Proton Electrochemical Gradient in *Escherichia coli* Cells and Its Relation to Active Transport of Lactose†

Dan Zilberstein, Shimon Schuldiner,* and Etana Padan

ABSTRACT: The electrochemical potential gradients of protons ($\Delta\tilde{\mu}_{H^+}$) and lactose ($\Delta\tilde{\mu}_{lac}$) maintained by respiring *Escherichia coli* ML 308-225 ($i^-z^-y^+a^+$) cells have been measured as a function of the external pH. The proton gradient (ΔpH) was determined from the distribution across the cell membrane of the weak acid 5,5-dimethyloxazolidine-2,4-dione (DMO) after a rapid centrifugation step. The accumulation of tetraphenylphosphonium (TPP^+; a lipophylic cation) and $^{86}Rb^+$ in the presence of valinomycin by EDTA-treated cells incubated in a flow dialysis system has been used to calculate the membrane potential ($\Delta\psi$). Simultaneous measurements of $\Delta\tilde{\mu}_{H^+}$ and lactose gradient were conducted using [^{14}C]lactose and [^{3}H]TPP^+. Respiring EDTA-treated cells maintain, at neutrality, a $\Delta\tilde{\mu}_{H^+}$ of 170 mV (ΔpH = 35 mV, $\Delta\psi$ = 135 mV, interior negative and alkaline). When the external pH was changed from 6.0 to 8.0, $\Delta\psi$ increased from 95 to 150 mV, while ΔpH decreased from 1.8 to −0.2 units (at pH 6 interior alkaline and at pH 8 interior acid). Thus, $\Delta\psi$ significantly compensates for the decrease in ΔpH, yielding a $\Delta\tilde{\mu}_{H^+}$ of 200 mV at pH 6 and 140 mV at pH 8. In contrast to intact cells, inverted membrane vesicles from *E. coli* maintain a large ΔpH at external pH 8.0, while $\Delta\psi$ was undetectable. Lactose steady-state gradients were determined in cells maintaining different $\Delta\tilde{\mu}_{H^+}$ levels in the presence of various concentrations of the uncoupler FCCP at pH 8. The results indicate that one proton is translocated with each molecule of lactose at pH 8 similar to the case at pH 6. This conclusion is supported by direct measurements of H^+ and lactose fluxes induced by a lactose pulse in nonmetabolizing cells.

It is now well established that an electrochemical proton gradient[1] ($\Delta\tilde{\mu}_{H^+}$) is maintained across the cytoplasmic membrane of bacterial cells. This gradient is created by outward translocation of protons coupled to respiration and/or ATP hydrolysis (Mitchell, 1968; Greville, 1969; Harold, 1972, 1977). Furthermore, as suggested by Mitchell (1968), the $\Delta\tilde{\mu}_{H^+}$ drives endergonic processes such as phosphorylation and active transport.

The electrochemical proton gradient is composed of the chemical gradient (ΔpH) and the membrane potential ($\Delta\psi$) according to the relation

$$\Delta\tilde{\mu}_{H^+}\ (\text{mV}) = \Delta\psi - \frac{2.3RT\Delta\text{pH}}{F}$$

The methods employed for estimation of the $\Delta\tilde{\mu}_{H^+}$ in microscopic systems (reviews in Rottenberg 1975, 1978) are based on the determination of concentration gradients of either weak acids and bases or freely permeant ions (for ΔpH or $\Delta\psi$, respectively). A conventional direct method for measurement of the differences between the internal and external concentrations of the monitor species involves rapid separation of cells or organelles from the reaction medium by centrifugation or filtration. Although the sensitivity of these methods is very high, changes in conditions occurring during the separation step may lead to underestimation of the concentration gradients. Using the flow dialysis technique (Colowick & Womack, 1969; Ramos et al., 1976) or fluorescent probes (Hoffman & Laris, 1974; Schuldiner et al., 1972), the separation step is avoided and the concentration gradient is calculated from changes in the concentration of the external medium. These techniques, however, are limited to conditions in which high changes in external concentrations occur during the reaction.

Employing the centrifugation technique, it was shown that respiring *Escherichia coli* cells maintain at neutral extracellular pH a $\Delta\tilde{\mu}_{H^+}$ of −115 mV (ΔpH = 0.56, $\Delta\psi$ = 82 mV, internal alkaline and negative) (Padan et al., 1976). When the external pH was changed between 6.0 and 8.0 the $\Delta\psi$ was hardly affected. However, ΔpH markedly changed, decreasing from 1.8, at the lower pH, to −0.2 at the basic pH, with the inversion point at 7.7.

Ramos et al. (1976) compared different techniques of $\Delta\tilde{\mu}_{H^+}$ determination in "right-side-out" membrane vesicles isolated from *E. coli* cells. A constant $\Delta\psi$ of 75 mV (negative inside) was measured in respiring vesicles at a broad range of external pH values, irrespective of whether a separation step was included or not. However, ΔpH (internal alkaline) could be measured only by the flow dialysis technique, yielding a ΔpH of 2.0 at pH 5.5, decreasing to 0 at pH 7.5 and remaining 0 up to pH 8. Membrane vesicles inverted with respect to the native orientation have been prepared from many sources. These membrane preparations (e.g., submitochondrial particles and chromatophores) differ from the native membranes not only in the orientation of proton movements but also in the relative contribution of ΔpH and $\Delta\psi$ to $\Delta\tilde{\mu}_{H^+}$ (Chance & Montal, 1971; Rottenberg, 1978). Hence, differences in the estimation of $\Delta\tilde{\mu}_{H^+}$ are observed in isolated membranes using different techniques or employing membrane preparations with different polarities.

Precise values of $\Delta\tilde{\mu}_{H^+}$ may bear upon the understanding of the active transport mechanisms of a solute which is cotransported with protons. The concentration gradient of such a solute at steady state is determined by the $\Delta\tilde{\mu}_{H^+}$ and the stoichiometry between protons and solute (Rottenberg, 1976). In right-side-out membrane vesicles isolated from *E. coli*, gradients of accumulated solutes do not vary dramatically with external pH while the $\Delta\tilde{\mu}_{H^+}$ does. Hence, it was proposed that the stoichiometry between hydrogen ions and the different solutes (proline and lactose) increases at the basic pH values from a value of 1 at pH 6 to a value of 2 at pH 7.5 (Ramos & Kaback, 1977). Measuring the rates of lactose transport

† From the Department of Microbiological Chemistry and Molecular Biology, Hebrew University–Hadassah Medical School, Jerusalem, Israel. *Received August 4, 1978.* This research was supported by grants from the United States–Israel Binational Foundation (BSF), Jerusalem, Israel, and the Israel Commission for Basic Research.

[1] Abbreviations used: TPP, tetraphenylphosphonium; DMO, 5,5-dimethyloxazolidine-2,4-dione; carbonic anhydrase, carbonate hydro-lyase (EC 4.2.1); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCC, *N,N'*-dicyclohexylcarbodiimide; $\Delta\tilde{\mu}_{H^+}$, the electrochemical proton gradient; $\Delta\psi$, membrane potential; $\Delta\text{pH} = \text{pH}_{in} - \text{pH}_{out}$.

0006-2960/79/0418-0669$01.00/0

and the coupled proton movement in *E. coli* cells at pH 7, West & Mitchell (1973) observed a stoichiometry of 1.

Using the flow dialysis technique for measuring concentration gradients established by respiring *E. coli* cells, the present work shows that $\Delta\tilde{\mu}_{H^+}$ as well as the chemical potential gradient of lactose is maintained relatively constant and coupled with a stoichiometry of 1 at a wide external pH range between 6 and 8. Whereas $\Delta\psi$ is the main component of $\Delta\tilde{\mu}_{H^+}$ at basic pH values in both intact and right-side-out isolated vesicles, ΔpH is the main contributor to $\Delta\tilde{\mu}_{H^+}$ of inverted "inside-out" membrane vesicles throughout the external pH range from 6 to 8.

Experimental Procedure

Methods

Bacteria and Growth Conditions. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) kindly provided by H. R. Kaback (Roche Institute of Molecular Biology, Nutley, NJ) was used in this work. The growth medium M-9 (Adams, 1959) or medium A (Davis & Mingioli, 1950) was supplemented with 16 mM disodium succinate. Growth conditions were as previously described (Padan et al., 1976).

Preparations of Inverted E. coli Membrane Vesicles. Inverted membrane vesicles were prepared according to Tsuchiya & Rosen (1975) as previously described (Schuldiner & Fishkes, 1978).

Determination of $\Delta\psi$ and $\Delta\mu_{lac}$. $\Delta\psi$ was evaluated from the distribution across the cell membrane of ^{86}RbCl in the presence of valinomycin or [^{3}H]tetraphenylphosphonium (TPP^+). Accumulation of the labeled ions was measured in EDTA-treated cells (Padan et al., 1976) by flow dialysis (Ramos et al., 1978). The upper and lower chamber of the flow dialysis apparatus was separated by dialysis tubing (12000 molecular weight cutoff; Thomas Technological Service). Buffer was pumped from the lower chamber at a rate of 6 mL/min using a Pharmacia pump (Model P3). Fractions of 1.8 mL were collected in a Gilson fraction collector. Samples of 1 mL from the appropriate fraction were mixed with 3 mL of toluene–Triton scintillation liquid and counted in a Tri-Carb scintillation counter (Packard).

When the lactose gradient was determined with $\Delta\psi$, [^{14}C]lactose and [^{3}H]TPP^+ were added to the same reaction medium.

Determination of ΔpH. ΔpH was determined by following the accumulation of [^{14}C]-5,5-dimethyloxazolidine-2,4-dione (DMO) with the same procedure described for $\Delta\psi$ or by the centrifugation method as previously described (Padan et al., 1976).

Direct Measurement of H^+/Lactose Stoichiometry. Proton influx induced by a lactose pulse and lactose transport were determined essentially as described by West & Mitchell (1973). Cells were depleted of endogenous energy sources by incubation at room temperature under continuous N_2 flushing in a 4-mL reaction mixture containing 0.15 M choline chloride, 0.05 M KSCN, 0.001 M KCl, 0.1 mg of carbonic anhydrase, 1 mM iodoacetate, and 100 μM DCC at a cell protein concentration of 2 mg/mL. After 55 min, the suspension was centrifuged. The pellet was resuspended in fresh medium of identical composition and transferred to a closed electrode vessel, thermostated at 25 °C. After an additional 3-min incubation in an N_2 atmosphere, 40 μL of 0.33 M oxygen-free lactose was added, and the pH was monitored using a GK 2301 C radiometer combined pH electrode, a pH meter, and a Goertz Model RE541 recorder. Calibration of the pH change was performed at the end of each experiment by addition of

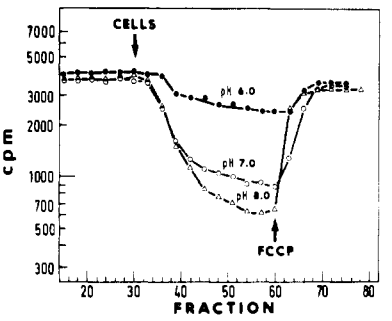

FIGURE 1: Measurement of $\Delta\psi$ by the flow dialysis technique. Accumulation of [^{3}H]TPP was measured by flow dialysis as described under Experimental Procedure. At the onset of the experiment, [^{3}H]TPP (888 Ci/mol) was added to the upper chamber of the flow dialysis apparatus to a final concentration of 13 μM. The upper chamber contained, in a final volume of 0.8 mL, 0.15 M choline chloride, 0.016 M disodium succinate, and 0.005 M Tris-Mes buffer adjusted to pH 6.0 (●), 7.0 (○), or 8.0 (△), and the same medium was continuously pumped through the lower chamber at a rate of 6 mL/min. Both the upper chamber and the buffer pumped through the lower chamber were continuously oxygenated with water-saturated oxygen. After 30 fractions were collected, *E. coli* cells (2 mg of protein), treated with EDTA as described under Experimental Procedure, were added to the upper chamber, and fractions were further collected and assayed for radioactivity. Where indicated, FCCP was added to a final concentration of 25 μM.

5 μL of a 10 mM oxygen-free HCl solution (in 0.1 M KCl). When transport was measured, [^{14}C]lactose (0.33 M, 0.99 Ci/mol) was used. Samples of 0.1 mL were incubated under N_2, and at different time intervals after the addition of lactose uptake was terminated by rapid addition of 2 mL of a nitrogen-saturated solution containing 0.15 M choline chloride, 0.05 M KSCN, 0.001 M KCl, and 0.1 mM *p*-hydroxymercuribenzoate and immediate filtration through membrane filters (Schleicher and Schuell; 0.45-μm pore size). The filters were then washed with another 2 mL of the stopping solution, dried, and assayed for radioactivity. In all the experiments, the pH was adjusted by adding oxygen-free KOH or HCl solutions.

Determination of ΔpH in Inverted E. coli Membrane Vesicles. ΔpH was determined by measuring fluorescence of 9-aminoacridine as previously described (Schuldiner & Fishkes, 1978).

Materials

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was obtained from Du Pont; valinomycin was from Calbiochem or Sigma; *N,N'*-dicyclohexylcarbodiimide, *p*-hydroxymercuribenzoate, and carbonic anhydrase were from Sigma; [^{14}C]methylamine, [^{86}Rb]rubidium chloride, [^{14}C]lactose, and ^3H_2O were from Radiochemical Centre (Amersham); [^{14}C]-5,5-dimethyloxazolidine-2,4-dione was from New England Nuclear; and [^{3}H]tetraphenylphosphonium was a gift from Professor S. R. Caplan, Weizmann Institute.

Results and Discussion

$\Delta\tilde{\mu}_{H^+}$ in EDTA-Treated Cells. The data shown in Figure 1 illustrate a typical flow dialysis experiment carried out to determine $\Delta\psi$ in EDTA-treated *E. coli* cells at different external pH values. [^{3}H]Tetraphenylphosphonium (TPP^+) was added to the reaction medium to the upper chamber at the onset of the experiment. After about 3 min (fraction 10) the maximum amount of radioactivity appeared in the dialysate (not shown), which then decreased at a slow constant rate. When the cells were added to the upper chamber (fraction 30), they accumulated TPP^+, and radioactivity in the dialysate sharply decreased and reached a new steady-state

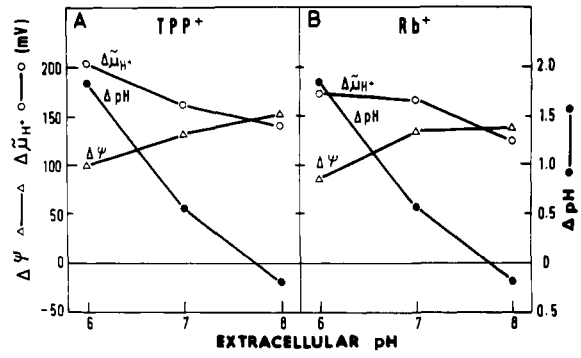

FIGURE 2: ΔpH, Δψ, and $\Delta\tilde{\mu}_{H^+}$ in intact cells as a function of the external pH. Δψ was calculated from the distribution of [^{3}H]TPP or ^{86}Rb as described in Figure 1. When ^{86}Rb was used, valinomycin (2 μg/mg of protein) was added immediately after the EDTA treatment as previously described (Padan et al., 1976). The concentration of ^{86}RbCl in the reaction medium was 50 μM (467 Ci/mol). ΔpH was measured as described under Experimental Procedure in the same reaction media as Δψ except that [^{14}C]DMO was added to a final concentration of 500 μM (9 Ci/mol). $\Delta\tilde{\mu}_{H^+}$ was calculated according to the equation $\Delta\tilde{\mu}_{H^+} = \Delta\psi - 58\Delta pH$ (in mV).

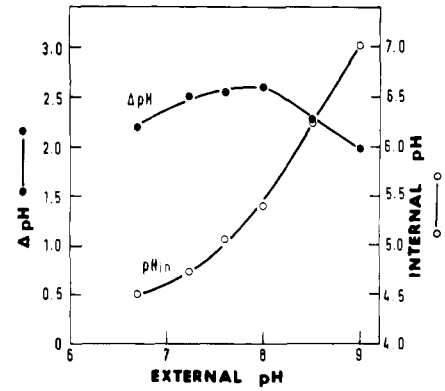

FIGURE 3: ΔpH and internal pH in inside-out membrane vesicles as a function of external pH. Inverted *E. coli* membrane vesicles (0.2 mg of protein) were added to a medium containing, in a final volume of 2.5 mL, 0.15 M KCl, 0.01 M $MgSO_4$, 10 μM 9-aminoacridine, and 0.01 M Tris-HCl at the indicated pH. After recording the initial fluorescence value, dipotassium succinate was added to a final concentration of 10 mM. The fluorescence decreased and reached a new steady-state value after approximately 8 min. The decrease in fluorescence was used to calculate the ΔpH as described (Schuldiner et al., 1972). The osmotic volume, found to be 5.6 μL/mg of protein, was used for the calculations. The internal pH was calculated from the value of the external pH and the ΔpH.

Table I: $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_{lac}$ at Different External pH Values[a]

external pH	Δψ (mV)	ΔpH (mV)	$\Delta\tilde{\mu}_{H^+}$ (mV)	$\Delta\mu_{lac}$ (mV)	$\Delta\mu_{lac}/\Delta\tilde{\mu}_{H^+}$
6.0	102 ± 7.0	105 ± 1.1	207 ± 6.6	162 ± 5.8	0.78
8.0	152 ± 4.0	−12 ± 1.6	138 ± 4.6	142 ± 6.2	1.03

[a] Δψ, ΔpH, and $\Delta\tilde{\mu}_{H^+}$ were measured as described in Figures 1 and 2. $\Delta\mu_{lac}$ was measured using flow dialysis at the same conditions as Δψ except that [^{14}C] lactose (0.125 mM, 5 Ci/mol) was added instead of [^{3}H] TPP$^+$.

value. Addition of FCCP, a proton ionophore, caused a quantitative release of the accumulated [^{3}H]TPP$^+$, and the external radioactivity returned to the control levels (without cells). This observation supports the notion that accumulated TPP$^+$ is free in solution and readily distributes across the membrane in response to the membrane potential, which thus may be calculated from its concentration gradient (Skulachev, 1971; Heinz et al., 1975).

In a parallel experiment, the internal volume of the cells was found to be 5 μL/mg of protein, and thus the concentration ratios of TPP$^+$ observed at the respective pH values are 53 at pH 6, 207 at pH 7, and 385 at pH 8; the respective calculated Δψ values (negative inside) are 100, 134, and 150 mV (Figure 2A). The same values were obtained in the presence of tetraphenylboron or when valinomycin (2 μg/mg of cell protein) was added in the absence of K$^+$. Similar Δψ values were obtained when ^{86}Rb$^+$ in the presence of valinomycin was used instead of [^{3}H]TPP$^+$ (Figure 2B). Upon increasing the external KCl concentration in this experimental system, the Δψ decreased in a pattern similar to that previously described (Padan et al., 1976). These Δψ values, obtained by the flow dialysis technique, are significantly higher than those obtained by the centrifugation method (Padan et al., 1976). The difference most probably stems from leakage of the radioactive probe used (^{86}Rb$^+$) during the centrifugation step.

When ΔpH was measured by flow dialysis using [^{14}C]DMO, the results obtained were similar to those determined by the centrifugation technique (Padan et al., 1976). However, since the amount of acid taken up is very low, particularly above pH 6, the results are on the borderline of significance, and we therefore chose to use the centrifugation technique to assess ΔpH (Figure 2). As previously shown (Padan et al., 1976), ΔpH is a function of the external pH; the ΔpH changes from 1.8 (internal alkaline) at external pH 6 to −0.2 units (internal acidic) at external pH 8.

From the results presented in Figure 2, the $\Delta\tilde{\mu}_{H^+}$ at the different pH values can be calculated. The highest $\Delta\tilde{\mu}_{H^+}$ (205 mV) is observed at pH 6.0 and it decreases only slightly at the basic pH values. Thus, the marked change in ΔpH as a function of external pH is partially compensated by a parallel increase of Δψ. At pH 8, where the ΔpH is −0.2, $\Delta\tilde{\mu}_{H^+}$ is still 140 mV, as measured with TPP$^+$ (Figure 2A), or 125 mV, when measured with ^{86}Rb in the presence of valinomycin (Figure 2B). Similar $\Delta\tilde{\mu}_{H^+}$ values are obtained when choline chloride was replaced with potassium phosphate (0.05 M) and $MgSO_4$ (0.01 M) throughout the whole procedure.

$\Delta\tilde{\mu}_{H^+}$ in Inside-Out Membrane Preparations. In contrast to intact cells, the $\Delta\tilde{\mu}_{H^+}$ measured in isolated right-side-out cytoplasmic membrane vesicles from *E. coli* is a steep function of the external pH (Ramos et al., 1976). Thus, although ΔpH changes with pH in a pattern similar to that detected in whole cells, Δψ is constant throughout the pH range tested.

The data shown in Figure 3 illustrate the ΔpH values measured at different external pH values in inverted inside-out membrane vesicles from *E. coli*. The pattern shown is completely different from that observed both in intact cells and in right-side-out vesicles; Δψ, as measured with [^{14}C]SCN, was undetectable while large ΔpH values (from 2.0 to 2.5) were measured throughout the whole pH range. Thus, also in marked contrast with the constant internal pH of intact cells and right-side-out vesicles, the internal pH of the inverted vesicles changes from 4.4 at an external pH of 6.6 to 7.0 at external pH 9.0. It is not clear whether these differences between intact cells and membrane preparations are due to changes in permeability or functionality of some membrane component or polarity per se in the case of the inverted membrane vesicles.

Stoichiometry of Lactose/H$^+$ Symport as Determined by $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_{lac}$ Measurements. In the intact *E. coli* cell, the $\Delta\tilde{\mu}_{H^+}$, which does not change significantly with the external pH, accounts for the lactose gradients observed with a stoichiometry of 1 throughout the pH range tested (Table I). Similar results were obtained at neutral pH by Flagg & Wilson (1977). The results at the basic pH values markedly differ from those obtained in the right-side-out isolated membrane

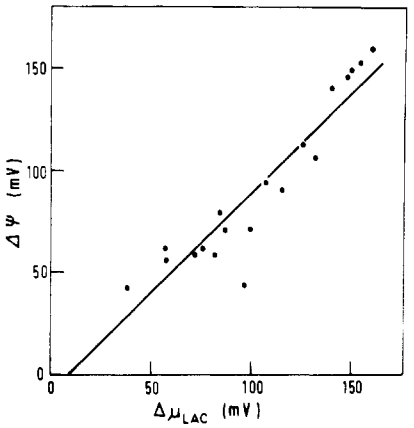

FIGURE 4: Dependence of $\Delta\mu_{lac}$ on $\Delta\tilde{\mu}_{H^+}$. Accumulation of [^{3}H]TPP and [^{14}C]lactose was measured as described in Figure 1 except that [^{14}C]lactose and [^{3}H]TPP were simultaneously present. [^{14}C]Lactose concentration was 0.125 mM (5 Ci/mol) and [^{3}H]TPP was 13 μM (888 Ci/mol). $\Delta\tilde{\mu}_{H^+}$ values were changed by increasing concentrations of FCCP (between 1 and 10 μM).

vesicles in which a stoichiometry of 2 has been suggested (Ramos & Kaback, 1977). Therefore, we further investigated the stoichiometry between protons and lactose at pH 7.8 by systematically changing $\Delta\tilde{\mu}_{H^+}$ (Figure 4). $\Delta\tilde{\mu}_{H^+}$ (which at this pH is comprised solely of $\Delta\psi$) was altered by increasing concentrations of FCCP, and $\Delta\psi$ and $\Delta\mu_{lac}$ were simultaneously measured. The data best fit a straight line with a slope of 0.98 and an intercept of 9 mV on the $\Delta\mu_{lac}$ axis (when $\Delta\psi$ = 0; obtained by linear regression with a correlation coefficient of 0.95). The fact that the intercept is slightly different from zero may be explained by the limit of accuracy of the technique; $\Delta\psi$ values below 40 mV represent a decrease in external concentration of about 10%, which is within the limits of detection.

Direct Measurement of H^+/Lactose Stoichiometry. To further support our results, a direct approach for determination of the lactose/H^+ symport, previously described by West & Mitchell (1973), was used. Figures 5A,B illustrate the results of such an experiment conducted at an external pH of 6.4 and 7.9. As previously shown by West & Mitchell (1973), addition of an anaerobic solution of lactose to an anaerobic suspension of energy-depleted nonmetabolizing *E. coli* cells induces an effective movement of protons and of lactose into the cell (Figures 5A,B, respectively). At both pH values a rapid linear flux of lactose into the cells is detected during the first 10 s, which slowly decreases and reaches equilibrium after 30 s, when the lactose concentration inside approximates the external one. In the parallel experiment, an equivalent inflow of H^+ is observed initially but it slows sooner than that of lactose. Eventually the hydrogen ions return to the medium, somewhat faster at pH 7.8. The stoichiometric ratio of effective H^+ translocation/lactose translocation is plotted in Figure 5C. At the earliest experimental point, the ratio is 0.57 H^+/lactose at the basic pH and 0.68 at the acid pH. Extrapolation of the curve back to zero time gives a stoichiometric ratio of 0.91 H^+/lactose at pH 6.4 and 0.76 at pH 7.8. Within the limitations of the technique, these values are quite close to each other and are in good agreement with the value obtained at pH 7.0 by West & Mitchell (1973). Furthermore, they are in agreement with those obtained by comparing steady-state values of the proton and lactose gradients (see Table I and Figure 4).

It should be noted that although stoichiometry is best measured directly, as described above, severe limitations exist when one of the fluxes measured is coupled to more than one

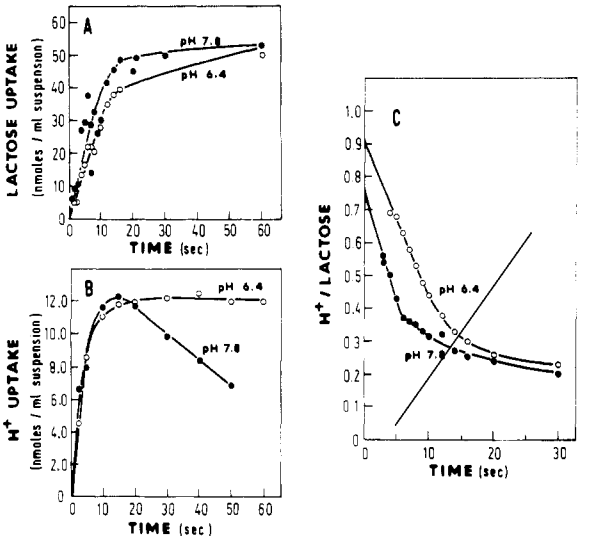

FIGURE 5: Direct measurement of H^+/lactose stoichiometry. Intact *E. coli* cells (8 mg of protein) were energy depleted by incubation in a medium containing 0.15 M choline chloride, 0.05 M KSCN, 0.001 M KCl, 0.1 mg of carbonic anhydrase, 1 mM iodoacetate, and 100 μM DCC in a total volume of 4 mL. After 55 min under continuous N_2 flushing, the suspension was centrifuged. The pellet was resuspended in fresh medium of identical composition and divided into two aliquots. One of them was transferred to a closed electrode vessel, thermostated at 25 °C. After an additional 3-min incubation in a N_2 atmosphere, 40 μL of 0.33 M oxygen-free lactose was added and pH was followed as described under Experimental Procedure (B). The remainder was divided into tubes (0.1 mL each) which were continuously flushed with N_2 for 3 min. The reaction was started by addition of 40 μL of [^{14}C]lactose (0.33 M, 0.99 Ci/mol) and uptake assayed as described under Experimental Procedure.

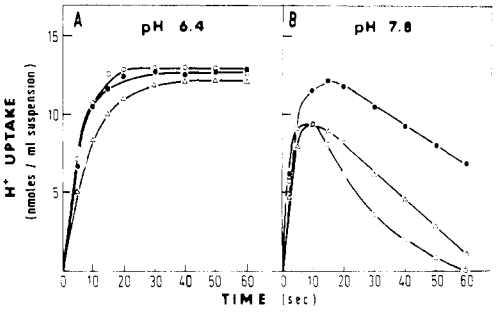

FIGURE 6: Effect of sodium ions on the lactose-induced pH change. Intact *E. coli* cells were energy depleted as described in Figure 5. After 55 min an aliquot was transferred to a closed electrode vessel. After an additional 3-min incubation in a N_2 atmosphere, 40 μL of 0.33 M oxygen-free lactose was added and pH was followed as described under Experimental Procedure (Δ). The rest of the suspension was centrifuged and resuspended in fresh medium of identical composition and the lactose-induced pH changes were followed (●). In a parallel experiment NaCl was added to a final concentration of 20 mM 2 min prior to the addition of lactose (○).

transport system. In such cases, leaks through other systems may interfere with the measurements. Thus, for example, in order to obtain reliable and reproducible pH traces at the basic pH, it was essential to wash the energy-depleted cells prior to the experiment. When unwashed cells were used, a much faster back flux of protons was observed as compared to washed cells (Figure 6B). Since a permeant anion (SCN^-) is present throughout the experiment, the difference in the rate of H^+ exit is not due simply to the removal of a counterion. The functioning of cation/H^+ antiport may explain these results. Washing of the cells removes the cations that leak to the medium during energy depletion. In the absence of an external cation, the protons taken up during the lactose pulse return to the medium by passive diffusion across the mem-

brane. In the presence of the proper cation, the internal protons may flow out through the carrier-mediated antiport mechanism. Na^+/H^+ antiport system was described both in intact *E. coli* cells and in membrane preparations (West & Mitchell, 1974; Schuldiner & Fishkes, 1978). Indeed, addition of 20 mM NaCl to the washed cells 2 min before lactose induces an increased exit of H^+ during the lactose pulse at pH 7.9 (Figure 6B). At pH 6.4, a slow exit rate was observed regardless of whether the cells were washed or whether Na^+ was added externally (Figure 6A). These results imply that the Na^+/H^+ antiport mechanism in *E. coli* cells is more effective at the basic pH values. A similar pH dependence was previously proposed to explain a different phenomenon: acidification of the cytoplasm at basic external pH while the primary energy transducing event is still proton extrusion from the cell.

The results described in this communication pinpoint several differences in the behavior of $\Delta\tilde{\mu}_{H^+}$ and lactose transport in intact cells as compared to isolated membrane vesicles. Thus, in intact cells, $\Delta\psi$ values increase when the external pH is raised and significantly compensate for the decrease in ΔpH, yielding a $\Delta\tilde{\mu}_{H^+}$ value relatively constant through the pH range tested. From a comparison of the lactose steady-state gradients and $\Delta\tilde{\mu}_{H^+}$, we conclude that in intact cells one proton is translocated with each molecule of lactose, both at pH 6 and pH 8. This is supported by direct measurements of H^+ and lactose fluxes.

It is still too early to conclude whether the differences observed are only apparent and due to the differing complexities of the two systems or if they are due to the functioning of some regulatory mechanism present only in one of them.

References

Adams, M. H., Ed. (1959) *Bacteriophages*, pp 446, Interscience, New York.
Chance, B., & Montal, M. (1971) *Curr. Top. Membr. Transp. 2*, 99.
Colowick, S., & Womack, F. C. (1969) *J. Biol. Chem. 244*, 774.
Davis, G. D., & Mingioli, E. S. (1950) *J. Bacteriol. 60*, 17.
Flagg, J. L., & Wilson, H. (1977) *J. Membr. Biol. 31*, 233.
Greville, G. D. (1969) *Curr. Top. Bioenerg. 3*, 1.
Harold, F. M. (1972) *Bacteriol. Rev. 36*, 172.
Harold, F. M. (1977) *Curr. Top. Bioenerg. 6*, 84.
Heinz, E., Geck, P., & Pietrzyk, C. (1975) *Ann. N.Y. Acad. Sci. 264*, 428.
Hoffman, J. F., & Laris, P. C. (1974) *J. Physiol. (London) 239*, 519.
Mitchell, P. (1968) in *Chemiosmotic Coupling and Energy Transduction*, Glynn Research Ltd., Bodmin, Cornwall, U.K.
Padan, E., Zilberstein, D., & Rottenberg, H. (1976) *Eur. J. Biochem. 63*, 533.
Ramos, S., & Kaback, H. R. (1977) *Biochemistry 16*, 4271.
Ramos, S., Schuldiner, S., & Kaback H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1892.
Ramos, S., Schuldiner, S., & Kaback, H. R. (1978) *Methods Enzymol.* (in press).
Rottenberg, H. (1975) *J. Bioenerg. 7*, 61.
Rottenberg, H. (1976) *FEBS Lett. 66*, 159.
Rottenberg, H. (1978) *Methods Enzymol.* (in press).
Schuldiner, S., & Fishkes, H. (1978) *Biochemistry 17*, 706.
Schuldiner, S., Rottenberg, H., & Avron, M. (1972) *Eur. J. Biochem. 25*, 64.
Skulachev, V. P. (1971) *Curr. Top. Bioenerg. 4*, 127.
Tsuchiya, T., & Rosen, B. P. (1975) *J. Biol. Chem. 250*, 7687.
West, C. I., & Mitchell, P. (1973) *Biochem. J. 132*, 587.
West, C. F., & Mitchell, P. (1974) *Biochem. J. 144*, 87.

Isolation and Characterization of Baby Hamster Kidney (BHK-21) Cell Modulator Protein†

Marie-Jeanne Yerna,* David J. Hartshorne,‡ and Robert D. Goldman

ABSTRACT: A Ca^{2+}-dependent modulator protein has been isolated from BHK-21 cells. The purification requires heat treatment, ion-exchange chromatography, and gel filtration. The protein appears homogeneous on sodium dodecyl sulfate–polyacrylamide and isoelectric focusing gels. The protein comigrates with purified smooth muscle and brain modulators. BHK-21 modulator is characterized by a high content of aspartic and glutamic acids and by a high phenylalanine/tyrosine ratio. It lacks both cysteine and tryptophan. The protein is effective in activating brain-modulator-deficient phosphodiesterase. It can also be used in assay systems to generate Ca^{2+}-sensitive actin activation of both BHK-21 and smooth muscle myosins. Therefore, it is proposed that the BHK-21 modulator protein is a component of the Ca^{2+}-dependent mechanism involved in the regulation of actin–myosin interactions in BHK-21 cells.

Modulator protein has been described as a cofactor for phosphodiesterase activity in rat brain by Cheung (1970) and Kakiuchi et al. (1970). Following these initial observations, similar proteins have been isolated from a wide variety of tissues (see Wang, 1977, for review). Further studies have indicated that the modulator protein binds Ca^{2+} and is involved in the Ca^{2+}-mediated regulation of several aspects of cyclic nucleotide metabolism (Cheung et al., 1975; Brostrom et al., 1975; Lin & Cheung, 1975; Teo & Wang, 1973; Stevens et al., 1976; Watterson et al., 1976a,b).

The central role of Ca^{2+} in the regulation of muscle contraction has also been extensively documented (Weber &

† From the Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213. *Received July 5, 1978; revised manuscript received November 20, 1978.* This work represents partial fulfillment for the requirements of Marie-Jeanne Yerna's Ph.D. degree. This work has been supported by National Cancer Institute funds granted to Robert D. Goldman (No. 1-R01-CA-17210-03).

‡ Present address: Department of Nutrition and Food Science, Agricultural Sciences Building, University of Arizona, Tucson, AZ 85721.

0006-2960/79/0418-0673$01.00/0