# Mechanism of Lactose Translocation in Proteoliposomes Reconstituted with *lac* Carrier Protein Purified from *Escherichia coli*. 1. Effect of pH and Imposed Membrane Potential on Efflux, Exchange, and Counterflow†

Maria Luisa Garcia, Paul Viitanen, David L. Foster,‡ and H. Ronald Kaback*

ABSTRACT: Proteoliposomes reconstituted with purified *lac* carrier by octyl glucoside dilution and freeze–thaw/sonication [Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1981) *J Biol. Chem. 256*, 11804; Foster, D. L., Garcia, M. L., Newman, M. J., Patel, L., & Kaback, H. R. (1982) *Biochemistry*, *21*, 5634] are unilamellar, 50–150 nm in diameter, and impermeable to ions. By use of this preparation, carrier-mediated lactose efflux down a concentration gradient was used to probe the mechanism of β-galactoside translocation. The maximum rate of efflux is pH dependent, increasing about 20-fold from pH 5.5 to 7.5. 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 extremely fast relative to efflux. Proton symport occurs during lactose efflux, resulting in transient formation of a membrane potential ($\Delta\psi$, interior negative). Thus, the ionophores valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone enhance the rate of efflux, and the proteoliposomes exhibit efflux-dependent accumulation of rubidium in the presence of valinomycin. Furthermore, imposition of a $\Delta\psi$ (interior negative) retards the maximum rate of efflux by as much as 10-fold with no effect on apparent $K_m$. Comparison of efflux and exchange reactions suggests that the rate-determining step for efflux corresponds to a reaction involving 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 pHs 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 counterflow. In contrast, however, when external lactose is saturating, the initial rate of counterflow and the magnitude of the overshoot are essentially unaffected by pH. Moreover, the transient formation of $\Delta\psi$ observed during lactose efflux is abolished under these conditions. The results confirm and extend earlier observations with right-side-out membrane vesicles which led to the suggestion that lactose efflux is an ordered mechanism and that the loaded carrier recycles in the protonated form during counterflow and exchange [Kaczorowski, G. J., & Kaback, H. R. (1979) *Biochemistry 18*, 3691; Kaczorowski, G. J., Robertson, D. E., & Kaback, H. R. (1979) *Biochemistry 18*, 3697]. Additional support for these arguments is presented in the following paper [Viitanen, P., Garcia, M. L., Foster, D. L., Kaczorowski, G. J., & Kaback, H. R. (1983) *Biochemistry* (following paper in this issue)] where the effects of deuterium oxide on lactose transport in proteoliposomes reconstituted with purified *lac* carrier are described.

As postulated in 1963 (Mitchell, 1963), the β-galactoside transport system in *Escherichia coli* catalyzes translocation of substrate across the membrane in symport with hydrogen ion [see Kaback (1981) for a recent review]. Thus, in the presence of a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$,[1] interior negative and alkaline), lactose is accumulated against a concentration gradient at the expense of the electrical potential ($\Delta\psi$) or the pH ($\Delta$pH) gradient across the membrane (Schuldiner & Kaback, 1975; Ramos & Kaback, 1977a,b) by a mechanism that exhibits a low apparent $K_m$ (Kaczorowski et al., 1979; Robertson et al., 1980). The system also catalyzes lactose translocation down a concentration gradient, and although this process has a higher apparent $K_m$ (Kaczorowski et al., 1979; Robertson et al., 1980; Ghazi & Shechter, 1981), symport of protons is observed (West, 1970; West & Mitchell, 1972, 1973; Kaczorowski & Kaback, 1979; Patel et al., 1982; Foster et al., 1982). Thus, in the presence of $\Delta\bar{\mu}_{H^+}$, downhill translocation of protons drives uphill transport of substrate, and conversely, downhill translocation of substrate in the absence of $\Delta\bar{\mu}_{H^+}$ drives uphill transport of protons.

Mechanistic studies with right-side-out membrane vesicles (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979) suggest a model in which the overall rate of carrier-mediated lactose efflux down a concentration gradient is limited either by deprotonation of the carrier on the outer surface of the membrane or by a step corresponding to the return of the unloaded carrier to the inner surface of the membrane. In addition, the experimental observations are consistent with the following: (i) efflux occurs by an ordered mechanism in which lactose is released first from the carrier, followed by loss of the symported proton; (ii) the carrier recycles in the protonated form during exchange and counterflow; (iii) reactions catalyzed by the unloaded carrier involve net movement of negative charge.

Recently, the *lac* carrier protein was purified extensively and reconstituted into proteoliposomes in a functional state, and it was demonstrated that this protein, the product of the *lac y* gene, is the only polypeptide species in the membrane required for lactose/proton symport (Newman et al., 1981;

† From the Laboratory of Membrane Biochemistry, Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received December 14, 1982.*

‡ Present address: Donner Laboratory, University of California, Berkeley, CA 94720.

[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, the proton electrochemical gradient across the membrane; $\Delta\psi$, membrane potential; $\Delta$pH, the pH gradient across the membrane; pCMBS, *p*-(chloromercuri)benzenesulfonate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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Foster et al., 1982). Clearly, in order to ascertain whether or not the purified protein reconstituted into an artificial membrane behaves in the same manner as the protein in its native environment, more detailed studies are needed. Furthermore, since proteoliposomes are considerably more impermeable to ions and other solutes than biological membranes, studies with reconstituted *lac* carrier might provide a more rigorous system in which to test some of the hypotheses presented.

The experiments presented here indicate that purified, reconstituted *lac* carrier protein behaves in a manner remarkably similar to that observed in the bacterial membrane with respect to efflux, exchange, and counterflow. In addition, the results confirm and extend previous observations with right-side-out membrane vesicles (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979).

## Experimental Procedures

*Materials.* [$^{86}$Rb]Rubidium chloride was purchased from New England Nuclear. *p*-(Chloromercuri)benzenesulfonate (pCMBS) was obtained from Sigma. Nigericin was generously provided by Dr. John Wesley, Hoffmann-La Roche, Inc. All other materials were obtained as described previously (Foster et al., 1982).

*Purification of lac Carrier Protein.* Purification of the *lac* carrier protein was accomplished by using a modification (Foster et al., 1982) of the procedure of Newman et al. (1981).

*Reconstitution of lac Carrier.* The *lac* carrier was reconstituted into proteoliposomes by a modification (Foster et al., 1982) of the procedure described by Newman & Wilson (1980). Bath sonicated liposomes (7.5 mL), prepared as described by Newman & Wilson (1980) but without lactose, were mixed with 0.65 mL of 15% (w/v) octyl glucoside (in 50 mM potassium phosphate, pH 7.5) and 25 mL of column fraction diluted with column buffer to contain 40 μg of purified *lac* carrier protein/mL. The mixture was blended on a vortex mixer and incubated on ice for 20 min. The suspension was then squirted into a beaker containing 1135 mL of 50 mM potassium posphate, pH 7.5, and 1 mM dithiothreitol at room temperature. After detergent dilution, the suspension was stirred, and proteoliposomes were collected by centrifugation for 4 h at 35 000 rpm (143000$g_{max}$) in a Beckman Type 35 rotor, and resuspended in 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol to a final protein concentration of 0.08 mg of protein/mL (the lipid concentration was 37.5 mg/mL). The proteoliposomes were frozen and stored in liquid nitrogen.

For studies at different pHs, proteoliposomes were thawed at room temperature, sonicated for about 15 s by using a bath sonicator as described (Foster et al., 1982), and resuspended in a 100-fold excess of a given buffer at the desired pH. After the suspension stood at room temperature for 30 min, the proteoliposomes were collected by centrifugation for 1 h at 45 000 rpm (175000$g_{max}$) in a Beckman Type 50 Ti rotor and resuspended to a final protein concentration of 0.065 mg of protein/mL. A small aliquot of labeled or unlabeled lactose was added to the suspension to yield a final concentration of 10 mM, and the sample was incubated at room temperature for 1 h in order to allow lactose to equilibrate with the intravesicular space.

*Transport Assays.* Lactose efflux, exchange, and counterflow assays were performed in the following manner: An aliquot (1 μL) of proteoliposomes, equilibrated with lactose as described, was drawn into a 10-μL syringe (Hamilton No. 801) and diluted 200-fold into a given buffer in the presence or absence of lactose, as indicated. The suspension was immediately blended on a vortex mixer and incubated at 25 °C. At a given time, 3 mL of ice-cold 50 mM potassium phosphate, pH 7.5, was added, the sample was filtered immediately, and the filter (type GSTF, 0.2 μm, Millipore Filter Co.) was washed once with the same cold buffer. Radioactivity retained on the filters was determined by liquid scintillation spectrometry.

For experiments involving $^{86}Rb^+$ efflux, proteoliposomes in 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol were treated with 20 μM valinomycin and equilibrated with 7.5 mM $^{86}$RbCl (33 mCi/mmol). Equilibraton took place within 10 min. Experiments were initiated by diluting 1 μL of proteoliposomes into 200 μL of appropriate media. At given times, the reaction was quenched by addition of 3 mL of ice-cold 50 mM sodium phosphate, pH 7.5, and the reaction mixture was filtered and assayed as described above.

*Determination of Internal Volume of Proteoliposomes.* Proteoliposomes reconstituted with purified *lac* carrier protein were suspended in 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol, and valinomycin was added to a final concentration of 20 μM. The proteoliposomes were then equilibrated with either 10 mM [1-$^{14}$C]lactose (12 mCi/mmol) or 10 mM $^{86}$RbCl (40 mCi/mmol) by incubation at 25 °C for at least 1 h. At this point, aliquots (1 μL) were rapidly diluted into 3 mL of ice-cold 50 mM potassium phosphate (for [1-$^{14}$C]lactose) or 50 mM sodium phosphate (for $^{86}Rb^+$) at pH 7.5, and the samples were filtered and assayed as described in order to obtain zero time values. Other 1-μL aliquots were diluted 200-fold into 50 mM potassium phosphate, pH 7.5, and incubated at 25 °C for 60 min prior to filtration and assay (cf. Figures 3 and 4 for $Rb^+$ and lactose efflux, respectively, under the conditions described). Relative to the samples assayed at zero time, after dilution and 60-min incubation, more than 99% of the trapped radioactive solute was released. From the loss of radioactivity and the specific activity of the solute, the amount of solute lost can be determined and internal volume calculated by comparison of this value to the concentration of radioactive solute in the reaction mixture. Identical results were obtained with [1-$^{14}$C]lactose and $^{86}Rb^+$, and 1 μL of proteoliposomes containing 0.08 μg of protein and 37.5 μg of lipid had an internal volume of 0.056 ± 0.0002 μL ($N$ = 40).

*Protein Determinations.* Protein was assayed by a modification of the method of Schaffner & Weissmann (1973) with bovine serum albumin as a standard (Newman et al., 1981).

## Results

*Morphology and Ion Permeability of Proteoliposomes Reconstituted with lac Carrier Protein.* The electron micrographs shown in Figure 1 demonstrate that proteoliposomes prepared as described are unilamellar vesicles that exhibit no internal structure. Moreover, when the cross-sectional diameters of the vesicles are measured, about 80% of the proteoliposomes fall between 50 and 150 nm (Figure 2). Examination of preparations that were frozen and thawed but left unsonicated (electron micrographs not shown) exhibits cross-sectional diameters that distribute over a considerably broader range. Therefore, octyl glucoside dilution followed by freeze–thaw/sonication is the method of choice for the type of studies presented here.

When proteoliposomes equilibrated with $^{86}Rb^+$ are treated with valinomycin and diluted 200-fold into sodium phosphate, efflux of the cation occurs very slowly, and at 20 min, the proteoliposomes still retain at least 80% of the label (Figure 3). On addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which increases permeability to protons specifically,

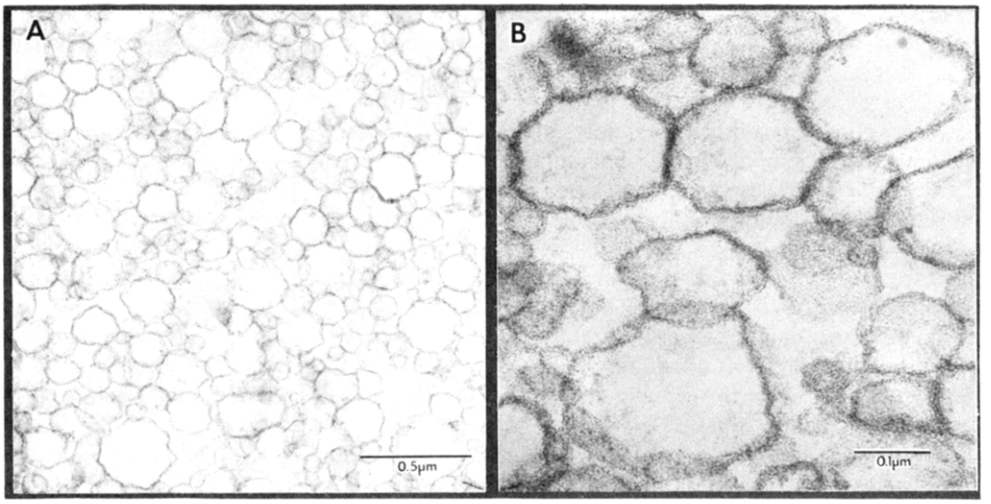


FIGURE 1: Electron micrographs of reconstituted proteoliposomes. Proteoliposomes containing purified *lac* carrier were fixed with 2% glutaraldehyde for 1 h at 20 °C, postfixed with 1% $OsO_4$ in 50 mM cacodylate, pH 6.8, and embedded in Epon.

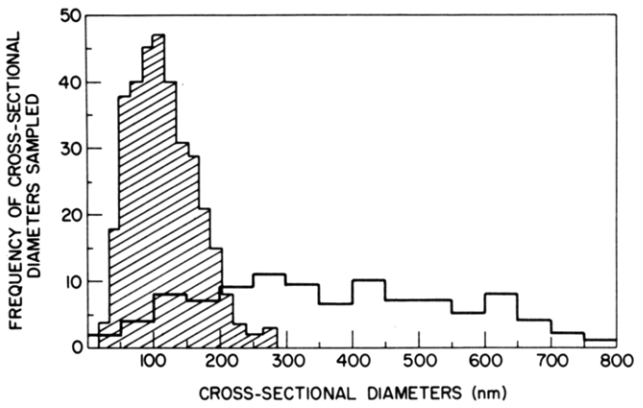


FIGURE 2: Distribution of cross-sectional diameters of proteoliposomes. The number of liposomes exhibiting given cross-sectional diameters was measured from electron micrographs such as those shown in Figure 1. The broad distribution profile corresponds to proteoliposomes subjected to a freeze–thaw step without sonication. The narrower peak (crosshatched) corresponds to the distribution of cross-sectional diameters observed for the same proteoliposomes following a brief period of sonication (cf. Figure 1).

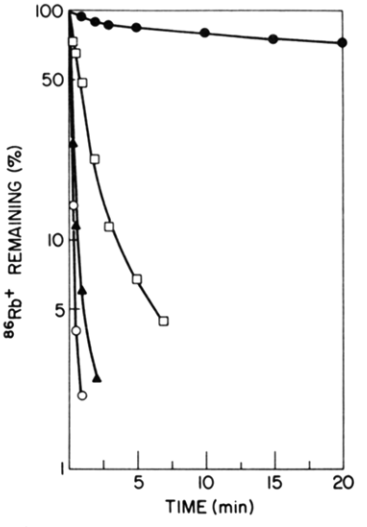


FIGURE 3: $^{86}Rb^+$ efflux from proteoliposomes. Proteoliposomes containing purified *lac* carrier in 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol were treated with 20 μM valinomycin and equilibrated with 7.5 mM $^{86}RbCl$ (33 mCi/mmol). Aliquots (1 μL) were then diluted 200-fold into 50 mM phosphate buffer, pH 7.5, at 25 °C. At the time indicated, samples were diluted with 3 mL of ice-cold 50 mM sodium phosphate, pH 7.5, and immediately filtered as described under Experimental Procedures. Experiments were carried out in 50 mM sodium phosphate, pH 7.5, alone (●), 50 mM sodium phosphate, pH 7.5, containing 1 μM nigericin (▲), or 50 mM sodium phosphate, pH 7.5, containing 20 μM CCCP (□). In another experiment, proteoliposomes were diluted into 50 mM potassium phosphate, pH 7.5 (○).

a marked increase in the rate of $Rb^+$ efflux is evident. Furthermore, even more rapid efflux rates are observed when potassium or nigericin is added because of electroneutral exchange of $^{86}Rb^+$ with potassium (via valinomycin) or protons (via nigericin). If the same experiments are performed in the absence of valinomycin, $Rb^+$ efflux is almost negligible, and addition of CCCP or potassium has no significant effect (data not shown). The observations demonstrate, albeit indirectly, that the proteoliposomes are highly impermeable to the ions present in the reaction mixture (i.e., hydrogen ion, sodium, chloride, and phosphate). Thus, the slow rate of $Rb^+$ efflux observed in the presence of valinomycin is caused by the generation of a $\Delta\psi$ (interior negative) that is maintained for long periods of time because of the impermeability of the proteoliposomes to counterions. Addition of CCCP, on the other hand, provides a pathway for protons and results in dissipation of $\Delta\psi$ with rapid downhill movement of $Rb^+$.

*pH Dependence of Lactose Efflux and Exchange.* When proteoliposomes reconstituted with purified *lac* carrier protein are equilibrated with 10 mM [1-$^{14}$C]lactose [a concentration that is approximately 5-fold in excess of the apparent $K_m$ for passive, carrier-mediated efflux (cf. Figure 7 and Kaczorowski et al., 1979)], treated with valinomycin, and diluted rapidly 200-fold into media devoid of lactose, efflux rates are pseudo first order until the internal concentration approaches the apparent $K_m$ for efflux (Figure 4A). At this point, the rates slow and the semilog plots deviate from linearity. As shown previously (Kaczorowski & Kaback, 1979), the overall rate of efflux increases markedly as a function of pH ($t_{1/2}$ = 20, 3, and 1 min at pH 5.5, 6.6, and 7.5, respectively), and the results shown here with the reconstituted carrier are considerably more dramatic (i.e., with right-side-out vesicles, the rate of efflux increases only 3-fold from pH 5.5 to pH 7.5).[2] The increase in efflux with pH is not due simply to an increase in passive membrane permeability, as the rate of lactose efflux

[2] It is also noteworthy that the initial, rapid loss of lactose from right-side-out membrane vesicles which is unrelated to *lac* carrier activity (Kaczorowski & Kaback, 1979) is not observed here.

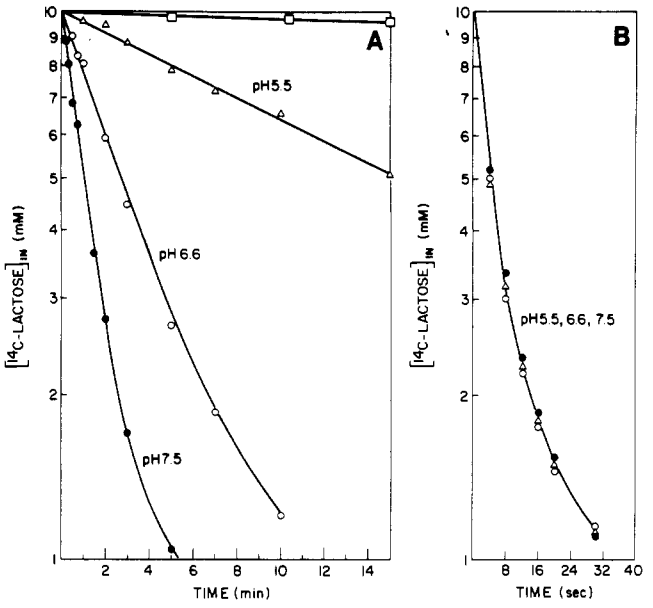


FIGURE 4: Effect of pH on lactose efflux (A) and exchange (B). (A) Proteoliposomes containing purified *lac* carrier were resuspended in 50 mM potassium phosphate and 1 mM dithiothreitol, at the pH values given. A small aliquot of [1-$^{14}$C]lactose (11.8 mCi/mmol) was added to each suspension to a final concentration of 10 mM, and valinomycin was added to a final concentration of 20 $\mu$M. After equilibration at room temperature for 1 h, 1-$\mu$L aliquots were rapidly diluted into 200 $\mu$L of 50 mM potassium phosphate at the appropriate pH at 25 °C. At the time indicated, samples were diluted with 3 mL of ice-cold 50 mM potassium phosphate, pH 7.5, and immediately filtered as described under Experimental Procedures. The experiments were conducted at pH 5.5 (△), 6.6 (○), and 7.5 (●), and the data are presented as the concentration of lactose (mM) retained within the proteoliposomes as a function of time. The zero time values were determined in triplicate by dilution of equilibrated proteoliposomes directly into ice-cold 50 mM potassium phosphate, pH 7.5, followed by immediate filtration as described under Experimental Procedures. Where indicated (□), identical experiments were carried out with proteoliposomes that were treated with 2 mM pCMBS for 30 min in the absence of dithiothreitol and then sonicated in the presence of 10 mM [1-$^{14}$C]lactose. (B) Experiments were performed as described in (A), except that 10 mM unlabeled lactose was included in the medium into which the proteoliposomes were diluted. Experiments were carried out at pH 5.5 (△), 6.6 (○), and 7.5 (●).

is negligible at all pHs examined when the *lac* carrier is inactivated with pCMBS.

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; (4) return of the unloaded carrier. In order to determine whether the return of the unloaded carrier is affected by pH, loss of internal [1-$^{14}$C]lactose was studied under conditions where the external medium contained equimolar concentrations of unlabeled lactose (i.e., under exchange conditions). As shown (Figure 4B), the rate of loss under these conditions is very fast ($t_{1/2} < 5$ s) relative to the rate of efflux and exhibits no dependence on pH. The results imply that a step involving 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 $\Delta\psi$ during Carrier-Mediated Lactose Efflux.* Lactose efflux from right-side-out membrane vesicles results in the generation of a $\Delta\psi$ (interior negative), and imposition of $\Delta\psi$ and/or $\Delta$pH alters the rate of efflux with no effect on exchange (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979). However, the overall rate of efflux is not influenced by ionophores for reasons that are not readily apparent (Kaczorowski & Kaback, 1979). In contrast, with reconstituted proteoliposomes, the rate of lactose efflux is enhanced 2-fold by addition of valinomycin or CCCP (Figure 5A). On the other hand, nigericin does not alter the rate of efflux significantly, indicating that a $\Delta$pH is not developed. The absence of $\Delta$pH is probably due to the high buffering capacity of potassium phosphate at pH 7.5, as efflux is accelerated by nigericin at more alkaline pHs (unpublished observations). Although data are not shown, it is also particularly notable that none of the ionophores tested has any effect whatsoever on the exchange reaction, thus supporting the notion that translocation in this mode does not involve net proton movement.

As shown (Kaczorowski & Kaback, 1979), carrier-mediated lactose efflux down a concentration gradient occurs in symport with protons, as evidenced by the generation of a $\Delta\psi$ (interior negative). Similarly, lactose efflux from proteoliposomes reconstituted with purified *lac* carrier and treated with valinomycin is associated with transient accumulation of $^{86}Rb^+$ (Figure 5B). Thus, immediately following the establishment of a downhill lactose gradient, the internal $Rb^+$ concentration of the proteoliposomes increases rapidly and linearly for about 2 min, achieving a maximum internal level of more than 3 mM which corresponds to a calculated $\Delta\psi$ of −43 mV. The $Rb^+$ concentration gradient is then maintained for the next few minutes and subsequently decays to the equilibration level (i.e., 0.5 mM) over about 1 h. In addition, it is evident that lactose efflux-induced $Rb^+$ uptake is abolished by CCCP, and importantly, by addition of lactose to the medium. Finally, $^{86}Rb^+$ uptake under these conditions is not observed when the *lac* carrier protein is inactivated with pCMBS.

*Effect of Imposed $\Delta\psi$ on Lactose Efflux and Exchange.* Since the rate-determining step for lactose efflux down a concentration gradient appears to involve return of the unloaded carrier to the inner surface of the membrane, conditions that perturb this step should influence the rate of lactose efflux without affecting exchange. The results presented in Figure 6 describe initial rates of lactose efflux or exchange from proteoliposomes equilibrated with 10 mM [1-$^{14}$C]lactose and 50 mM potassium phosphate (pH 7.5), treated with valinomycin, and then diluted into media containing various proportions of potassium and sodium phosphate without (efflux) or with (exchange) unlabeled lactose. By this means, the effect of $\Delta\psi$s (interior negative) of various magnitudes on the initial rates of efflux and exchange can be determined, and it is aparent that imposition of $\Delta\psi$ dramatically inhibits the initial rate of efflux. Thus, efflux is retarded by 50% when a potassium diffusion gradient ($K_{in} \rightarrow K_{out}$) of about 5 ($\Delta\psi \simeq -40$ mV) is imposed across the proteoliposome membrane and by over 90% by a potassium diffusion potential of about −150 mV. Although we have made no attempt to quantitate the magnitude of the $\Delta\psi$ generated, the data suggest that the relationship between potassium diffusion gradient and $\Delta\psi$ is reasonably close. That is, lactose efflux gives rise to a $\Delta\psi$ of about −43 mV (Figure 5B), collapse of this potential stimulates the rate of efflux 2-fold (Figure 5A), and imposition of a potassium diffusion potential corresponding to −40 mV inhibits the rate of efflux by a factor of 2 (cf. arrow, Figure 6).

In light of previous observations (Kaczorowski et al., 1979; Robertson et al., 1980), it is important to determine the effect of imposed $\Delta\psi$ on the kinetic characteristics of the efflux process. Toward this end, proteoliposomes were equilibrated with appropriate media containing various concentrations of lactose and treated with valinomycin. Following 200-fold dilution under specified conditions, initial rates of efflux were

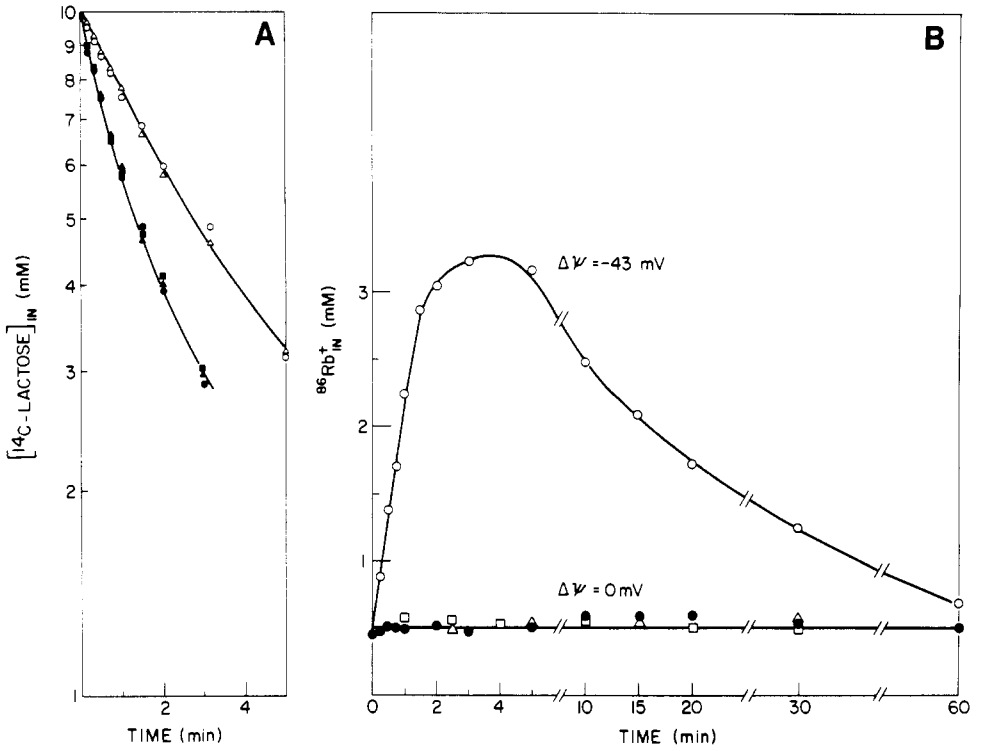


FIGURE 5: Effect of ionophores on lactose efflux (A) and lactose efflux-dependent $^{86}Rb^{+}$ accumulation (B). (A) Proteoliposomes containing purified *lac* carrier in 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol were equilibrated with 10 mM [1-$^{14}$C]lactose (11.8 mCi/mmol) as described under Experimental Procedures. Aliquots (1 μL) were then diluted into 200 μL of 50 mM potassium phosphate, pH 7.5, at 25 °C, and loss of lactose was monitored by filtration. The internal concentration of lactose retained was determined as described in the legend to Figure 4. Experiments were carried out with proteoliposomes to which the following additions were made: none (○); 2 μM nigericin (△); 20 μM valinomycin (●); 2 μM nigericin and 20 μM valinomycin (▲); 20 μM CCCP (■). (B) Proteoliposomes containing purified *lac* carrier were reconstituted in 50 mM sodium phosphate, pH 7.5, and 1 mM dithiothreitol, resuspended to a concentration 5-fold greater than that specified for the reconstitution under Experimental Procedures, and loaded by freeze–thaw/sonication with 10 mM lactose and 0.5 mM $^{86}$RbCl (35.1 mCi/mmol). Valinomycin was added to a final concentration of 20 μM, and aliquots (1 μL) were diluted into 200 μL of 50 mM sodium phosphate, pH 7.5, and 1 mM dithiothreitol, containing 0.5 mM $^{86}$RbCl (35.1 mCi/mmol) (○). At given times, samples were diluted with 3 mL of ice-cold 50 mM sodium phosphate, pH 7.5, and filtered as described under Experimental Procedures. Where indicated, samples were diluted into the same buffer containing 10 mM lactose (●), 40 μM CCCP (△), or 2 mM pCMBS (□).

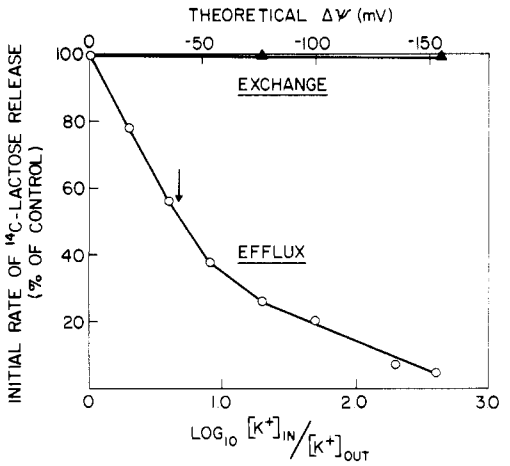


FIGURE 6: Effect of Δψ (interior negative) on lactose efflux and exchange. Proteoliposomes containing purified *lac* carrier in 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol were treated with 20 μM valinomycin and equilibrated with 10 mM [1-$^{14}$C]lactose (11.8 mCi/mmol) as described under Experimental Procedures. Aliquots (1 μL) were diluted into 200 μL of media (pH 7.5) containing various proportions of potassium and sodium phosphate (50 mM, final concentration) at 25 °C. At given times, samples were diluted with 3 mL of ice-cold 50 mM sodium phosphate, pH 7.5, and immediately filtered as described under Experimental Procedures. Initial rates of efflux, relative to the control (i.e., proteoliposomes diluted into 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol), are plotted as a function of the log of the potassium concentration gradient. Theoretical Δψs calculated from the potassium diffusion gradients are shown at the top of the figure. (○) Proteoliposomes diluted into media without lactose (efflux); (▲) proteoliposomes diluted into media containing 10 mM lactose (exchange).

measured and the data plotted in double-reciprocal fashion (Figure 7). In the absence of a diffusion potential, $V_{max}$ is about 8 μmol $min^{-1}$ (mg of protein)$^{-1}$, and the apparent $K_m$ is 2–3 mM. Imposition of a potassium diffusion potential corresponding to −136 mV causes a 10-fold decrease in $V_{max}$ to 0.8 μmol $min^{-1}$ (mg of protein)$^{-1}$ with a change in apparent $K_m$ that is probably insignificant. Although the magnitude of the effects described here are considerably more marked than those obtained previously with right-side-out membrane vesicles (Kaczorowski et al., 1979), the overall phenomena are very similar.

*Effect of pH on Lactose Counterflow.* When intact *E. coli* (Wong & Wilson, 1970; Bentaboulet & Kepes, 1977), right-side-out membrane vesicles (Kaczorowski & Kaback, 1979) or proteoliposomes containing the *lac* carrier protein (Newman & Wilson, 1980; Newman et al., 1981; Foster et al., 1982) are loaded with appropriate transport substrates and subsequently diluted into media containing the same substrate in radioactive form, transient influx of radioactive substrate is observed. 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 proteoliposomes equilibrated with 10 mM lactose are diluted 200-fold into media containing 0.8 mM [1-$^{14}$C]-lactose, a concentration that approaches saturation,[3] rapid

[3] The apparent $K_m$ for entrance counterflow in proteoliposomes reconstituted with purified *lac* carrier protein is about 0.65 mM (unpublished information).

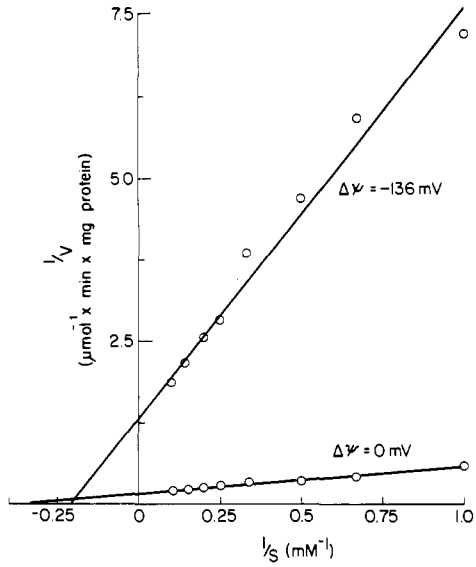


FIGURE 7: Kinetic analysis of the effects of imposed $\Delta\psi$ (interior negative) on lactose efflux. Proteoliposomes containing purified *lac* carrier in 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol were treated with 20 $\mu$M valinomycin and equilibrated at room temperature with [1-$^{14}$C]lactose at concentrations ranging from 0.5 to 10 mM. Initial rates of efflux were measured by diluting 1-$\mu$L aliquots into 200 $\mu$L of either 50 mM potassium phosphate, pH 7.5 (0 mV), or 50 mM sodium phosphate, pH 7.5 (-136 mV), at 25 °C, and the reactions were terminated as described under Experimental Procedures. Data are presented in the form of a double-reciprocal plot. *S*, substrate concentration in mM; *V*, initial velocity in $\mu$mol min$^{-1}$ (mg of protein)$^{-1}$.

transient uptake of radioactive substrate is observed at pH 5.5, 6.6, and 7.5 (Figure 8A). Under these conditions, the initial rate and peak level achieved are essentially independent of pH, although the rate of decay of the "overshoot" exhibits a pH dependence similar to that observed for lactose efflux (cf. Figure 4A). Furthermore, the internal lactose concentration at the peak height approximates 10 mM at each pH studied, indicating that the coupling efficiency between efflux of unlabeled lactose and influx of radioactive lactose is 1:1.

In contrast, when counterflow is repeated under identical conditions with subsaturating external lactose concentrations, the results are strikingly different (Figure 8B). In these studies, the proteoliposomes were loaded with 10 mM lactose again and diluted 200-fold, but the external [1-$^{14}$C]lactose concentration was 0.075 mM. Under these circumstances, the overshoot phenomenon observed is markedly sensitive to pH, and the peak level decreases from pH 5.5 to 6.6 to 7.5. Interestingly, despite the decrease in the level of the overshoot with increasing pH, the initial rate of counterflow remains essentially constant.

## Discussion

The observations presented in this paper are important on two levels: *first*, they provide more detailed experimental support for the argument that highly purified *lac* carrier protein incorporated into proteoliposomes behaves in a manner similar to that observed in the bacterial membrane; *second*, the observations extend previous findings (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979) concerning the mechanisms of carrier-mediated translocation down a chemical gradient, exchange, and counterflow.

Clearly, the proteoliposomes employed here are almost ideally suited to the studies described. Morphologically, the preparation consists of a population of unilamellar, closed, unit membrane-bound sacs that are relatively uniform in diameter and contain no internal structure, findings that correlate nicely with the pseudo-first-order efflux and exchange kinetics observed for both lactose and $Rb^+$. Furthermore, it is apparent that the proteoliposomes are passively impermeable to many ions, a property that is highly advantageous with regard to the study of proton/solute symport. Thus, certain aspects of proton/lactose symport that were impossible to document with right-side-out vesicles (i.e., stimulation of efflux by ionophores) are readily elucidated with the reconstituted system. Generally, proteoliposomes reconstituted with the *lac* carrier exhibit all of the phenomena described in right-side-out mem-

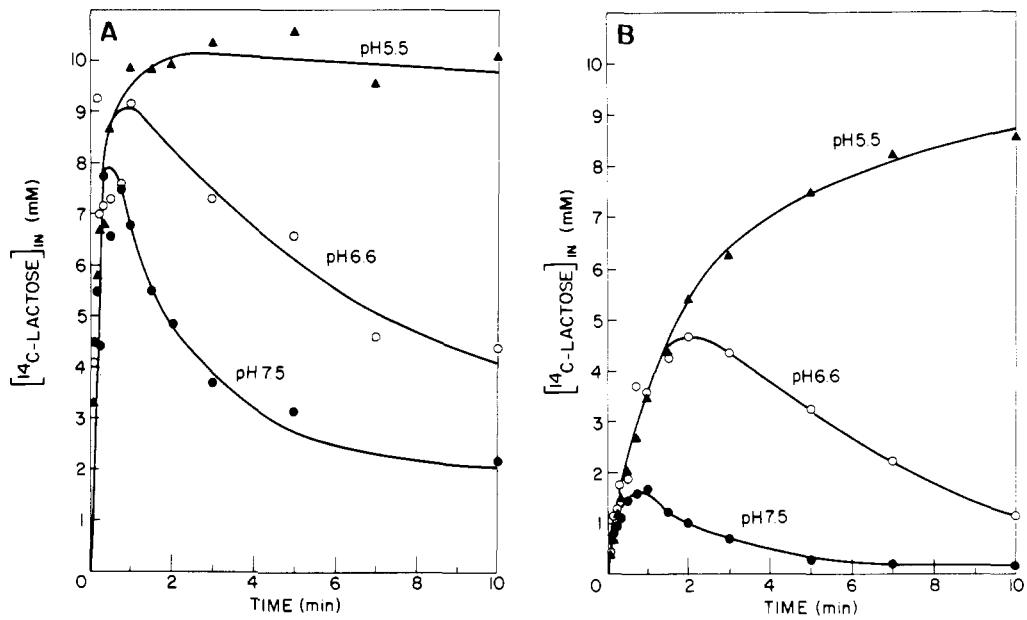


FIGURE 8: pH dependence of lactose counterflow at saturating (A) and subsaturating (B) external lactose concentrations. (A) Proteoliposomes containing purified *lac* carrier were suspended in 50 mM potassium phosphate and 1 mM dithiothreitol at given pH values, and valinomycin was added to a final concentration of 20 $\mu$M. The proteoliposomes were equilibrated with 10 mM lactose as described under Experimental Procedures, and aliquots (1 $\mu$L) were diluted into 200 $\mu$L of 50 mM potassium phosphate at the same pH containing 0.8 mM [1-$^{14}$C]lactose (7.38 mCi/mmol). Counterflow was assayed at given times at 25 °C as described under Experimental Procedures. Experiments were conducted at pH 5.5 (▲), 6.6 (○), and 7.5 (●). (B) Experiments were carried out exactly as described in (A) except that the dilution medium contained 0.075 mM [1-$^{14}$C]lactose (19.5 mCi/mmol). Experiments were conducted at pH 5.5 (▲), 6.6 (○), and 7.5 (●).

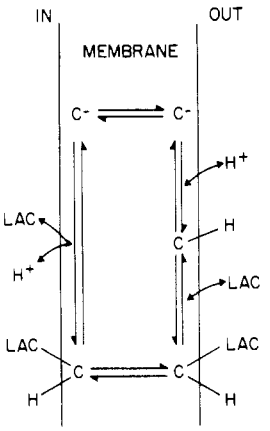


FIGURE 9: Schematic representation of reactions involved in lactose efflux, exchange, and counterflow. C represents the *lac* carrier protein. The order of substrate binding at the inner surface of the membrane is not implied. From Kaczorowski & Kaback (1979).

brane vesicles, but the results are significantly more clear-cut and provide firmer support for certain ideas concerning reaction mechanisms.

The evidence, taken as a whole, provides virtually incontrovertible support for the concept that proton/lactose symport is the mechanism of β-galactoside transport and that this activity is catalyzed by a single polypeptide species in the membrane of *E. coli*. Furthermore, when carrier-mediated lactose efflux down a chemical gradient, exchange, and entrance counterflow are utilized to drive carrier turnover in a highly purified system, further evidence for the following conclusions is presented: (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 on the outer surface may be limiting; (3) during exchange and counterflow, the carrier recycles without deprotonation.

Transient accumulation of $Rb^+$ during lactose efflux in the presence of valinomycin argues strongly for the coupled translocation of a charged species with lactose which leads to the generation of a $\Delta\psi$ (interior negative). In addition to the phenomenon itself, the process is abolished by the protonophore CCCP, and efflux-induced $Rb^+$ uptake is blocked by pCMBS, a sulfhydryl reagent that completely inactivates the *lac* carrier protein. Furthermore, the rate of lactose efflux is enhanced by ionophores that collapse $\Delta\psi$, and artificial imposition of $\Delta\psi$ dramatically slows the rate of efflux with no significant change in apparent $K_m$. Although it has not been demonstrated here that the charged species moving in symport with lactose is hydrogen ion, considerable evidence supporting this contention has been presented (cf. Kaback, 1981). In particular, downhill influx of lactose in proteoliposomes reconstituted with purified *lac* carrier protein occurs with concomitant alkalinization of the external medium (Foster et al., 1982).

Comparison of efflux and exchange rates from the data presented in Figure 4 demonstrates that exchange is very rapid, at least 10 times faster than efflux. Therefore, the rate-determining step for efflux must involve a reaction corresponding 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. Under the assumption that loss of lactose and protons from the carrier is necessary for reinitiation of an efflux cycle (cf. Figure 9), external pH would influence the rate of turnover in either of two ways. First, deprotonation could be slow and thereby limit the overall rate of efflux in a pH-dependent manner. Although proton transfers between accessible amino acid residues and water in soluble enzymes are usually fast (Eigen, 1963), little is known about such reactions with hydrophobic membrane proteins. Alternatively, pH could alter the equilibrium between protonated and unprotonated forms of the *lac* carrier, favoring the unprotonated form at more alkaline pH. Since it is assumed that only the deprotonated form of the carrier can recycle, the rate of efflux would be at least partially controlled by external pH, and the rate-determining 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 4A) is consistent with either possiblity. In contrast, if deprotonation of the carrier is not obligatory for exchange, protons might remain bound to the carrier during this mode of translocation, rendering exchange insensitive to pH. Evidence that is consistent with this argument is presented in Figure 4B. If efflux is an ordered mechanism in which 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 efflux.

The counterflow experiments presented in Figure 8 are consistent with an ordered reaction mechanism (cf. Figure 9). With concentrations of external lactose that approach saturation, variations in pH have relatively little effect on the magnitude of the overshoot. On the other hand, with subsaturating external lactose concentrations, counterflow is progressively inhibited as pH becomes more alkaline. The results can be interpreted in the following way. On initiation of efflux, lactose and protons bind to the carrier on the inner surface of the membrane (in an unspecified order) and are translocated to the outer surface. Lactose is released from the carrier, but in the presence of excess labeled substrate, rebinding and influx occur rapidly before deprotonation occurs. Under these conditions, therefore, proton release is infrequent, and pH has no effect on the overall phenomenon. When [$^{14}$C]lactose is limiting, however, rebinding of labeled substrate is less frequent, allowing deprotonation and return of the unloaded carrier. Moreover, as pH is increased, deprotonation and return of the unloaded carrier are enhanced, resulting in further diminution of counterflow. Inhibition of efflux-generated $\Delta\psi$ formation by external lactose is also readily explained by this scheme. When lactose is 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 $\Delta\psi$ is abolished.

In the following paper (Viitanen et al., 1983), some of these considerations are pursued in more detail by examining the effects of deuterium oxide on the system.

## Acknowledgments

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**Registry No.** Lactose, 63-42-3; hydrogen ion, 12408-02-5.

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# Mechanism of Lactose Translocation in Proteoliposomes Reconstituted with *lac* Carrier Protein Purified from *Escherichia coli*. 2. Deuterium Solvent Isotope Effects†

Paul Viitanen, Maria Luisa Garcia, David L. Foster,‡ Gregory J. Kaczorowski,§ and H. Ronald Kaback*

ABSTRACT: Various modes of lactose translocation catalyzed by purified *lac* carrier protein reconstituted into proteoliposomes were studied in water ($H_2O$) and deuterium oxide ($D_2O$). Initial rates of carrier-mediated efflux down a chemical gradient are retarded over 3-fold in deuterium relative to protium over a pH (pD) range from 5.5 to 7.5 (pD = pH + 0.4), and the maximum velocity of the reaction is altered with no effect on apparent $K_m$. In contrast, the exchange reaction proceeds at the same rate in protium and deuterium, and remarkably, the initial rate of membrane potential driven uphill transport is identical in $H_2O$ and $D_2O$. To test the hypothesis that efflux is an ordered reaction limited either by the rate of deprotonation or by a pH-dependent equilibrium, counterflow was studied in $H_2O$ and $D_2O$. With external lactose at concentrations approaching saturation, the initial rate of counterflow and the extent of overshoot are independent of solvent from pH (pD) 5.5 to 7.5. On the other hand, at external lactose concentrations below the apparent $K_m$, the overshoot is enhanced in deuterium. Furthermore, the stimulatory effect of the isotope decreases from pH (pD) 7.5 to 6.6 and is no longer evident at pH (pD) 5.5. In order to distinguish between a kinetic isotope effect and a p$K_a$ effect, efflux was studied over an extended range of pH (pD) values, from 5.5 to 10.5. In $H_2O$, the initial rate of efflux increases sigmoidally with pH, exhibiting a sharp rise between pH 7.5 and pH 9.5 with a midpoint at about pH 8.3. The rate is maximal at pH 9.5 and above and more than 6 times greater than the rate at pH 7.5. The pD profile for efflux is shifted to the right by 0.4–0.5 pH (pD) unit and is essentially parallel to the pH profile. Thus, the deuterium isotope effect on efflux decreases above pH 7.5 and disappears at pH (pD) 9.5. Over the same extended range of pH (pD) values, exchange remains constant, independent of solvent, and at pH 9.5 and above, the rate of exchange is only about 25–30% faster than the rate of efflux. Since the maximum rate of efflux at pH (pD) 9.5 and above is the same in $H_2O$ and $D_2O$, it cannot be concluded with certainty that the solvent isotope effect observed at lower pH (pD) represents a true kinetic isotope effect. In any event, the results taken as a whole provide strong support for the kinetic model presented previously [Kaczorowski, G. J., & Kaback, H. R. (1979) *Biochemistry 18*, 3691; Garcia, M. L., Viitanen, P., Foster, D. L., & Kaback, H. R. (1983) *Biochemistry* (preceding paper in this issue)] and indicate further that reactions involving protonation or deprotonation of the *lac* carrier are not rate determining for uphill lactose transport in the presence of a proton electrochemical gradient.

In the preceding paper (Garcia et al., 1983), proteoliposomes reconstituted with purified *lac* carrier protein were utilized to provide additional support for the concept that carrier-mediated lactose efflux down a concentration gradient is an ordered symport mechanism. The salient features of the model presented are the following [cf. Figure 9 in the preceding paper (Garcia et al., 1983)]: (1) release of lactose from the carrier on the outer surface of the membrane occurs prior to loss of the symported proton; (2) during exchange and counterflow, the loaded carrier recycles in the protonated form; (3) either proton loss or the immediately succeeding step (i.e., return of the unloaded carrier) is rate determining for the efflux reaction.

Many enzyme reactions involve proton transfer in the rate-determining step, and as a consequence, these reactions

† From the Laboratory of Membrane Biochemistry, Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received December 14, 1982.*
‡ Present address: Donner Laboratory, University of California, Berkeley, CA 94720.
§ Present address: Merck Sharp & Dohme Laboratories, Rahway, NJ 07065.

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