Mechanism of Lactose Translocation in Membrane Vesicles from *Escherichia coli*. 1. Effect of pH on Efflux, Exchange, and Counterflow†

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ABSTRACT: Carrier-mediated lactose efflux down a concentration gradient was used to probe the mechanism of β-galactoside translocation in *Escherichia coli* membrane vesicles, with particular emphasis on proton/lactose symport. The maximal rate of efflux is dependent on pH, increasing about threefold from pH 5.5 to 7.5 ($t_{1/2}$ = 45, 27, and 15 s at pH 5.5, 6.6, and 7.5, respectively). In contrast, experiments performed under identical conditions with equimolar lactose in the external medium (i.e., under exchange conditions) demonstrate that the exchange reaction is insensitive to pH and very fast relative to efflux ($t_{1/2}$ < 2 s). Proton symport occurs during lactose efflux, resulting in the transient formation of a membrane potential (ΔΨ, interior negative) as demonstrated by efflux-dependent accumulation of rubidium (in the presence of valinomycin) and active transport of proline, both of which are abolished by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone. Moreover, the magnitude of the ΔΨ generated increases with pH in much the same manner as the rate of lactose efflux, suggesting tight coupling between the processes. Comparison of the efflux and exchange reactions suggests that the rate-determining step for efflux involves the return of the unloaded carrier to the inner surface of the membrane and that either loss of the symported proton from the carrier or translocation of the unloaded carrier may be limiting. Counterflow experiments conducted at various pH values reveal that external lactose affects proton loss from the carrier. When external lactose is present at concentrations below the apparent K_m of the carrier, counterflow is pH dependent and decreases from pH 5.5 to 7.5, indicating that deprotonation of the carrier occurs frequently under these conditions to limit the counterflow process. When the external lactose concentration is saturating, however, counterflow is unaffected by pH. Moreover, the transient formation of ΔΨ observed during lactose efflux is abolished under these conditions. The observations are consistent with an ordered mechanism for efflux whereby lactose is released first, followed by loss of a proton. In addition, it is postulated that the loaded carrier recycles in the protonated form during counterflow and exchange.

The chemiosmotic hypothesis proposed by Mitchell (1961, 1966, 1968, 1973, 1977) has stimulated widespread interest in the role of the "protonmotive force" in bioenergetic processes. According to this hypothesis, energy derived from respiration or photochemical reactions is transformed into a transmembrane electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$)[1] that represents the immediate driving force for the synthesis of ATP, active transport, and certain other energy-dependent processes. In 1963, Mitchell postulated explicitly that $\Delta\bar{\mu}_{H^+}$ drives the accumulation of β-galactosides in *Escherichia coli* and that the active transport of these substrates occurs via coupled movements with protons (i.e., symport) (Mitchell, 1963). By this means, a β-galactoside-specific membrane protein (the product of the *lac y* gene) translocates substrate with protons, the substrate moving against and the proton(s) with their respective electrochemical gradients.

E. coli membrane vesicles which retain the same configuration as the plasma membrane in the intact cell (Owen & Kaback, 1978, 1979a,b) have contributed increasingly to an understanding of chemiosmotic phenomena, particularly with respect to active transport (Kaback, 1974, 1976; Kaback et al. 1977; Harold, 1976; Konings & Boonstra, 1977). Recent experiments with this system have clarified considerably the relationship between the electrical and chemical components of $\Delta\bar{\mu}_{H^+}$ and the accumulation of specific transport substrates (Ramos et al., 1976; Ramos & Kaback, 1977a–c; Tokuda & Kaback, 1977). Moreover, the concept of proton/substrate symport has been supported by the demonstration that active lactose accumulation leads to partial collapse of the electrical potential (ΔΨ) (Schuldiner & Kaback, 1975) and the pH gradient (ΔpH) (Ramos & Kaback, 1977b) across the vesicle membrane, by studies of proton/substrate stoichiometry (Ramos & Kaback, 1977c), and by monitoring *lac* carrier function in response to ΔΨ and ΔpH with fluorescent and photoreactive probes (Schuldiner & Kaback, 1977; Kaczorowski et al., 1979).

Several laboratories have investigated proton/β-galactoside symport in intact *E. coli* by studying substrate-induced proton fluxes. West & Mitchell (West, 1970; West & Mitchell, 1972, 1973) demonstrated that addition of lactose to deenergized cells causes alkalinization of the medium, and Wilson's laboratory showed that transient accumulation of methyl 1-thio-β-D-galactopyranoside (TMG) can be coupled to an artificially generated ΔΨ (interior negative) or ΔpH (interior alkaline) in starved cells (Flagg & Wilson, 1977). Similarly, Flagg & Wilson (1978) observed that TMG efflux can drive proline uptake due to the ability of the *lac* carrier to catalyze proton translocation as TMG moves down its concentration gradient. Although these studies provide strong evidence for proton/substrate symport, the mechanism of the reaction is unknown.

One limitation inherent in studying passive processes in intact cells is difficulty achieving the completely deenergized state that is essential for quantitation of passive flux data. In

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[1] Abbreviations used: $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; ΔΨ, membrane potential; ΔpH, pH gradient; TMG, methyl 1-thio-β-D-galactopyranoside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\bar{\mu}_{Pro}$, concentration gradient of proline expressed in millivolts.

0006-2960/79/0418-3691$01.00/0

view of this limitation, we have studied proton/β-galactoside symport in membrane vesicles since they are totally deenergized unless appropriate electron donors are added exogenously. In this and the following paper, passive lactose movements were used to drive turnover of the *lac* carrier protein in order to study the symport reaction and to probe the mechanism of translocation.

Experimental Section

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) and mL 30 ($i^+z^+y^+a^+$) were grown on a minimal salts medium (Davis & Mingioli, 1959) containing 1.0% disodium succinate (hexahydrate), and membrane vesicles were prepared as described previously (Kaback, 1971; Short et al., 1975).

For experiments at various pHs, vesicles prepared in 0.1 M potassium phosphate (pH 6.6) and stored in liquid N_2 were thawed and resuspended in a 50-fold excess of a given buffer at the desired pH. After standing at room temperature for 30 min, the vesicles were collected by centrifugation (45000*g* for 30 min), washed once with the same buffer, and resuspended in a minimal volume to give as concentrated a suspension as possible (25–35 mg of protein/mL). A small aliquot of labeled or unlabeled lactose was added to this concentrated suspension to yield a final concentration of 10 mM, and the sample was incubated at room temperature for 3 h to allow lactose to equilibrate with the intravesicular space.

Transport Assays. Efflux, exchange, and counterflow assays were performed in the following way. An aliquot of vesicles that had been concentrated and equilibrated with lactose was drawn into a 10-μL syringe (Hamilton No. 801) and then diluted rapidly 200-fold into a given buffer in the presence or absence of an appropriate substrate as indicated. The suspension was immediately agitated by means of a maximixer (Sybron-Thermoline Corp.), and at given times 1.5 mL of 0.1 M lithium chloride, with or without 0.1 M potassium phosphate (pH 5.5), was added rapidly (i.e., the pH of the quench solution made no significant difference). The sample was filtered immediately, and the filter was washed once with the same salt solution. Cellulose acetate filters (0.45 μm, Millipore Filter Corp.) were used for assays of cation uptake, while nitrocellulose filters (0.45 μm) were employed for all other assays. Radioactivity was determined by liquid scintillation spectrometry with typical efficiencies of 80–90% for ^{14}C and ^{86}Rb.

Calculations. The internal concentration of solutes accumulated by the vesicles was calculated by using a value of 2.2 μL of intravesicular fluid/mg of membrane protein (Kaback & Barnes, 1971). The concentration gradient determined from ratios of internal and external concentrations was then converted to millivolts by using the Nernst equation [mV = −59 log (concentration gradient)].

Protein. Protein was measured as described by Bradford (1976) by using bovine serum albumin as a standard.

Materials

Rubidium chloride and [U-^{14}C]proline were purchased from New England Nuclear and [1-^{14}C]lactose from Amersham-Searle. [^{3}H]Tetraphenylphosphonium bromide was prepared by The Isotope Synthesis Group at Hoffmann-La Roche, Inc., under the direction of Dr. Arnold Liebman as described (S. Ramos, L. Patel, H. R. Kaback, unpublished experiments), and 3,3′-dipentyloxacarbocyanine iodide was the generous gift of Dr. Hansruedi Kiefer of The Basel Institute of Immunology. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and valinomycin were obtained from Calbiochem.

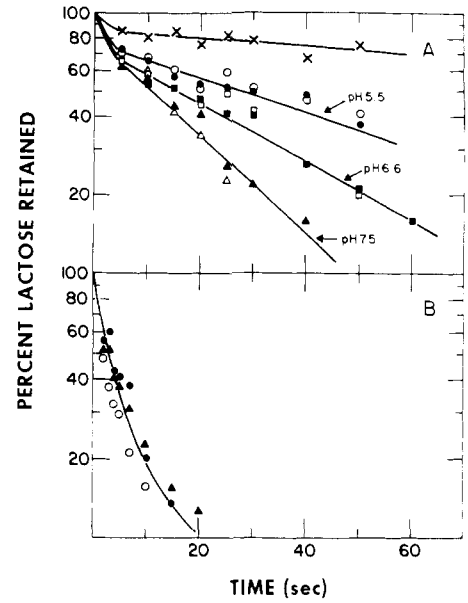

FIGURE 1: Effect of pH on lactose efflux (A) and exchange (B). (A) Membrane vesicles prepared from *E. coli* ML 308-225 and uninduced ML 30 were concentrated to 35 mg of protein/mL in 0.1 M potassium phosphate at the pH values given. A small aliquot of [1-^{14}*C*]lactose (3 mCi/mmol) was added to each suspension to a final concentration of 10 mM. After equilibrating for 3 h at room temperature, 2-μL aliquots were rapidly diluted into 400 μL of 0.1 M potassium phosphate at the appropriate pH at 25 °C. At the times shown, samples were diluted rapidly with 1.5 mL of 0.1 M potassium phosphate (pH 5.5) containing 0.1 M lithium chloride and immediately filtered as described under Methods. The experiments with *E. coli* ML 308-225 were carried out at pH 5.5 (●), 6.6 (■), and 7.5 (▲), and the open symbols represent the same experiments performed in the presence of 10 μM CCCP (added to the vesicles and dilution buffer from a concentrated stock solution). Similar experiments were carried out with uninduced *E. coli* ML 30 membrane vesicles at the three given pHs (×). Data are expressed as a percentage of lactose retained by using zero time points for normalization. The zero time values were determined by dilution of equilibrated vesicles directly into 0.1 M potassium phosphate (pH 5.5) containing 0.1 M lithium chloride, followed by immediate filtration, and absolute values of 22 nmol/mg of protein ± 10% were obtained. Each time point represents the average of two assays. (B) Experiments were carried out as described in part A, but 10 mM unlabeled lactose was included in the medium into which the concentrated, equilibrated vesicles were diluted and the temperature was maintained at 18 °C. Experiments were performed at pH 5.5 (●), 6.6 (○), and 7.5 (▲). Data are normalized to zero time points determined as described in part A, and each point represents the average of two assays.

Results

pH Dependence of Lactose Efflux and Exchange. When concentrated *E. coli* ML 308-225 membrane vesicles are equilibrated with 10 mM [1-^{14}C]lactose [a concentration that is approximately fivefold over the K_m for passive, carrier-mediated efflux (Kaczorowski et al., 1979)] and then diluted rapidly 200-fold into media devoid of lactose, the rate of efflux is first order[2] and increases as a function of pH ($t_{1/2}$ = 45, 27, and 15 s at pH 5.5, 6.6, and 7.5, respectively) (Figure 1A). Moreover, the protonophore CCCP does not influence the rate of efflux at any of the pH values tested. The increase in efflux with pH is not due simply to an increase in passive membrane permeability as evidenced by studies with vesicles that do not

[2] The reason for the apparent initial, rapid loss of lactose is unknown and varies from one experiment to another. However, it is clear that the phenomenon is unrelated to carrier-mediated lactose efflux since it is also observed with vesicles prepared from uninduced *E. coli* ML 30 (Figure 1A).

contain the *lac* transport system. When vesicles prepared from uninduced *E. coli* ML 30 are subjected to identical treatment, the rate of lactose efflux is very slow, exhibiting a $t_{1/2}$ of more than 5 min that is unaffected by pH (Figure 1A).

Kinetically, efflux consists of a minimum of four steps: (1) binding of substrate to the carrier on the inner surface of the membrane; (2) translocation across the membrane; (3) release of substrate; and (4) return of the unloaded carrier. In order to determine whether the return of the unloaded carrier is affected by pH, we studied the loss of intravesicular [1-^{14}C]lactose under conditions where the external medium contained equimolar concentrations of unlabeled lactose (i.e., under conditions of exchange). Thus, vesicles equilibrated with 10 mM [1-^{14}C]lactose were diluted 200-fold into media containing 10 mM unlabeled lactose, and loss of labeled sugar from the intravesicular pool was monitored at 18 °C so that rates could be measured accurately (Figure 1B). As shown, the rate of loss under these conditions (i.e., exchange) is very fast ($t_{1/2}$ = 3 s) relative to the rate of efflux and exhibits no dependence on pH. At 25 °C (i.e., the temperature at which the efflux measurements were made; Figure 1A), although exchange is so rapid ($t_{1/2}$ < 2 s) that accurate determinations are prohibited, it is clear that the rate of exchange is at least 10-fold greater than the rate of efflux and that it is insensitive to pH. The results imply that a step involving the return of the unloaded carrier is rate-determining for efflux since the rate of translocation is markedly enhanced when the carrier is occupied by substrate on the external surface of the membrane.

Generation of ΔΨ during Carrier-Mediated Lactose Efflux. If the *lac* carrier protein catalyzes the simultaneous translocation of protons and lactose, an increase in proton efflux should be induced by the movement of lactose down its concentration gradient, and this should be reflected by the generation of a ΔΨ (interior negative) and/or a ΔpH (interior alkaline). When ML 308-225 vesicles prepared in sodium phosphate are equilibrated with 10 mM lactose, treated with excess valinomycin, and then diluted 200-fold into sodium phosphate containing ^{86}RbCl, the results shown in Figure 2 are obtained. Clearly, transient ^{86}Rb uptake is observed at pH 5.5, 6.6, and 7.5, demonstrating that a ΔΨ (interior negative) is indeed established (Schuldiner & Kaback, 1975). Moreover, at each pH studied, the time course of the reaction reflects the rate of lactose efflux. That is, the rates of ^{86}Rb uptake, the maximum levels of accumulation, and the rates of decay increase with pH in a manner that approximates the increase in lactose efflux with pH (cf. Figure 1A). In addition, lactose efflux-induced ^{86}Rb uptake at each pH tested is abolished by CCCP, and importantly by addition of lactose to the medium. Finally, ^{86}Rb uptake under these conditions is not observed with vesicles that are treated with 15 μM methyl methanethiosulfonate (Smith et al., 1975), a rapidly reacting sulfhydryl reagent that blocks *lac* carrier function (G. J. Kaczorowski & H. R. Kaback, unpublished experiments). It is apparent, therefore, that downhill translocation of lactose via the *lac* carrier protein leads to the generation of a ΔΨ (interior negative). On the other hand, all attempts to measure pH changes directly using a sensitive, rapidly responding pH electrode were negative.

Calculation of ΔΨ values from the time points at which the Rb concentration gradients are maximal (Figure 2) reveals that the magnitude of ΔΨ increases with pH from a value of −31 mV at pH 5.5 to −44 mV at pH 6.6 to −51 mV at pH 7.5. Although data will not be presented, these values for ΔΨ do not appear to be limited by passive membrane permeability

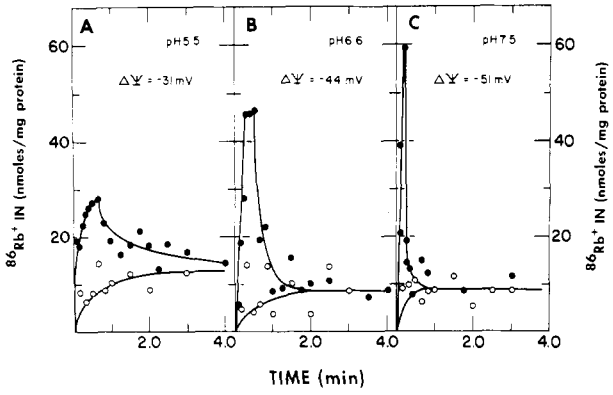

FIGURE 2: Generation of ΔΨ (interior negative) during lactose efflux in ML 308-225 membrane vesicles. Vesicles were concentrated to 28 mg of protein/mL in 0.1 M sodium phosphate at given pH values and equilibrated with 10 mM lactose as described in Methods. In addition, valinomycin was added to a final concentration of 2 nmol/mg of protein. Aliquots (2 μL) of vesicles were then diluted 200-fold into 0.1 M sodium phosphate containing 3.8 mM ^{86}RbCl (7.5 mCi/mmol) at pH 5.5 (A), 6.6 (B), and 7.5 (C) (closed symbols). ^{86}Rb accumulation was monitored by filtration with cellulose acetate filters by using 0.1 M lithium chloride to terminate the reaction (Lombardi et al., 1973). Identical experiments were also performed in the presence of 10 μM CCCP and with 10 mM lactose present in the medium into which the concentrated, equilibrated vesicles were diluted (open symbols). Concentration gradients were calculated by using a value of 2.2 μL/mg of protein for the intravesicular volume (Kaback & Barnes, 1971), and ΔΨ was determined from the Nernst equation ($\Delta\Psi = -59 \log([Rb^+_{in}]/[Rb^+_{out}])$). Each time point represents the average of five assays.

to protons or other ions. Thus, similar values are obtained under the same conditions with vesicles that were treated with *N,N'*-dicyclohexylcarbodiimide (Patel et al., 1975) and when the experiments were carried out in a weakly permeant buffer system [i.e., choline/2-(*N*-morpholino)ethanesulfonic acid]. It is also noteworthy that although ^{86}Rb uptake was chosen as a probe for ΔΨ because its response is rapid in the presence of excess valinomycin (Lombardi et al., 1973; Schuldiner & Kaback, 1975), similar results were obtained at pH 6.6 with two other probes that respond relatively rapidly to ΔΨ: [^{3}H]tetraphenylphosphonium, a permeant lipophilic cation (S. Ramos, L. Patel, and H. R. Kaback, unpublished experiments), and 3,3'-dipentyloxacarbocyanine iodide, a fluorescent cyanine dye (Sims et al., 1974; Bramhall et al., 1976).

The ability of external lactose to inhibit the generation of ΔΨ (interior negative) induced by lactose efflux is related to the apparent affinity of the carrier on the external surface of the membrane and not to diminution of the lactose concentration gradient across the membrane (Figure 3). In these experiments, generation of ΔΨ was investigated under conditions identical with those described in Figure 2, except that increasing concentrations of lactose were added to the external medium. When the maximal ΔΨ generated is plotted as a function of the external lactose concentration, it is clear that 50% inhibition is observed at an external lactose concentration of ~0.3 mM and that complete inhibition occurs at ~1 mM lactose. Thus, the ability of lactose to inhibit the generation of ΔΨ under these conditions correlates reasonably well with the apparent K_m of the carrier and not with the lactose gradient across the membrane (i.e., even at 1 mM lactose, there is a 10-fold concentration gradient across the vesicle membrane).

Since lactose efflux clearly leads to the generation of ΔΨ (interior negative) and artificial imposition of a ΔΨ of the same polarity has been shown to drive proline uptake in the vesicle system (Hirata et al., 1973, 1974), it follows that lactose efflux should drive proline accumulation. Evidence supporting this

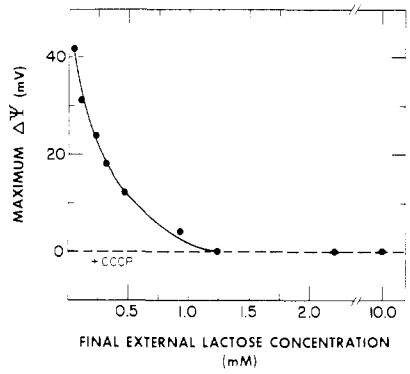

FIGURE 3: Effect of external lactose on $\Delta\Psi$ (interior negative) generated during lactose efflux. Vesicles were concentrated to 40 mg of protein/mL in 0.1 M choline phosphate, pH 6.6, and then equilibrated with 10 mM lactose for 3 h at room temperature. Valinomycin was also added to a final concentration of 2 nmol/mg of protein. Aliquots (2 μL) of vesicles were then diluted 200-fold into 0.1 M choline phosphate, pH 6.6, containing 3.6 mM ^{86}RbCl (8.2 mCi/mmol) and various concentrations of lactose. ^{86}Rb accumulation during a 30-s time period was monitored by filtration as described in Methods. Similar experiments were performed in the presence of 10 μM CCCP. The concentration gradients for ^{86}Rb uptake and the corresponding values of membrane potential were calculated as described in the legend to Figure 2. Each point reported represents the average of eight separate determinations.

Table I: pH Dependence of Lactose Efflux-Stimulated Proline Uptake in *E. coli* ML 308-225 Membrane Vesicles[a]

parameter	pH 5.5	pH 6.6	pH 7.5
time at which peak accumulation is observed (s)	~40	~30	~20
peak accumulation (pmol/mg of protein)	55.7	70.6	108
$\Delta\bar{\mu}_{Pro}$ (mV)	−24	−30	−41
peak accumulation in the presence of 10 $\bar{\mu}$M CCCP (pmol/mg of protein)	26	25	23
$\Delta\mu_{Pro}$ in the presence of 10 μM CCCP (mV)	<−4	<−3	<−1

[a] Vesicles were concentrated to 30–33 mg of protein/mL in 0.1 M potassium phosphate at pH 5.5, 6.6, or 7.5. After equilibration with 10 mM lactose, 2-μL aliquots of the membrane suspension were diluted into 400 μL of 0.1 M potassium phosphate at the appropriate pH containing 10.1 μM [U-^{14}C]-L-proline (240 mCi/mmol). The time course of amino acid uptake was determined by filtration as described in Methods, and peak accumulation was recorded (cf. Figure 3). The concentration gradient for proline was calculated from this value and converted into millivolts (mV) (i.e., $\Delta\bar{\mu}_{Pro}$) by using the Nernst equation [mV = −59 log (concentration gradient)]. Similar experiments were carried out in the presence of 10 μM CCCP.

suggestion has been presented with intact cells (Flagg & Wilson, 1978), and experiments with membrane vesicles that confirm and extend the observations are presented in Figure 4 and Table I. When ML 308-225 vesicles equilibrated with lactose at pH 6.6 are diluted into media containing [U-^{14}C]-L-proline, the amino acid is transiently accumulated threefold over the equilibrium concentration, and the effect is blocked by CCCP (Figure 4). Moreover, proline accumulation under these conditions is abolished by addition of equimolar concentrations of lactose to the medium or by addition of saturating concentrations of *p*-nitrophenyl α-D-galactopyranoside (Rudnick et al., 1976).

Data from pH studies of lactose efflux-induced proline uptake are presented in Table I. At each pH tested, translocation of lactose down a concentration gradient drives proline accumulation and the time courses observed reflect the rate of lactose efflux and ^{86}Rb uptake as functions of pH (cf. Figures 1A and 2). The concentration gradient established for proline increases with pH, an observation that is consistent

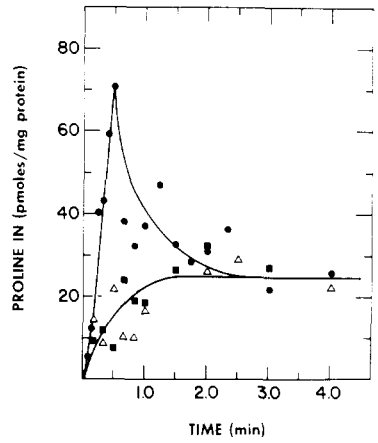

FIGURE 4: Lactose efflux-stimulated proline accumulation. *E. coli* ML 308-225 membrane vesicles were concentrated to 30 mg of protein/mL in 0.1 M potassium phosphate (pH 6.6) and equilibrated with 10 mM lactose as described in Methods. Aliquots (2 μL) were diluted into 400 μL of 0.1 M potassium phosphate (pH 6.6) containing 10 μM [U-^{14}C]-L-proline (240 mCi/mmol), and accumulation was assayed as described in Methods (●). Similar experiments were performed in the presence of 10 μM CCCP (△) or with 0.3 mM *p*-nitrophenyl α-D-galactopyranoside (■) present in the medium into which the concentrated, equilibrated vesicles were diluted. Although not shown, the same results were obtained when 10 mM lactose was added to the medium.

with the increase in $\Delta\Psi$ with pH, and accumulation is completely blocked by CCCP at each pH tested. Although the coupling between lactose efflux and proline accumulation is technically difficult to quantitate, calculation of $\Delta\bar{\mu}_{Pro}$ yields values that are in accord with $\Delta\Psi$ (Table I).

Effect of pH on Lactose Counterflow. When intact *E. coli* containing the β-galactoside transport system are loaded with appropriate transport substrates and subsequently diluted into media containing the same substrate in radioactive form, transient influx of the radioactive substrate is observed (Wong & Wilson, 1970; Bentaboulet & Kepes, 1977). The phenomenon is called "entrance counterflow", and its efficiency, in kinetic terms, is due in part to the frequency with which the carrier returns from the outer to the inner surface of the membrane in the loaded vs. the unloaded form. When ML 308-225 vesicles equilibrated with 10 mM lactose are diluted 200-fold into media containing 0.4 mM [1-^{14}C]lactose, a concentration 2 times higher than the K_m for influx under energized conditions (Barnes & Kaback, 1970; Kaczorowski et al., 1979), rapid transient uptake of radioactive substrate is observed at pH 5.5, 6.6, and 7.5 (Figure 5). Under these conditions (i.e., saturating external lactose concentrations), the initial rate and peak level of radioactivity achieved are independent of pH, although the rate of decay of the "overshoot" exhibits a pH dependence similar to that observed for lactose efflux (cf. Figure 1A). With an intravesicular volume of 2.2 μL/mg of membrane protein (Kaback & Barnes, 1971), it can be calculated that the intravesicular concentration of [1-^{14}C]lactose at the peak height is ~10 mM at each pH studied (i.e., the coupling efficiency between efflux of unlabeled lactose and influx of radioactive lactose is 1:1). Finally, it is apparent that counterflow under these conditions is completely insensitive to concentrations of CCCP that dissipate $\Delta\bar{\mu}_{H^+}$ (Ramos et al., 1976).

In contrast to the experiments presented in Figure 5, when counterflow is repeated under identical conditions with subsaturating external lactose concentrations, the results are strikingly different. Thus, in the studies presented in Figure 6, the vesicles were loaded with 10 mM lactose again and diluted 200-fold, but the external [1-^{14}C]lactose concentration

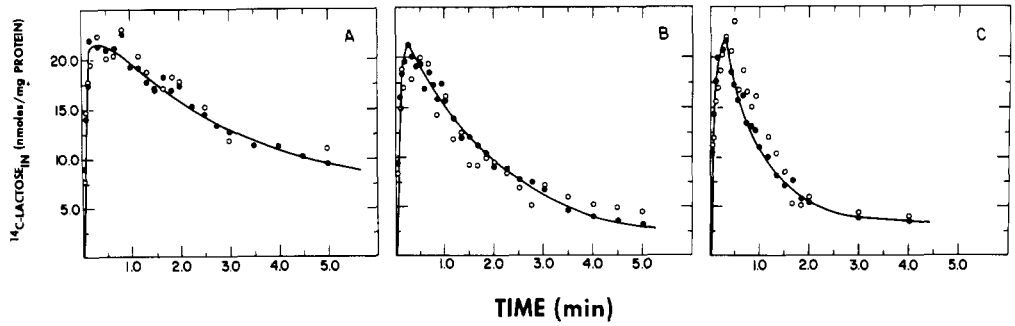

FIGURE 5: pH dependence of lactose counterflow at saturating external lactose concentrations. *E. coli* ML 308-225 membrane vesicles were concentrated to 27.2 mg of protein/mL in 0.1 M potassium phosphate at given pH values and equilibrated with 10 mM lactose as described in Methods. Aliquots (2 μL) were then diluted into 400 μL of 0.1 M potassium phosphate at the same pH containing 0.43 mM [1-^{14}C]lactose (10 mCi/mmol), and counterflow was assayed (●). The experiments were conducted at pH 5.5 (A), 6.6 (B), and 7.5 (C). Parallel experiments were performed in the presence of 10 μM CCCP (○).

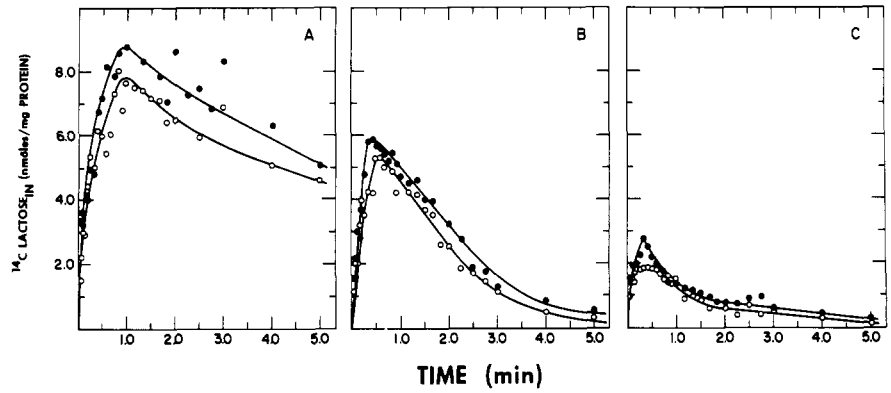

FIGURE 6: pH dependence of lactose counterflow at subsaturating external lactose concentrations. *E. coli* ML 308-225 membrane vesicles were concentrated to 35 mg of protein/mL in 0.1 M potassium phosphate at given pH values and equilibrated with 10 mM lactose as described in Methods. Aliquots (2 μL) were then diluted into 400 μL of 0.1 M potassium phosphate at the same pH containing 0.075 mM [1-^{14}C]lactose (18.5 mCi/mmol), and counterflow was assayed (●). The experiments were conducted at pH 5.5 (A), 6.6 (B), and 7.5 (C). Parallel experiments were performed in the presence of 10 μM CCCP (○).

was 0.075 mM, a concentration that is about threefold lower than the K_m for influx under energized conditions (Barnes & Kaback, 1970; Kaczorowski et al., 1979). Clearly, under these circumstances, the overshoot phenomenon observed is now markedly sensitive to pH, and the peak level of radioactivity decreases with pH 5.5 to 7.5. The maximum intravesicular concentration achieved at the peak of the overshoot is reduced from 21.5 nmol/mg of protein (Figure 5) to 8.8 nmol/mg of protein at pH 5.5 (a coupling efficiency of 0.4:1), 6.0 nmol/mg of protein at pH 6.6 (a coupling efficiency of 0.27:1), and 2.8 nmol/mg of protein at pH 7.5 (a coupling efficiency of 0.13:1). In addition, there is a small, but possibly significant, inhibitory effect of CCCP, suggesting that an efflux-generated $\Delta\Psi$ may make a minor contribution to the overall phenomenon under these conditions.

Discussion

The experiments presented in this paper strongly support the concept that proton/substrate symport is the basic mechanism of β-galactoside transport in *E. coli* (Mitchell, 1963) and provide additional insight into the nature of the catalytic role of the *lac* carrier protein. Carrier-mediated lactose efflux down a chemical gradient, exchange, and entrance counterflow were used to drive carrier turnover in order to study the translocation reaction in the absence of an imposed $\Delta\bar{\mu}_{H^+}$. Utilizing these experimental manipulations in membrane vesicles isolated from *E. coli*, we have presented evidence consistent with the following conclusions: (1) the *lac* carrier protein catalyzes the coupled movements of lactose and protons; (2) the rate-determining step for lactose translocation down a chemical gradient is associated with the return of the unloaded carrier to the inner surface of the membrane, and deprotonation of the *lac* carrier protein on the outer surface may be limiting; and (3) during exchange and entrance counterflow, the carrier may recycle without being deprotonated.

Clearly, the transient generation of a $\Delta\Psi$ (interior negative) observed during carrier-mediated lactose efflux provides strong evidence for coupled movement of a charged species with lactose. The phenomenon has been documented extensively by demonstrating transient uptake of ^{86}Rb in the presence of valinomycin and transient accumulation of proline, both of which increase with pH in a manner that reflects the increase in the rate of lactose efflux with pH. Moreover, both processes are abolished by the protonophore CCCP, and efflux-induced Rb uptake is blocked by a sulfhydryl reagent that inactivates the *lac* carrier protein, emphasizing further the involvement of the *lac* carrier. In addition, limited studies on the uptake of [^{3}H]tetraphenylphosphonium and quenching of 3,3′-dipentyloxacarbocyanine fluorescence provide further support for the argument. Although it has not been possible to elicit lactose efflux-induced changes in external pH, it seems highly likely that the charged species involved is hydrogen ion since the $\Delta\Psi$ created during efflux is abolished by CCCP and is independent of the ionic composition of the medium [i.e., ^{86}Rb uptake is observed in either sodium phosphate or choline/2-(*N*-morpholino)ethanesulfonic acid, and proline uptake as well as [^{3}H]tetraphenylphosphonium uptake and quenching of 3,3′-dipentyloxacarbocyanine fluorescence is observed in potassium phosphate]. In addition, this conclusion is consistent with studies in intact cells (West, 1970; West & Mitchell, 1972, 1973; Flagg & Wilson, 1978; Bentaboulet et al., 1979).

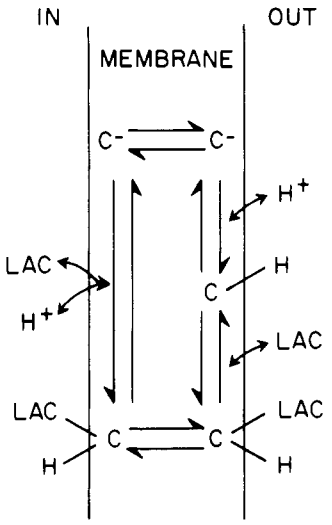

FIGURE 7: Schematic representation of reactions involved in lactose efflux. C represents the lactose carrier protein. The order of substrate binding at the inner surface of the membrane is not implied.

On the other hand, it should be emphasized that none of these studies can differentiate between proton/lactose symport and hydroxide/lactose antiport, a matter that is obviously important when considering the functional groups that may be involved in charge translocation.

Comparison of efflux and exchange rates from the data presented in Figure 1 indicates that the exchange process is very rapid, at least 10 times faster than efflux. Thus, the rate-determining step for efflux must involve a reaction that corresponds to the return of the unloaded carrier to the inner surface of the membrane since this is the only step by which efflux and exchange differ. Assuming that the charged species translocated with lactose is protons (i.e., proton/lactose symport) and that loss of both substrates from the carrier is necessary for reinitiation of an efflux cycle (cf. Figure 7 for a schematic representation of the reactions involved), we might expect the external pH to influence the rates of turnover in either of two ways. First, deprotonation of the carrier could be slow and thereby limit the overall efflux rates in a pH-dependent fashion. Although proton transfers between accessible amino acid residues and water in soluble enzyme systems are typically very fast (Eigen, 1963), little is known about such processes with proteins embedded in a hydrophobic milieu. Alternatively, pH could alter the equilibrium between protonated and unprotonated forms of the carrier (i.e., two different conformations), favoring the presence of the unprotonated form at higher pH. Since it is presumed that only the deprotonated carrier can recycle, the rate of efflux would be partially controlled by external pH and the rate-limiting step might then involve "movement" of the unloaded carrier to the inner surface of the membrane. The observation that the rate of lactose efflux increases with pH (Figure 1A) is consistent with either of these possibilities. On the other hand, if deprotonation of the carrier is not obligatory for exchange, protons might remain bound to the carrier during this reaction, rendering it insensitive to pH. Evidence supporting this argument is presented in Figure 1B. Given an ordered mechanism whereby the carrier releases lactose first, followed by loss of a proton, deprotonation and/or return of the unloaded carrier could be slow and appear as the limiting step for carrier turnover during efflux.

The counterflow experiments presented in Figures 5 and 6 are consistent with the ordered mechanism proposed. When entrance counterflow is carried out at saturating external lactose concentrations, variations in pH have no effect on the magnitude of the overshoot phenomenon described. However, when external lactose is limiting, counterflow is progressively inhibited as the pH is increased. These results can be interpreted in the following manner. As efflux is initiated, lactose and protons bind to the carrier on the inner surface of the membrane and are translocated to the exterior. Lactose is released from the carrier, but in the presence of excess labeled substrate rebinding and influx occur rapidly before deprotonation can occur. Thus, under these circumstances, proton release is infrequent and pH has no effect on the overall phenomenon. In contrast, when [^{14}C]lactose is limiting externally, rebinding of radioactive substrate is less frequent, allowing deprotonation and return of the unloaded carrier. As a result, influx of [^{14}C]lactose is diminished and counterflow is inhibited. Moreover, when the pH is increased, deprotonation and return of the unloaded carrier are enhanced, resulting in further diminution of counterflow. Inhibition of lactose efflux-generated $\Delta\Psi$ formation [i.e., ^{86}Rb uptake in the presence of valinomycin (Figures 2 and 3) and proline uptake (Figure 4)] by external lactose and/or *p*-nitrophenyl α-D-galactopyranoside is now readily understood. When these substrates are present externally at saturating concentrations, release of lactose from the carrier and rebinding of substrate occur rapidly before deprotonation can occur, and the ability of the system to generate a $\Delta\Psi$ (interior negative) is abolished.

Finally, it should be noted that some of the findings can be used to argue that release of lactose and protons by a random mechanism is probably unlikely. If protons are released before lactose during a significant number of turnovers, (1) exchange would not be completely insensitive to pH, (2) it would be difficult to explain the ability of external lactose at concentrations approximating the apparent K_m of the carrier to completely inhibit the generation of $\Delta\Psi$ during lactose efflux, and (3) CCCP would be expected to inhibit counterflow to a significant extent, especially under conditions where the external lactose concentration is limiting.

If return of the unloaded carrier to the inner surface of the membrane is the rate-determining step for lactose efflux down a concentration gradient, conditions that perturb this step should influence the overall rate of lactose efflux. Moreover, under the same conditions, the rate of exchange should be unaffected. Results that support and extend these conclusions are presented in the following paper (Kaczorowski et al., 1979).

Acknowledgments

We are indebted to Dr. Hagai Rottenberg for calling our attention to the paper by Bentaboulet & Kepes (1977) and thus stimulating the inception of these experiments. We also thank Dr. Dan E. Robertson for many useful discussions during the course of this work.

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Mechanism of Lactose Translocation in Membrane Vesicles from *Escherichia coli*. 2. Effect of Imposed $\Delta\Psi$, ΔpH, and $\Delta\bar{\mu}_{H^+}$[†]

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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* ML 308-225 vesicles leads to a marked, transient increase in the fluorescence of 6′-(*N*-dansyl)aminohexyl 1-thio-β-D-galactopyranoside. The maximum increase in fluorescence appears to be a linear function of the magnitude of the imposed $\Delta\Psi$ or ΔpH, and the effect of each parameter is additive. Imposition of $\Delta\Psi$ or ΔpH also alters the rate of carrier-mediated lactose efflux from the intravesicular pool, and the effects are dependent upon the polarity of the imposed $\Delta\Psi$ or ΔpH. The rate of efflux is diminished with $\Delta\Psi$ (interior negative) or ΔpH (interior alkaline) and enhanced with $\Delta\Psi$ (interior positive) or ΔpH (interior acid). These effects are also additive, and importantly kinetic experiments demonstrate that $\Delta\Psi$ and ΔpH alter the maximum velocity of efflux without a significant effect on the apparent K_m of the process. Strikingly, moreover, imposition of $\Delta\Psi$, ΔpH, or $\Delta\bar{\mu}_{H^+}$ of either polarity has no effect whatsoever on the rate of exchange. The data provide support for the suggestion [Kaczorowski, G. J., & Kaback, H. R. (1979) *Biochemistry* (preceding paper in this issue)] that the rate-limiting step for carrier-mediated lactose efflux down a concentration gradient involves a step that is associated with the return of the carrier to the inner surface of the membrane. In addition, the results are consistent with the notion that the loaded carrier (i.e., the ternary complex between the carrier, protons, and lactose) is neutral while the unloaded carrier is negatively charged. Finally, comparative studies of the effects of $\Delta\Psi$ and ΔpH on influx and efflux demonstrate that the translocation reactions catalyzed by the *lac* carrier are kinetically asymmetrical.

In the preceding paper (Kaczorowski & Kaback, 1979), evidence supporting the concept that carrier-mediated lactose efflux down a concentration gradient involves proton/lactose symport is presented. In addition, some of the observations stimulated the following suggestions: (1) lactose efflux is an ordered reaction in which dissociation of lactose from the porter on the outer surface of the membrane precedes the loss of protons; (2) a step associated with the return of the unloaded porter to the inner surface of the membrane is limiting for efflux; and (3) the loaded carrier recycles in the protonated form during exchange and counterflow. In the experiments presented here, the reactions catalyzed by the *lac* carrier protein were studied by monitoring the effect of imposed $\Delta\Psi$'s and ΔpH's on influx, efflux, and exchange. The results support

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0006-2960/79/0418-3697$01.00/0