initial, linear phase of the response (i.e., over the first 15 s), glucose-6-P is taken up at a rate of ~24 nmol/(mg of membrane protein min), a value approximating the V_{max} of the transport system in this vesicle preparation in the presence of reduced PMS.[3]

A number of independent experiments demonstrate that glucose-6-P transport under these conditions is directly related to the generation of $\Delta\Psi$ (interior negative). (1) Under the same conditions, uptake of [^{3}H]TPP$^+$, a lipophilic cation that distributes across the membrane in accordance with $\Delta\Psi$ (Lichtshtein et al., 1979a,b; Porter et al., 1979; Felle et al.,

[3] Kinetic studies of the initial rate of glucose-6-P transport in the vesicle preparations used in these experiments reveal an apparent K_m of 0.28 mM and a V_{max} of 28 nmol/(mg of membrane protein min) in the presence of reduced PMS (data not shown).

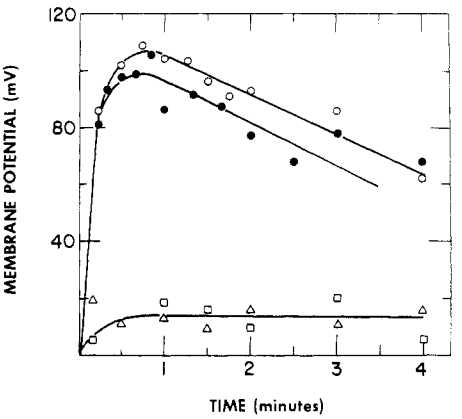

FIGURE 2: Uptake of [^{3}H]TPP$^+$ induced by outwardly directed potassium diffusion gradients in the presence of valinomycin. Experiments were performed as described in Figure 1 except that DF2000 vesicles were diluted 200-fold into solutions (cf. below) containing 15 μM [^{3}H]TPP$^+$ (2.5 Ci/mmol). (○) Vesicles equilibrated with 100 mM potassium phosphate (pH 7.5) and 10 mM magnesium sulfate diluted into 100 mM sodium phosphate (pH 7.5) containing 10 mM magnesium sulfate and 2×10^{-6} M valinomycin; (●) vesicles equilibrated with 100 mM potassium phosphate (pH 5.5) and 10 mM magnesium sulfate diluted into 100 mM sodium phosphate (pH 5.5) containing 10 mM magnesium sulfate and 2×10^{-6} M valinomycin; (△) vesicles equilibrated with 100 mM potassium phosphate (pH 7.5 or 5.5) and 10 mM magnesium sulfate diluted into 100 mM potassium phosphate (pH 7.5 or 5.5, respectively) containing 10 mM magnesium sulfate and 2×10^{-6} M valinomycin; (□) vesicles equilibrated with 100 mM potassium phosphate (pH 7.5 or 5.5), 10 mM magnesium sulfate, and CCCP at a final concentration of 4 nmol/mg of membrane protein diluted into 100 mM sodium phosphate (pH 7.5 or 5.5, respectively) containing 10 mM magnesium sulfate, 2×10^{-6} M valinomycin, and 5×10^{-6} M CCCP.

1980), exhibits a time course that is virtually identical with that observed with glucose-6-P (Figure 2, open circles). (2) When potassium-loaded vesicles are treated with valinomycin and diluted 200-fold into medium containing an equimolar potassium concentration (i.e., under conditions where there is no potassium diffusion gradient), transient accumulation of neither glucose-6-P (Figure 1, open squares) nor TPP$^+$ (Figure 2, open squares) is observed and the uptake of both substrates under these conditions approximates simple equilibration with the external medium. (3) When potassium-loaded vesicles are treated with valinomycin and diluted 200-fold into sodium phosphate in the presence of the protonophore CCCP, both glucose-6-P (Figure 1, open triangles) and TPP$^+$ (Figure 2, open triangles) accumulation are abolished. Although not shown, it is noteworthy that the rate at which the glucose-6-P concentration gradient dissipates from a maximum value at 1 min is greatly accelerated by the addition of 5×10^{-6} M CCCP (i.e., when CCCP is added at 1 min, the intravesicular concentration of glucose-6-P approximates that of the control samples within 30 s). Since a corresponding collapse of the TPP$^+$ gradient is also observed, it is apparent that the time course of glucose-6-P accumulation is controlled by the magnitude of $\Delta\Psi$. Taken together, these results demonstrate that glucose-6-P transport at pH 7.5 under the conditions described is electrogenic (i.e., coupled to the $\Delta\Psi$ across the vesicle membrane).

In contrast, when the same operations are performed at pH 5.5, both the initial rate of glucose-6-P uptake and the maximum level of accumulation are diminished considerably (Figure 1, closed circles). The initial rate of uptake is reduced to about 5 to 6 nmol/(mg of membrane protein min), while the maximum level of accumulation at 1 min is reduced to ~4 nmol/mg of membrane protein (a concentration gradient of only threefold). Importantly, however, the time course and

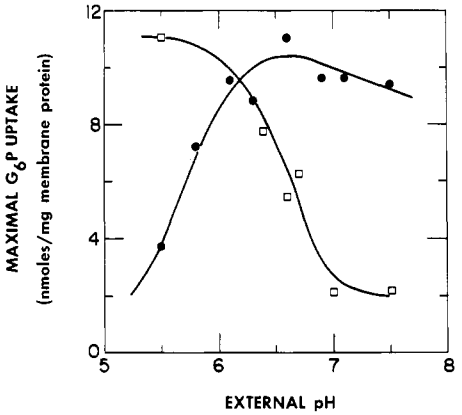

FIGURE 3: Effect of pH on the magnitude of $\Delta\Psi$- and ΔpH-driven glucose-6-P accumulation by DF2000 membrane vesicles. Time courses of transient glucose-6-P accumulation induced by artificially imposed $\Delta\Psi$ (interior negative) and ΔpH (interior alkaline) were measured at given pH values as described in Figures 1 and 4, and the maximum levels accumulated are plotted as a function of pH. (●) $\Delta\Psi$-driven glucose-6-P accumulation; (□) ΔpH-driven glucose-6-P accumulation.

particularly the magnitude of TPP^+ accumulation are very similar to those observed at alkaline pH (Figure 2, closed circles). Thus, a maximum $\Delta\Psi$ (interior negative) of approximately −105 mV is generated at pH 7.5, and at pH 5.5 this value is reduced only marginally to about −100 mV (compare open and closed circles in Figure 2). Since the magnitude of the driving force for glucose-6-P transport is similar at pH 5.5 and 7.5, it seems likely that the difference in transport activity at these pH values is due to an effect of pH on the glucose-6-P porter itself.

When time courses of $\Delta\Psi$-driven glucose-6-P transport are studied at various pH values between pH 5.5 and 7.5 and the maximum levels of accumulation are plotted as a function of external pH, the data presented in Figure 3 (closed circles) are obtained. As shown, the maximum level of accumulation increases from ~4 nmol/mg of membrane protein at pH 5.5 to 10 to 11 nmol/mg of membrane protein at pH 6.6 and above and half-maximal stimulation by $\Delta\Psi$ is observed at approximately pH 6.0.

Effect of pH on ΔpH-Driven Glucose-6-P Transport. Recent studies with the *lac* transport system in *E. coli* membrane vesicles demonstrate that an artificially imposed ΔpH (interior alkaline) drives lactose accumulation (Lancaster & Hinkle, 1977) and leads to an increase in the fluorescence of Dns^6-Gal (Kaczorowski et al., 1979). Vesicles are equilibrated with relatively high concentrations of acetate and diluted into a medium containing a less permeant anion such as gluconate in order to generate a ΔpH of this polarity artificially. Since acetate is permeant in its protonated form only (i.e., as acetic acid; Lancaster & Hinkle, 1977; Ramos & Kaback, 1977a), this manipulation leads to a net loss of protons from the intravesicular space, giving rise to a ΔpH (interior alkaline). As shown in Figure 4 closed circles), when DF2000 vesicles are equilibrated with 100 mM potassium acetate at pH 5.5 and diluted into a medium containing an isotonic concentration of potassium gluconate at pH 5.5, glucose-6-P is rapidly accumulated for ~30 s, at which time the vesicles achieve a maximum level of ~9.0 nmol/mg of membrane protein, corresponding to a sixfold concentration gradient. After 30 s glucose-6-P is lost from the vesicles, and by ~7 min (not shown) the level returns to that of the control samples. During the first 10–15 s of the response, glucose-6-P is taken up at a rate of ~24 nmol/(mg of membrane protein min).[3] Importantly, moreover, glucose-6-P accumulation above the

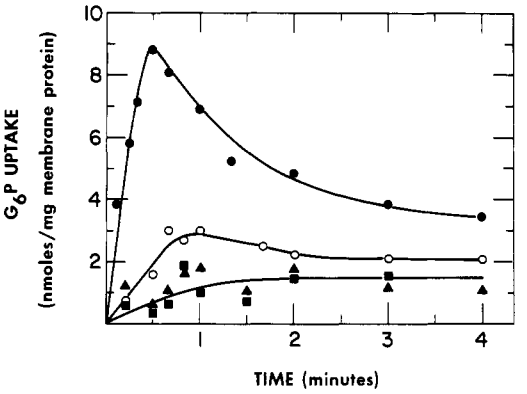

FIGURE 4: ΔpH-driven glucose-6-P transport at pH 5.5 and 7.5. Membrane vesicles prepared from *E. coli* DF2000 induced for the glucose-6-P transport system as described under Methods were equilibrated with buffered solutions of given composition and pH (cf. below) and concentrated to 35–40 mg of protein per mL, and valinomycin was added to a final concentration of 2 nmol/mg of membrane protein. An aliquot (2 μL) of the suspension was then diluted rapidly 200-fold into a solution of given composition (cf. below) containing 0.6 mM [U-^{14}C]glucose-6-P (3.3 mCi/mmol) that had been equilibrated previously to 25 °C. The samples were incubated at 25 °C, and at the time indicated the reactions were terminated and assayed as described under Methods. (●) Vesicles equilibrated with 100 mM potassium acetate, 5 mM potassium phosphate (pH 5.5), and 10 mM magnesium sulfate diluted into 100 mM potassium gluconate, 5 mM potassium phosphate (pH 5.5), and 10 mM magnesium sulfate; (○) vesicles equilibrated with 100 mM potassium acetate, 5 mM potassium phosphate (pH 7.5), and 10 mM magnesium sulfate diluted into 100 mM potassium gluconate, 5 mM potassium phosphate (pH 7.5), and 10 mM magnesium sulfate; (□) vesicles equilibrated with 100 mM potassium acetate, 5 mM potassium phosphate (pH 5.5 or 7.5), and 10 mM magnesium sulfate diluted into 100 mM potassium acetate, 5 mM potassium phosphate (pH 5.5 or 7.5, respectively), and 10 mM magnesium sulfate; (▲) vesicles equilibrated with 100 mM potassium acetate, 5 mM potassium phosphate (pH 5.5 or 7.5), 10 mM magnesium sulfate, and CCCP at a final concentration of 4 nmol/mg of membrane protein diluted into 100 mM potassium gluconate, 5 mM potassium phosphate (pH 5.5 or 7.5, respectively), 10 mM magnesium sulfate, and 5×10^{-6} M CCCP.

equilibration level is not observed when acetate-loaded vesicles are diluted into medium containing an equimolar acetate concentration (Figure 4, solid squares) or when an acetate diffusion gradient is imposed in the presence of CCCP (Figure 4, solid triangles). It is also important that the experiments were carried out in the presence of valinomycin and that no significant $\Delta\Psi$ is generated under these conditions (Kaczorowski et al., 1979). Clearly, therefore, an artificially imposed ΔpH (interior alkaline) can drive glucose-6-P accumulation at pH 5.5.

Interestingly, when the same manipulation is performed at pH 7.5, both the initial rate of glucose-6-P transport and the maximum level of accumulation are markedly reduced (Figure 4, open circles). The initial rate of transport is less than 4 nmol/mg of membrane protein min), and the maximum level of accumulation is only ~3 nmol/mg of membrane protein (a concentration gradient of only about twofold). Attempts to increase the amplitude of the response at pH 7.5 by increasing the magnitude of the acetate diffusion gradient, treating the vesicles with *N,N'*-dicyclohexylcarbodiimide (Patel et al., 1975; Patel & Kaback, 1976), or generating diffusion gradients with other permeant weak acids (i.e., acetylsalicylate or 5,5'-dimethyloxazolidine-2,4-dione) were repeatedly unsuccessful.

A direct method for quantitating the ΔpH generated by outwardly directed acetate diffusion gradients is not available. However, imposition of such gradients across the vesicle

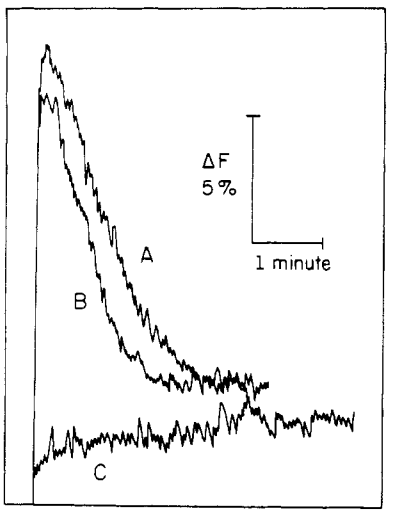

FIGURE 5: Effect of imposed ΔpH (interior alkaline) on Dns[6]-Gal fluorescence. *E. coli* ML 308-225 vesicles prepared as described under Methods were equilibrated with 100 mM potassium acetate and 5 mM potassium phosphate at the same pH as that used during the subsequent dilution and concentrated to ~30 mg of protein per mL as described in Figure 4. After valinomycin was added to a final concentration of 2 nmol/mg of membrane protein, 5-μL aliquots of the suspension were diluted into a cuvette containing 1.5 mL of a salt solution given below and 20 μM Dns[6]-Gal. Changes in fluorescence were monitored at 500 nm (excitation 340 nm) as described under Methods. (Curve A) Vesicles equilibrated with acetate at pH 7.5 were diluted into 100 mM potassium gluconate and 5 mM potassium phosphate (pH 7.5); (curve B) vesicles equilibrated with acetate at pH 5.5 were diluted into 100 mM potassium gluconate and 5 mM potassium phosphate (pH 5.5); (curve C) vesicles equilibrated with acetate at either pH 7.5 or pH 5.5 were diluted into 100 mM potassium acetate and 5 mM potassium phosphate (pH 7.5 or pH 5.5, respectively).

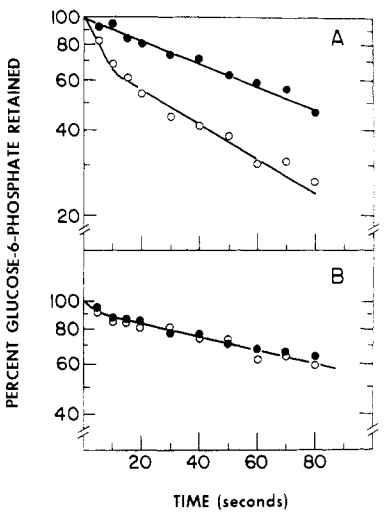

FIGURE 6: Effect of imposed ΔΨ (interior negative) on glucose-6-P efflux at pH 7.5 (panel A) and pH 5.5 (panel B). Experiments were performed with vesicles prepared from *E. coli* DF2000 induced for the glucose-6-P transport system as described under Methods. (A) Vesicles were equilibrated with 100 mM potassium phosphate (pH 7.5) and 10 mM magnesium sulfate and concentrated to 35–40 mg of membrane protein per mL. Valinomycin was added to a final concentration of 2 nmol/mg of membrane protein, and the suspension was equilibrated with [U-^{14}C]glucose-6-P (5 mCi/mmol) at a final concentration of 20 mM by incubation at room temperature for 3 h. Aliquots (2 μL) were then diluted 400-fold into either potassium phosphate (pH 7.5) and 10 mM magnesium sulfate (○) or sodium phosphate (pH 7.5) and 10 mM magnesium sulfate (●), and loss of intravesicular lactose was monitored by filtration as described under Methods. (B) Vesicles were treated as described in panel A, except that the pH values of the equilibration and dilution media were 5.5. (○) Potassium-loaded vesicles diluted into potassium; (●) potassium-loaded vesicles diluted into sodium. The percentage of glucose-6-P retained was determined by comparison with zero time points (44 nmol/mg of protein ± 5%).

membrane leads to a transient increase in Dns[6]-Gal fluorescence, the amplitude of which is directly related to the magnitude of the acetate diffusion gradient (Kaczorowski et al., 1979). Thus, we have used this phenomenon to assess the relative magnitude of the ΔpH generated at pH 5.5 and 7.5 (Figure 5). When acetate-loaded vesicles are diluted into a cuvette containing gluconate, there is a rapid, transient increase in Dns[6]-Gal fluorescence at both pH 5.5 and 7.5 that is not observed when the vesicles are diluted into equimolar acetate. Furthermore, the amplitude of the response is essentially the same at both pH values. In other words, although a significant ΔpH (interior alkaline) is apparently generated at pH 7.5, it does not seem to drive glucose-6-P transport very effectively at this pH.

The open squares in Figure 3 represent maximum levels of ΔpH-dependent glucose-6-P accumulation derived from a series of time courses carried out at given pH values, and it is apparent that the pH profile for ΔpH-driven glucose-6-P transport is almost the mirror image of that observed for ΔΨ-driven transport. Thus, a maximum level of accumulation of ~11 nmol/mg of membrane protein is obtained at pH 5.5, and this value decreases to a minimum of ~2 nmol/mg of membrane protein at pH 7.0 and above with a midpoint at about pH 6.5.

Since the pK_2 of glucose-6-P is 6.11, it is conceivable that the pH profile for ΔpH-dependent glucose-6-P accumulation reflects in some way a change in the concentration of the protonated form of glucose-6-P.[4] ΔpH-driven glucuronate transport was studied as a function of pH with vesicles prepared from *E. coli* ML 308-225 grown on glucuronate (Ramos & Kaback, 1977b) to assess this possibility. Although the data are not presented,[5] outwardly directed acetate difussion gradients affect glucuronate transport as a function of pH in a manner similar to that observed for glucose-6-P. Maximum levels of glucuronate accumulation of ~15 nmol/mg of membrane protein are observed at pH 5.0 and 5.5 that decrease to values of ~5 nmol/mg of membrane protein at pH 7.0 and 7.5, and half-maximum stimulation is observed at around pH 6.0. Since the pK of glucuronate is 3.02, it seems unlikely that these effects can be attributed to changes in the protonation state of the substrates. In addition, ΔΨ-driven glucuronate transport exhibits a pH profile that is similar to that observed for glucose-6-P. The amplitude of ΔΨ-driven glucuronate accumulation is minimal at pH 5.0, increases progressively between pH 5.0 and 6.5, and remains essentially constant from pH 6.5 to 7.5.

Effect of ΔΨ on Glucose-6-P Efflux at pH 5.5 and 7.5. Since previous results (Ramos & Kaback, 1977b,c) and those discussed above suggest that the glucose-6-P porter catalyzes an electrically neutral reaction at pH 5.5 and an electrogenic reaction at pH 7.5, imposition of a ΔΨ would be expected to influence the rate of passive, carrier-mediated glucose-6-P efflux at pH 7.5 but not at pH 5.5 (Kaczorowski et al., 1979). The results presented in Figure 6A describe the time course of glucose-6-P efflux from DF2000 vesicles equilibrated with 20 mM [U-^{14}C]glucose-6-P, treated with valinomycin, and then diluted 400-fold into media containing either potassium phosphate (no ΔΨ; open circles) or sodium phosphate (ΔΨ,

[4] This possibility seems unlikely a priori, since ΔΨ-driven glucose-6-P accumulation is observed at pH 7.5.

[5] G. Rimon and H. R. Kaback, unpublished experiments.

interior negative; closed circles) at pH 7.5. Clearly, imposition of $\Delta\Psi$ (interior negative) retards efflux and the $t_{1/2}$ is increased from about 25 to 70 s. When the identical experiment is performed at pH 5.5 (Figure 6B), on the other hand, imposition of $\Delta\Psi$ (interior negative) has no significant effect on the rate of efflux and $t_{1/2}$ values of 120 s are observed in the absence or presence of an imposed $\Delta\Psi$ (interior negative).

Discussion

The results presented in this paper provide independent experimental support for earlier observations (Ramos & Kaback, 1977b,c), indicating that there is an increase in proton/substrate stoichiometry with pH for a number of transport systems that are driven by ΔpH (interior alkaline) at pH 5.5. In the previous studies, the innate properties of the respiration-dependent $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) in *E. coli* membrane vesicles were utilized and it was demonstrated by means of pH studies and titrations with valinomycin and nigericin that the steady-state level of accumulation of glucose-6-P and certain other organic acids is in equilibrium with ΔpH at pH 5.5 but with $\Delta\Psi$ at pH 7.5 (Ramos & Kaback, 1977b). In a subsequent study (Ramos & Kaback, 1977c), more direct evidence for the putative stoichiometry change was provided by varying ΔpH and $\Delta\Psi$ systematically at pH 5.5 and 7.5, respectively, and relating the values to steady-state levels of glucose-6-P and lactate accumulation under the same conditions. In addition, models were formulated (Rottenberg, 1976; Ramos & Kaback, 1977c) that are consistent thermodynamically with the observations. In essence, it was suggested that at pH 5.5 glucose-6-P and certain other organic acids are translocated across the membrane in a protonated form and accumulate in the ionized state because of the relative alkalinity of the internal milieu (i.e., transport of these compounds at pH 5.5 is driven by ΔpH and is thus electrically neutral). Alternatively, due to the dissociation of important functional groups, the porters for these substrates would become negatively charged at pH 7.5, catalyzing the symport of a proton with the weak acid, and the systems would become electrogenic (i.e., coupled to $\Delta\Psi$).

In the present study, ΔpH (interior alkaline) and $\Delta\Psi$ (interior negative) were imposed artificially across the vesicle membrane and the following points which are important to the arguments outlined above are clearly established: (1) imposition of $\Delta\Psi$ (interior negative) by means of outwardly directed potassium diffusion gradients in the presence of valinomycin drives glucose-6-P (and glucuronate) transport at pH 7.5 but much less effectively at pH 5.5, although the magnitude of the $\Delta\Psi$ generated is comparable at both pH values; (2) imposition of $\Delta\Psi$ (interior negative) retards passive, carrier-mediated glucose-6-P efflux down a concentration gradient at pH 7.5 but not at pH 5.5; (3) imposition of ΔpH (interior alkaline) by means of outwardly directed acetate diffusion gradients drives glucose-6-P (and glucuronate) accumulation at pH 5.5. Therefore, glucose-6-P (and glucuronate) transport is electroneutral at pH 5.5 and electrogenic at pH 7.5. Moreover, given these conclusions, the most simple explanation for the observations within a chemiosmotic framework is that the proton/substrate stoichiometry increases with pH as discussed by Rottenberg (1976) and Ramos & Kaback (1977b,c).

The finding that ΔpH-driven accumulation of both glucose-6-P and glucuronate decreases from pH 5.0–5.5 to 7.5 is both unexpected and puzzling. If these transport systems become electrogenic by the mechanism postulated (Rottenberg, 1976; Ramos & Kaback, 1977c), they should exhibit the properties of a symport system at pH 7.5. That is, the systems would be expected to respond to both $\Delta\Psi$ (interior negative) and ΔpH (interior alkaline), as demonstrated for the *lac* system (Kaczorowski et al., 1979). This does not appear to be the case, however. Accumulation of glucose-6-P and glucuronate is driven by an artificially imposed ΔpH (interior alkaline) at pH 5.0–5.5, but as pH is increased the imposed ΔpH appears to drive transport less efficiently. An obvious explanation for this behavior is that the magnitude of the ΔpH generated by outwardly directed acetate diffusion gradients is diminished at alkaline pH, but it is difficult to establish this point experimentally since it is not possible to measure ΔpH (interior alkaline) directly under the conditions utilized. In contrast, moreover, from the Dns[6]-Gal fluorescence studies presented in Figure 5, it seems unlikely that this simplistic explanation is valid. Furthermore, numerous attempts to drive glucose-6-P accumulation at pH 7.5 with 5,5′-dimethyloxazolidine-2,4-dione gradients were also unsuccessful.[6] Finally, it is noteworthy that $\Delta\Psi$-driven glucose-6-P transport is demonstrable at pH 8.5 (data not shown), indicating that the relative inability of the imposed ΔpH to drive transport at pH 7.5 cannot be attributed to an effect of internal pH on the glucose-6-P porter. As discussed previously (Kaczorowski et al., 1979), although the *lac* transport system appears to respond equivalently to imposed $\Delta\Psi$ and ΔpH in a manner that is consistent with thermodynamic predictions, it is not clear that both parameters affect the same step in the overall translocation mechanism. In view of these considerations, the effects of imposed $\Delta\Psi$ and ΔpH on glucose-6-P and glucuronate accumulation at high pH may be important, as they suggest that these parameters may not be equivalent mechanistically. In any event, it should be emphasized that the differential effects of ΔpH at acid and alkaline pH are not critical to the arguments formulated previously (Rottenberg, 1976; Ramos & Kaback, 1977b,c) and above, since it is apparent that ΔpH drives transport very efficiently at pH 5.5, while $\Delta\Psi$ is relatively inefficient at this pH but drives transport effectively at pH 7.5.

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[6] Since the p*K* of acetic acid is 4.75, it could be argued that outwardly directed acetate diffusion gradients do not generate ΔpH effectively at pH 7.5 because the concentration of the protonated species is limiting at this pH. The p*K* of 5,5′-dimethyloxazolidine-2,4-dione is 6.3, however, and the concentration of the protonated form should not be limiting at pH 7.5.

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