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

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ABSTRACT: Imposition of a membrane potential ($\Delta\Psi$, interior negative) or a pH gradient ($\Delta$pH, 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 $\Delta$pH, and the effect of each parameter is additive. Imposition of $\Delta\Psi$ or $\Delta$pH 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 $\Delta$pH. The rate of efflux is diminished with $\Delta\Psi$ (interior negative) or $\Delta$pH (interior alkaline) and enhanced with $\Delta\Psi$ (interior positive) or $\Delta$pH (interior acid). These effects are also additive, and importantly kinetic experiments demonstrate that $\Delta\Psi$ and $\Delta$pH alter the maximum velocity of efflux without a significant effect on the apparent $K_m$ of the process. Strikingly, moreover, imposition of $\Delta\Psi$, $\Delta$pH, 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 $\Delta$pH 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 $\Delta$pH's on influx, efflux, and exchange. The results support

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and extend many of these suggestions.

## Experimental Section

### *Methods*

*Growth of Cells and Preparation of Membrane Vesicles.* *Escherichia coli* ML 308-225 ($i^-z^-y^+a^+$) and ML 30 ($i^+z^+y^+a^+$) were grown and membrane vesicles prepared as described in the preceding paper (Kaczorowski & Kaback, 1979). With the exception of the dansyl galactoside measurements, all of the experiments presented in this paper were carried out with the same vesicle preparation.

For experiments performed in various buffer systems, vesicles prepared in 0.1 M potassium phosphate (pH 6.6) and frozen in liquid $N_2$ were thawed and equilibrated with given media as described (Kaczorowski & Kaback, 1979). Kinetic measurements of lactose efflux were made after allowing vesicle suspensions to equilibrate with various concentrations of [1-$^{14}$C]lactose for 3 h at 25 °C.

*Transport Assays.* Efflux and exchange assays were performed as described (Kaczorowski & Kaback, 1979). Transport in the presence of ascorbate and phenazine methosulfate was monitored by using filtration assays (Kaback, 1974). Determinations of the membrane potential ($\Delta\Psi$)[1] and the pH gradient ($\Delta$pH) were accomplished by means of flow dialysis (Ramos et al., 1976, 1979; Ramos & Kaback, 1977a).

*Fluorescence Measurements.* Fluorescence of 6′-(*N*-dansyl)aminohexyl 1-thio-β-D-galactopyranoside (Dns$^6$-Gal) was observed at 90° from the excitation beam with a Perkin-Elmer MPF4 spectrophotofluorometer using 1 × 1 cm quartz covettes (Beckman) as described (Reeves et al., 1973). The sample chamber was maintained at 25 °C with a Lauda Model K-2/R circulating water bath (Brinkman). Emission and excitation slits were adjusted to 6 nm. Additions were made to the cuvettes with Hamilton microsyringes or with a Gilson Pipetman microliter pipet, and mixing was accomplished within 5 s by using a plastic stick (Calbiochem). Fluorescence of 3,3′-dipropylthiodicarbocyanine [diS-$C_3$-(5)] was measured as described by Sims et al. (1974).[2]

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

### *Materials*

[1-$^{14}$C]Lactose was purchased from Amersham-Searle. [$^3$H]Tetraphenylphosphonium bromide was synthesized by the Isotope Synthesis Group at Hoffmann-La Roche, Inc., under the direction of Dr. Arnold Liebman.[2] Dns$^6$-Gal was prepared as described (Schuldiner et al., 1975). DiS-$C_3$-(5) was the generous gift of Dr. Alan Waggoner of Amherst College. Nigericin was graciously supplied by Dr. J. Berger of Hoffmann-La Roche, Inc. Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Calbiochem. All other materials were reagent grade and obtained from commercial sources.

## Results

*Effect of Imposed $\Delta\Psi$, $\Delta pH$, and $\Delta\bar{\mu}_{H^+}$ on Dansyl Galactoside Fluorescence.* Previous experiments demonstrate that an artificially imposed $\Delta\Psi$ (interior negative) drives active transport in *E. coli* ML 308-225 membrane vesicles (Hirata et al., 1973, 1974; Schuldiner & Kaback, 1975) and causes a marked increase in the fluorescence of Dns$^6$-Gal (Schuldiner et al., 1975). Although recent observations (Overath et al., 1979) question the argument that the increase in fluorescence is due to binding specifically, as opposed to binding and translocation (Schuldiner & Kaback, 1977), there is general agreement that the dansyl galactosides are useful probes for *lac* carrier function.

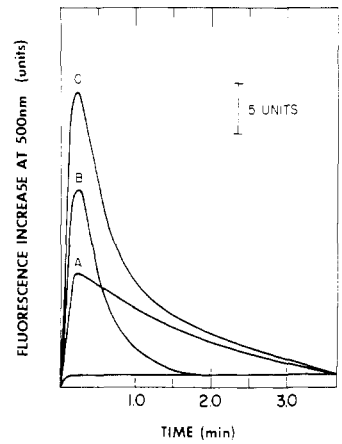


FIGURE 1: Effect of imposed $\Delta\Psi$ (interior negative), $\Delta$pH (interior alkaline), and $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) on Dns$^6$-Gal fluorescence. *E. coli* ML 308-225 membrane vesicles were equilibrated with 0.1 M potassium phosphate (pH 7.0) and 0.1 M potassium acetate and concentrated to ~35 mg of protein/mL as described (Kaczorowski & Kaback, 1979). After valinomycin was added to a final concentration of 2 nmol/mg of protein, 5-μL aliquots of the suspension were diluted into a cuvette containing 1.5 mL of a salt solution given below and 20 μM Dns$^6$-Gal. Changes in fluorescence were monitored at 500 nm (excitation, 340 nm). The following media were used for dilution: (curve A) 0.1 M sodium phosphate (pH 7.0) and 0.1 M sodium acetate; (curve B) 0.1 M potassium phosphate (pH 7.0) and 0.1 M potassium gluconate; and (curve C) 0.1 M sodium phosphate (pH 7.0) and 0.1 M sodium gluconate. As a control (lower line in the figure), vesicles were diluted into 0.1 M potassium phosphate (pH 7.0) and 0.1 M potassium acetate.

When *E. coli* ML 308-225 membrane vesicles are equilibrated with 100 mM potassium phosphate (pH 7.0) and 100 mM potassium acetate, treated with valinomycin, and then diluted 200-fold into a cuvette containing equimolar concentrations of sodium phosphate (pH 7.0) and sodium acetate, there is a rapid increase in Dns$^6$-Gal fluorescence at 500 nm that reaches a maximum in ~12 s and subsequently decays to the base line in 3–4 min (Figure 1, curve A). The increase in fluorescence is dependent upon addition of valinomycin and is not observed when the vesicles are diluted into medium containing equimolar concentrations of potassium nor when the potassium diffusion gradient is allowed to dissipate for 5 min prior to addition of Dns$^6$-Gal. Moreover, the effect is dependent upon the presence of functional *lac* carrier, as evidenced by the observations that Dns$^6$-Gal fluorescence remains constant when a similar experiment is carried out with *N*-ethylmaleimide-treated vesicles or with vesicles prepared from uninduced *E. coli* ML 30 (data not shown).

Although data will not be presented, the time course of the increase in Dns$^6$-Gal fluorescence correlates with the generation and dissipation of $\Delta\Psi$ (interior negative) under these conditions. When the vesicles are treated exactly as described for curve A (Figure 1), they rapidly accumulate the permeant lipophilic cation [$^3$H]tetraphenylphosphonium[2] to a maximum level within 10–15 s. Subsequently, the cation is lost from the vesicles and the level returns to base line within ~4 min. In addition, transient quenching of diS-$C_3$-(5) fluorescence (Sims et al., 1974) is observed[2] and the time course exhibits a pattern

[1] Abbreviations used: $\Delta\Psi$, membrane potential; $\Delta$pH, pH gradient; $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; Dns$^6$-Gal, 6′-(*N*-dansyl)-aminohexyl 1-thio-β-D-galactopyranoside; diS-$C_3$-(5), 3,3′-dipropylthiodicarbocyanine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

[2] S. Ramos, L. Patel, and H. R. Kaback, unpublished experiments.

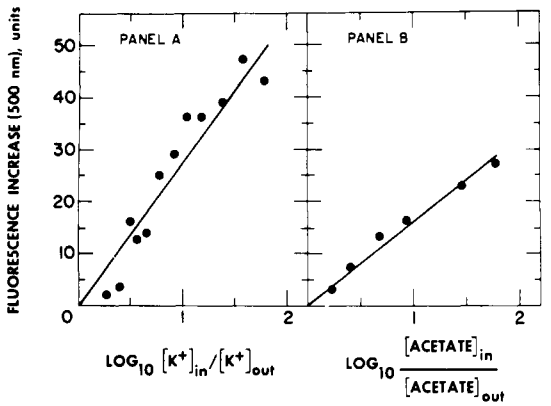


FIGURE 2: Effect of varying $\Delta\Psi$ (interior negative) and $\Delta$pH (interior alkaline) on maximum Dns$^6$-Gal fluorescence. Panel A: Dns$^6$-Gal fluorescence vs. magnitude of outwardly directed potassium diffusion gradients. ML 308-225 vesicles were equilibrated in 0.1 M potassium phosphate (pH 6.6) and 0.1 M potassium acetate and concentrated to 17 mg of protein/mL. Valinomycin was added to a final concentration of 1 nmol/mg of protein, and aliquots (25 $\mu$L) were diluted into a cuvette containing 1.5 mL of acetate–phosphate media at pH 6.6 containing various ratios of potassium and sodium and 20 $\mu$M Dns$^6$-Gal. The maximum change in Dns$^6$-Gal fluorescence (cf. Figure 1) is plotted as a function of the log of the potassium concentration gradient. Panel B: Dns$^6$-Gal fluorescence vs. magnitude of outwardly directed acetate diffusion gradients. In a series of experiments similar to those described in panel A, vesicles prepared as described above were diluted into media containing various ratios of acetate and gluconate and the same concentration of potassium. The maximum change in Dns$^6$-Gal fluorescence is plotted as a function of the log of the acetate diffusion gradient.

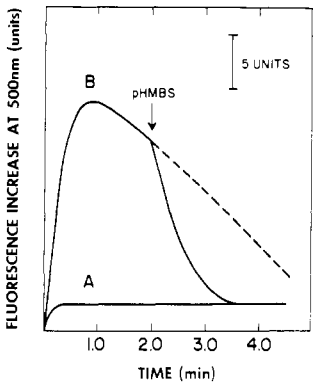


FIGURE 3: Nigericin-stimulated Dns$^6$-Gal fluorescence. ML 308-225 vesicles were equilibrated with 2 mM choline phosphate (pH 6.6) and 200 mM choline chloride and concentrated to ~20 mg of protein/mL. Nigericin was added to a final concentration of 0.05 nmol/mg of protein, and 20-$\mu$L aliquots were diluted into a cuvette containing 1.5 mL of either 2 mM choline phosphate (pH 6.6) and 200 mM choline chloride (A) or 2 mM potassium phosphate (pH 6.6) and 200 mM potassium chloride (B). In both instances, Dns$^6$-Gal was present at a final concentration of 20 $\mu$M and fluorescence was monitored at 500 nm (excitation, 340 nm). As indicated by the arrow, *p*-(hydroxymercuri)benzenesulfonic acid (pHMBS) was added to a final concentration of 20 $\mu$M at 2 min.

similar to that described for Dns$^6$-Gal fluorescence and [$^3$H]tetraphenylphosphonium accumulation. It is also noteworthy that a similar effect on diS-$C_3$-(5) fluorescence is observed with a thiocyanate diffusion gradient directed inward (Schuldiner et al., 1975).

Importantly, the maximum intensity of the Dns$^6$-Gal fluorescence increase induced by a potassium diffusion gradient in the presence of valinomycin is directly related to the magnitude of the imposed gradient (Figure 2A). In these experiments, vesicles equilibrated with 100 mM potassium phosphate (pH 6.6) and 100 mM potassium acetate were treated with valinomycin and diluted into isotonic media containing various ratios of sodium and potassium. When the maximum increase in Dns$^6$-Gal fluorescence is plotted as a function of the log of the potassium diffusion gradient, a linear relationship is obtained that passes through the origin.

An increase in Dns$^6$-Gal fluorescence is also observed when a $\Delta$pH (interior alkaline) is imposed across the vesicle membrane, and this phenomenon can be documented in at least two ways. First, in the experiment shown in Figure 1, curve B, vesicles equilibrated with 100 mM potassium phosphate (pH 7.0) and 100 mM potassium acetate were diluted 200-fold into isotonic medium devoid of acetate[3] so that a large acetate diffusion gradient is created ($Ac_{in} \rightarrow Ac_{out}$). Since acetate is permeant in its protonated form only (i.e., as acetic acid; Lancaster & Hinkle, 1977a,b; Ramos & Kaback, 1977a), this manipulation leads to a net loss of protons from the intravesicular space, giving rise to a $\Delta$pH (interior alkaline). As shown, there is a rapid increase in Dns$^6$-Gal fluorescence under these conditions that achieves a maximum in ~12 s and decays to the control level in ~2 min. Furthermore, when the magnitude of the acetate diffusion gradient is altered systematically by adding acetate to the external medium, the increase in Dns$^6$-Gal fluorescence varies linearly with the log of the acetate diffusion gradient and the function passes through the origin (Figure 2B).[4] It is important to note that these experiments were carried out in the presence of valinomycin and that the absence of $\Delta\Psi$ under the conditions employed was confirmed by independent studies of diS-$C_3$-(5) fluorescence (not shown).

Second, under appropriate conditions (Schuldiner et al., 1978), the ionophore nigericin can be used to generate a $\Delta$pH (interior alkaline). Thus, when vesicles are equilibrated with 2 mM choline phosphate (pH 6.6) and 200 mM choline chloride, treated with nigericin, and then diluted 100-fold into a cuvette containing 2 mM potassium phosphate (pH 6.6) and 200 mM potassium chloride, a rapid increase in Dns$^6$-Gal fluorescence is observed that decays to the base line in ~5 min (Figure 3). The effect is rapidly reversed by addition of *p*-(hydroxymercuri)benzenesulfonate at 2 min, and no change in Dns$^6$-Gal fluorescence is observed when the vesicles are diluted into 2 mM choline phosphate (pH 6.6) and 200 mM choline chloride. Since nigericin catalyzes the electroneutral exchange of potassium and protons (Pressman, 1976) (i.e., under the conditions described, 1 external potassium ion presumably exchanges for 1 intravesicular proton), it seems reasonable to conclude that the transient increase in Dns$^6$-Gal fluorescence is due to nigericin-mediated generation of $\Delta$pH (interior alkaline).

Since outwardly directed potassium diffusion gradients in the presence of valinomycin (i.e., generation of $\Delta\Psi$, interior negative) and acetate diffusion gradients (i.e., generation of $\Delta$pH, interior alkaline) independently stimulate turnover of the *lac* carrier, it was of interest to impose both gradients

[3] In order to generate $\Delta$pH by means of acetate diffusion gradients in the absence of undesired osmotic effects, we diluted vesicles equilibrated with acetate into media containing equimolar concentrations of gluconate or vice versa. Since gluconate is polyhydroxylated and induction of a specific transport system is required to observe uptake in the vesicles (Lagarde & Stoeber, 1974; Ramos & Kaback, 1977b), it is presumably much less permeant than acetate.

[4] The experiments shown in Figure 2 were carried out at pH 6.6 and those in Figure 1 at pH 7.0. For this reason, the effect of $\Delta$pH (interior alkaline) in Figure 1 appears to be smaller than the effect of $\Delta\Psi$ (interior negative) (Figure 2A), while the opposite effects are observed in Figure 1.

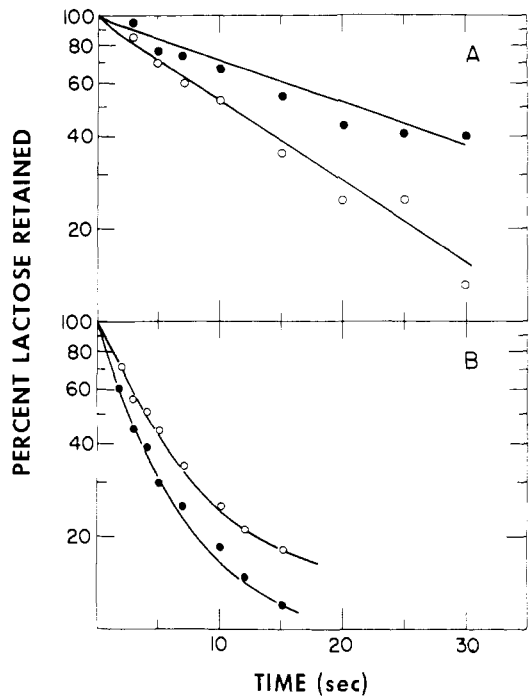


FIGURE 4: Lactose efflux in the presence of $\Delta\Psi$, interior negative (A) or positive (B). Panel A: vesicles were concentrated to 35 mg of protein/mL in 0.1 M potassium phosphate (pH 7.0), valinomycin was added to a final concentration of 2 nmol/mg of protein, and the suspension was equilibrated with 10 mM [1-$^{14}$C]lactose (6 mCi/mmol) by incubating for 3 h at 25 °C. Aliquots (2 $\mu$L) of the suspension were then rapidly diluted into 400 $\mu$L of either 0.1 M potassium phosphate, pH 7.0 (○), or 0.1 M sodium phosphate, pH 7.0 (●), and loss of intravesicular lactose was monitored by filtration. The percentage of lactose retained was determined by comparison with zero time points (22 nmol/mg of protein ± 5%), and the time required for loss of 50% of the intravesicular lactose ($t_{1/2}$) was calculated from the linear portion of the function. Panel B: vesicles were treated as described in panel A, except that they were initially equilibrated with 1.0 mM lactose and 0.1 M sodium phosphate (pH 7.0). Efflux was then initiated by diluting the suspension into either 0.1 M sodium phosphate, pH 7.0 (○), or 0.1 M potassium phosphate, pH 7.0 (●).

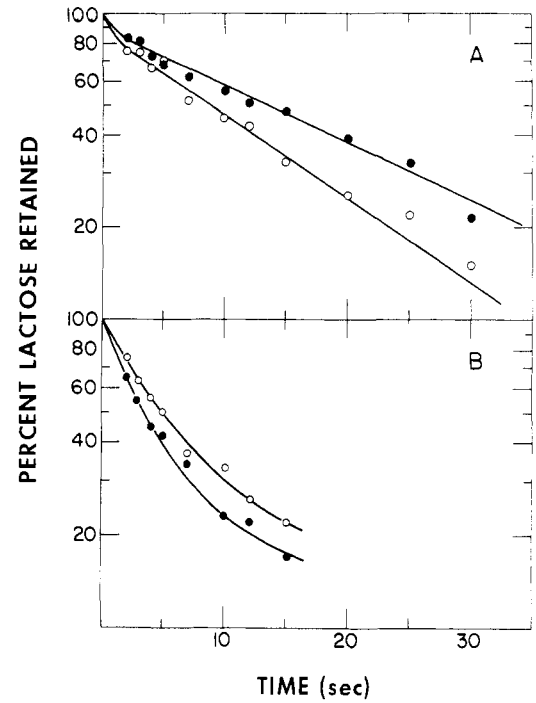


FIGURE 5: Lactose efflux in the presence of $\Delta$pH, interior alkaline (A) or acid (B). Panel A: vesicles were concentrated to 30 mg of protein/mL in 5 mM potassium phosphate (pH 7.0) and 100 mM potassium acetate, valinomycin was added to a final concentration of 2 nmol/mg of protein, and the suspension was equilibrated with 10 mM [1-$^{14}$C]lactose (6 mCi/mmol) by incubating for 3 h at 25 °C. Aliquots (2 $\mu$L) were then diluted 200-fold into either 5 mM potassium phosphate (pH 7.0) and 100 mM potassium acetate (○) or 5 mM potassium phosphate (pH 7.0) and 100 mM potassium gluconate (●), and loss of intravesicular lactose was monitored by filtration. The data were analyzed as described in Figure 4. Panel B: vesicles were treated as described in panel A, except that they were initially equilibrated with 1.5 mM lactose, 5 mM potassium phosphate (pH 7.0), and 100 mM potassium gluconate. Efflux was then initiated by diluting the suspension into either 5 mM potassium phosphate (pH 7.0) and 100 mM potassium gluconate (○) or 5 mM potassium phosphate (pH 7.0) and 100 mM potassium acetate (●).

simultaneously (Figure 1, curve C). In this instance, the vesicles were equilibrated with 100 mM potassium phosphate (pH 7.0) and 100 mM potassium acetate and treated with valinomycin as described for curves A and B, but diluted into a cuvette containing 100 mM sodium phosphate (pH 7.0) and 100 mM sodium gluconate so that potassium and acetate gradients were generated concurrently. As shown, the magnitude of the increase in Dns$^6$-Gal fluorescence observed under these conditions (curve C) is the arithmetic sum of curves A and B, and the time course of the fluorescence change follows the pattern expected for an additive phenomenon. Interestingly, moreover, the extent of the fluorescence increase observed at the maximum approximates that observed when D-lactate oxidation is used to generate the electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$; Schuldiner et al., 1975).

*Effect of* $\Delta\Psi$, $\Delta pH$, and $\Delta\bar{\mu}_{H^+}$ *on Carrier-Mediated Lactose Efflux and Exchange.* The observations presented in the accompanying paper (Kaczorowski & Kaback, 1979) indicate that the rate-determining step for lactose efflux down a concentration gradient is involved with the return of the unloaded carrier to the inner surface of the membrane, and it was suggested that conditions that perturb this step should influence the rate of lactose efflux without affecting exchange. The results presented in Figure 4A describe the time course of lactose efflux from ML 308-225 vesicles equilibrated with 10 mM [1-$^{14}$C]lactose and 100 mM potassium phosphate (pH 7.0), treated with valinomycin, and then diluted 200-fold into media containing either potassium phosphate (no $\Delta\Psi$; open symbols) or sodium phosphate ($\Delta\Psi$, interior negative; closed symbols) at pH 7.0. Clearly, generation of $\Delta\Psi$ (interior negative) retards efflux, and the $t_{1/2}$ is increased from about 12 s to 21 s. The corollary experiment performed with sodium-loaded vesicles diluted into sodium phosphate (no $\Delta\Psi$, open symbols) or potassium phosphate ($\Delta\Psi$, interior positive; closed symbols) is presented in Figure 4B. In addition, the vesicles were loaded with a lower concentration of [1-$^{14}$C]-lactose in order to accentuate the effect of the inwardly directed potassium diffusion gradient. Dilution of vesicles under conditions that do not produce $\Delta\Psi$ yields a $t_{1/2}$ for efflux of ~4 s, and when a $\Delta\Psi$ (interior positive) is generated the rate of efflux is enhanced (i.e., $t_{1/2}$ decreases to 2.5 s). The effect of $\Delta\Psi$ in this experiment is less pronounced than that observed in Figure 4A presumably because the potassium flux is directed into rather than out of the small intravesicular compartment.

In an analogous series of experiments, the effects of $\Delta$pH, interior alkaline or acid, on lactose efflux were monitored (Figure 5). In the experiments shown in panel A, vesicles equilibrated with 10 mM [1-$^{14}$C]lactose, 5 mM potassium phosphate (pH 7.0), and 100 mM potassium acetate were diluted 200-fold into media containing equimolar concentrations of either acetate (no $\Delta$pH; open symbols) or gluconate ($\Delta$pH, interior alkaline; closed symbols). In the absence of $\Delta$pH, the $t_{1/2}$ for efflux is ~11 s. When $\Delta$pH (interior alkaline) is imposed, the rate of efflux is reduced and $t_{1/2}$ in-

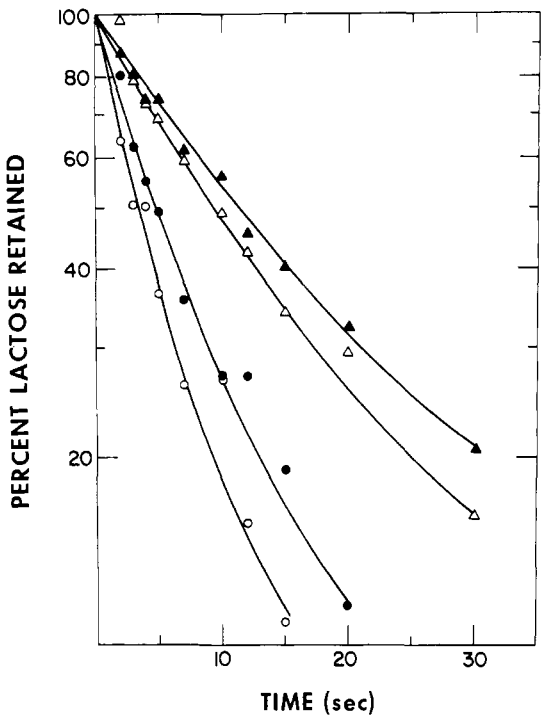


FIGURE 6: Effect of $\Delta\Psi$ (interior negative), $\Delta$pH (interior alkaline), and $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) on lactose efflux. Vesicles suspended in 0.1 M potassium phosphate (pH 7.0) and 0.1 M potassium acetate were concentrated to ~30 mg of protein/mL. Valinomycin was added to a final concentration of 2 nmol/mg of protein, and the suspension was equilibrated with 2 mM [1-$^{14}$C]lactose (15 mCi/mmol) by incubation at 25 °C for 3 h. Aliquots (2 $\mu$L) were then rapidly diluted into 400 $\mu$L of 0.1 M potassium phosphate (pH 7.0) and 0.1 M potassium acetate (○), 0.1 M sodium phosphate (pH 7.0) and 0.1 M sodium acetate ($\Delta\Psi$, interior negative; ●), 0.1 M potassium phosphate (pH 7.0) and 0.1 M potassium gluconate ($\Delta$pH, interior alkaline; △), or 0.1 M sodium phosphate (pH 7.0) and 0.1 M sodium gluconate ($\Delta\bar{\mu}_{H^+}$, interior negative and alkaline; ▲), and efflux was monitored by filtration. Each time point presented is the average of two assays.

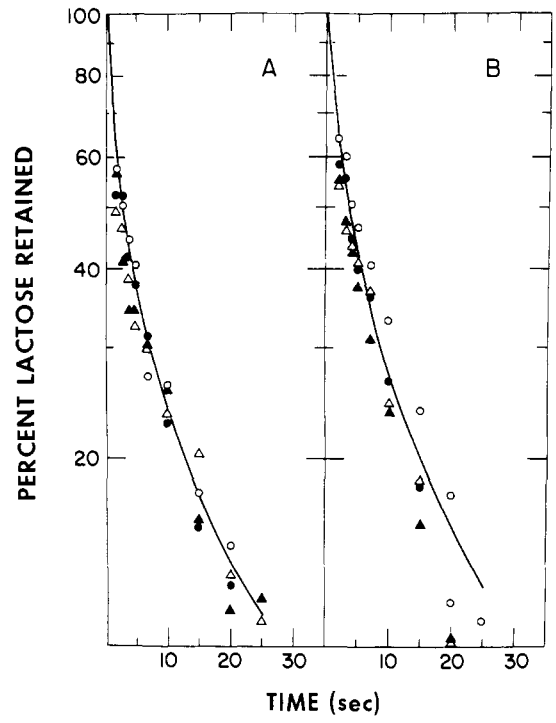


FIGURE 7: Effect of $\Delta\Psi$ (interior negative or positive) (A) and $\Delta$pH (interior alkaline or acid) (B) on lactose exchange in ML 308-225 vesicles. Panel A: vesicles were concentrated to ~30 mg of protein/mL in either 0.1 M potassium phosphate (pH 7.0) or 0.1 M sodium phosphate (pH 7.0). Valinomycin was added to a final concentration of 2 nmol/mg of protein, and the vesicles were equilibrated with 10 mM [1-$^{14}$C]lactose (6 mCi/mmol) by incubation at 25 °C for 3 h. Aliquots (2 $\mu$L) were then diluted 200-fold into media at 18 °C containing unlabeled lactose at a final concentration of 10 mM. Exchange was carried out with potassium-loaded vesicles by diluting into either 0.1 M potassium phosphate, pH 7.0 (●), or 0.1 M sodium phosphate, pH 7.0 (○). Similarly, sodium-loaded vesicles were diluted into either 0.1 M sodium phosphate, pH 7.0 (▲), or 0.1 M potassium phosphate, pH 7.0 (△). Panel B: vesicles were treated as described in panel A, except that the vesicles were initially equilibrated with 5 M potassium phosphate (pH 7.0) and 100 mM potassium acetate or 5 mM potassium phosphate (pH 7.0) and 100 mM potassium gluconate. Exchange experiments were carried out by diluting acetate-loaded vesicles into either acetate (●) or gluconate (○) and by diluting gluconate-loaded vesicles into either gluconate (▲) or acetate (△). As in panel A, the temperature of the reaction mixtures was 18 °C.

creases to 17 s. The data presented in panel B depict the converse experiment in which an acetate diffusion gradient was directed inward in order to generate a $\Delta$pH of opposite polarity (i.e., interior acid). As in Figure 4B, the vesicles were loaded with lower concentrations of [1-$^{14}$C]lactose. Although the effect is relatively small (cf. above), it is apparent that $\Delta$pH (interior acid) enhances the rate of efflux (i.e., $t_{1/2}$ decreases from about 5 to 3.5 s).

Since imposition of $\Delta\Psi$ (interior negative) and $\Delta$pH (interior alkaline) increases Dns$^6$-Gal fluorescence in an additive fashion (Figure 1), the effect of simultaneous imposition of outwardly directed potassium and acetate diffusion gradients on lactose efflux was investigated (Figure 6). Vesicles were equilibrated with 100 mM potassium phosphate (pH 7.0), 100 mM potassium acetate, and 2 mM [1-$^{14}$C]lactose, treated with valinomycin, and diluted into media appropriate for the generation of $\Delta\Psi$ (interior negative), $\Delta$pH (interior alkaline), or $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline). In the absence of imposed gradients, efflux proceeds rapidly with a $t_{1/2}$ of ~4 s. As expected, imposition of $\Delta\Psi$ (interior negative) or $\Delta$pH (interior alkaline) independently decreases the rate of efflux ($t_{1/2}$ = 6 and 10 s, respectively). Moreover, when both gradients are imposed concurrently, leading to generation of $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline), the rate of efflux is diminished even further and the effects of $\Delta\Psi$ and $\Delta$pH appear to be additive ($t_{1/2}$ = 12 s).

In marked contrast to the experiments presented above, outward or inward directed fluxes of potassium and/or acetate have no discernible effect on the loss of intravesicular lactose under exchange conditions (Figure 7). In these experiments, vesicles were equilibrated with 10 mM [1-$^{14}$C]lactose and appropriate salts, treated with valinomycin, and diluted 200-fold into media containing equimolar concentrations of unlabeled lactose. In addition, the composition of the media was manipulated in order to establish potassium and/or acetate diffusion gradients of defined polarities (cf. Figures 4–6), and the experiments were performed at 18 °C so that rates of exchange could be measured accurately (Kaczorowski & Kaback, 1979).[5] As shown in Figure 7A, exchange is extremely rapid ($t_{1/2}$ = 3 s) and unaffected by potassium diffusion gradients in either direction across the membrane (i.e., $\Delta\Psi$, interior negative or positive). Similarly, imposition of acetate diffusion gradients in either direction (i.e., $\Delta$pH, interior alkaline or acid) does not alter the rate of exchange (Figure 7B). Although not shown, simultaneous imposition of $\Delta\Psi$ (interior negative) and $\Delta$pH (interior alkaline) under conditions identical with those described in Figure 6 also has

[5] Efflux experiments carried out at 18 °C demonstrate that the effects of imposed $\Delta\Psi$ and $\Delta$pH are similar to those observed at 25 °C.

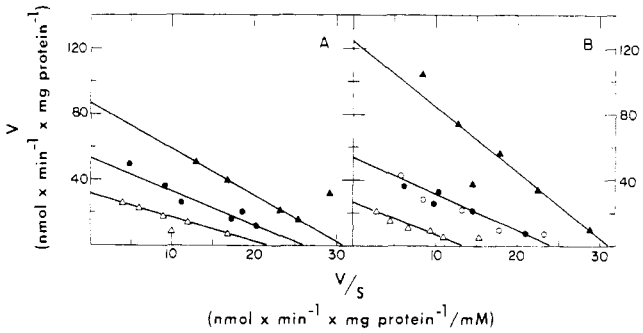


FIGURE 8: Kinetic analysis of the effects of imposed diffusion gradients on lactose efflux. Panel A: effect of $\Delta\Psi$ (interior negative or positive). Vesicles suspended in either potassium or sodium phosphate were concentrated and treated with valinomycin as described in Figure 4. The vesicles were then equilibrated with [1-$^{14}$C]lactose at concentrations ranging from 0.4 to 10 mM. Efflux was initiated by diluting vesicles 200-fold into appropriate media, and time points were taken at 2, 3, 4, and 5 s. The data were corrected for passive lactose efflux by performing identical experiments with vesicles prepared from uninduced *E. coli* ML 30. Kinetic parameters were determined from the Eadie–Hofstee plots shown. (●) Sodium-loaded vesicles diluted into sodium ($K_m$ = 2.0 mM); (▲) sodium-loaded vesicles diluted into potassium ($K_m$ = 2.8 mM); (△) potassium-loaded vesicles diluted into sodium ($K_m$ = 1.4 mM). Although not shown, potassium-loaded vesicles diluted into potassium exhibit a $K_m$ for efflux of 1.2 mM and a $V_{max}$ of 57 nmol/(min mg of protein). Panel B: effect of $\Delta$pH (interior alkaline or acid). Vesicles were treated as described in Figure 5 and equilibrated with [1-$^{14}$C]lactose concentrations ranging from 0.4 to 10 mM. Rates of efflux were then determined as described in panel A above. (○) Acetate-loaded vesicles diluted into acetate ($K_m$ = 2.2 mM); (△) acetate-loaded vesicles diluted into gluconate ($K_m$ = 2.0 mM); (●) gluconate-loaded vesicles diluted into gluconate ($K_m$ = 2.2 mM); (▲) gluconate-loaded vesicles diluted into acetate ($K_m$ = 3.9 mM).

no effect on the rate of exchange.

*Kinetic Analysis of the Effect of* $\Delta\Psi$ and $\Delta$*pH on lac Carrier Turnover.* For mechanistic considerations, it is important to determine the effect of imposed $\Delta\Psi$ and $\Delta$pH on the kinetic characteristics of the efflux process. Toward this end, vesicles were equilibrated with appropriate media containing various concentrations of lactose and treated with valinomycin. Following 200-fold dilution under specified conditions, we measured the rates of efflux over the first 5 s of the reaction (i.e., under initial rate conditions) and the data were corrected for non-carrier-mediated efflux by carrying out parallel studies with vesicles prepared from uninduced *E. coli* ML 30 [cf. Figure 1 in paper by Kaczorowski & Kaback (1979)]. Eadie–Hofstee plots of data obtained in this manner are presented in Figure 8. In the absence of imposed diffusion gradients (panels A and B, open and closed circles), the $V_{max}$ (taken from the intercept with the ordinate) is ~53 nmol/(min mg of protein), regardless of the salt composition of the reaction mixtures. Similarly, the apparent $K_m$ for efflux under control conditions (taken from the slope of the functions) approximates 2.1 mM (the variation observed is less than twofold and is probably insignificant). When $\Delta\Psi$ (interior negative) or $\Delta$pH (interior alkaline) is generated by means of outwardly directed potassium or acetate diffusion gradients (open triangles in panels A and B, respectively), $V_{max}$ is decreased to 31 and 26 nmol/(min mg of protein), respectively, while the apparent $K_m$ does not change appreciably. Moreover, when $\Delta\Psi$ (interior positive) or $\Delta$pH (interior acid) is generated by means of inwardly directed diffusion gradients (closed triangles in panels A and B, respectively), $V_{max}$ is increased to 88 and 124 nmol/(min mg of protein), respectively, and the apparent $K_m$ is not altered significantly. In other words, the data imply that $\Delta\Psi$ and $\Delta$pH alter the velocity of efflux but do not affect the apparent affinity of the carrier.

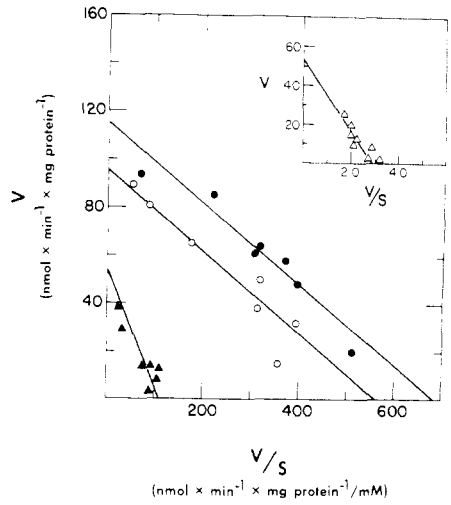


FIGURE 9: Kinetic analysis of lactose influx under energized and nonenergized conditions. Vesicles were assayed for lactose uptake as described previously (Kaback, 1974). The reaction mixtures (50 $\mu$L, final volume) contained 0.05 M potassium phosphate (pH 7.0), 0.01 M magnesium sulfate, 0.1 mg of membrane protein, 0.02 M potassium ascorbate (pH 7.0), 0.1 mM phenazine methosulfate, and concentrations of [1-$^{14}$C]lactose (60 mCi/mmol) ranging from 0.04 to 1.7 mM. The vesicles were preincubated at 25 °C with ascorbate and phenazine methosulfate for 30 s before addition of [1-$^{14}$C]lactose, and uptake was terminated 10 s later. The experiment was performed in the absence of ionophores (●), in the presence of 0.2 $\mu$M nigericin (○), and in the presence of 3 $\mu$M valinomycin (▲). Inset: kinetic analysis of facilitated diffusion. Vesicles were concentrated to 15.5 mg of protein/mL in 0.1 M potassium phosphate (pH 7.0), and CCCP was added to a final concentration of 5 $\mu$M. Aliquots (100 $\mu$L) were then incubated at 25 °C, and [1-$^{14}$C]lactose (60 mCi/mmol) was added at concentrations ranging from 0.5 to 12.5 mM. Initial rates of transport were determined from time points taken during the first 15 s of the reaction, and the data were corrected for passive permeability by performing identical experiments with vesicles prepared from uninduced *E. coli* ML 30. Because of the high protein concentration, 47-mm diameter filters were used for the assay.

To complete this study, kinetics of lactose influx were investigated under a variety of conditions. For facilitated diffusion measurements, vesicles were concentrated to ~15 mg of protein/mL and treated with CCCP in order to inhibit generation of $\Delta\Psi$ or $\Delta$pH induced by lactose/proton symport. Lactose uptake under these conditions proceeds rapidly for ~30 s and achieves a steady state in ~2 min, at which time the intravesicular lactose concentration equals that of the medium (not shown). Initial rates determined from time courses of lactose uptake at various lactose concentrations are presented in the form of an Eadie–Hofstee plot in the inset of Figure 9. The $V_{max}$ for facilitated diffusion [53 nmol/(min mg of protein)] is identical with that obtained for efflux under control conditions (cf. Figure 8). On the other hand, the apparent $K_m$ is significantly different from the apparent $K_m$ for efflux (18.9 mM for facilitated diffusion compared to 2.1 mM for efflux).

For the data presented in the body of Figure 9, reduced phenazine methosulfate was used to drive lactose accumulation at pH 7.0 and the vesicles were exposed to the electron donor for 30 s prior to addition of lactose to insure complete formation of $\Delta\bar{\mu}_{H^+}$ (Kaczorowski et al., 1977). Independent measurements of $\Delta\Psi$ and $\Delta$pH using techniques described previously (Ramos et al., 1976, 1979; Ramos & Kaback, 1977a) yield values of −120 and −60 mV, respectively, for these vesicles at pH 7.0 (data not shown). The initial rate of lactose influx (closed circles) exhibits a $V_{max}$ of ~115 nmol/(min mg of protein), a value approximately twice that of carrier-mediated lactose efflux. The apparent $K_m$ is 0.17 mM, ~100-fold

lower than the apparent $K_m$ for facilitated diffusion. In the presence of nigericin, ΔpH is abolished, the entirety of $\Delta\bar{\mu}_{H^+}$ is expressed as ΔΨ (Ramos et al., 1976; Ramos & Kaback, 1977a), and $V_{max}$ is diminished to 95 nmol/(min mg of protein) without a significant change in apparent $K_m$ (open circles). Valinomycin, on the other hand, collapses ΔΨ with an increase in ΔpH (Ramos et al., 1976; Ramos & Kaback, 1977a), and when this ionophore is added to the vesicles (closed triangles) $V_{max}$ approximates the value obtained for efflux and facilitated diffusion [i.e., 53 nmol/(min mg of protein)] and the apparent $K_m$ is altered to a degree that is probably insignificant (i.e., 0.48 mM). Addition of both ionophores abolishes active transport and reestablishes kinetics typical of facilitated diffusion (not shown). It is apparent from the results that the effects of ΔΨ and ΔpH on influx and efflux differ. In the first instance both $V_{max}$ and $K_m$ are affected, while in the second $V_{max}$ only is changed.

## Discussion

Notwithstanding recent experiments (Overath et al., 1979) indicating that (*N*-dansyl)aminoalkyl β-D-galactopyranosides may be translocated across the membrane, these molecules are extremely useful probes for assessing the effect of imposed diffusion gradients on *lac* carrier turnover (Figures 1–3). As shown previously (Schuldiner et al., 1975), imposition of ΔΨ (interior negative) across the vesicle membrane leads to an increase in Dns[6]-Gal fluorescence; however, attempts to duplicate this effect by imposing ΔpH (interior alkaline) failed. By use of acetate diffusion gradients or a potassium diffusion gradient ($K^+_{out} \rightarrow K^+_{in}$) in the presence of nigericin, this has now been accomplished. Moreover, it has been demonstrated that the effects of ΔΨ and ΔpH are linear with respect to Dns[6]-Gal fluorescence and that the effects are additive. Although the latter results and those demonstrating additive effects of ΔΨ and ΔpH on lactose efflux (Figure 6) may imply that both parameters affect the same step in the overall translocation reaction, it should be emphasized that this conclusion is premature. Attempts to achieve saturation of Dns[6]-Gal fluorescence or lactose efflux by means of imposed diffusion gradients have been unsuccessful. Thus, it has not been possible to test the additive effects of ΔΨ and ΔpH under conditions that are meaningful mechanistically (i.e., if the effect of ΔΨ and ΔpH on the rate of carrier turnover is additive under saturating conditions, it would suggest that the parameters do not affect the same step in the translocation process).

Since the rate-determining step for lactose efflux down a concentration gradient appears to involve a reaction associated with return of the unloaded carrier to the inner surface of the membrane, it was suggested (Kaczorowski & Kaback, 1979) that perturbations of this step should influence the overall rate of efflux. Clearly, this prediction is borne out by the data presented in Figures 4 and 5, where it is demonstrated that imposed ΔΨ's and ΔpH's alter the rate of lactose efflux. Although data were not shown, the effects of ΔΨ and ΔpH on efflux of galactosyl 1-thio-β-D-[6-$^3$H]galactopyranoside are similar to those shown for lactose. When the normal polarity of $\Delta\bar{\mu}_{H^+}$ across the vesicle membrane is considered, efflux is altered in the expected manner. Imposition of ΔΨ (interior negative) or ΔpH (interior alkaline) alone or simultaneously decreases the rate of efflux, while imposition of ΔΨ (interior positive) or ΔpH (interior acid) has the opposite effect. Importantly, moreover, the alterations in rate measured under these conditions are due to changes in $V_{max}$, an observation that is consistent with the notion that ΔΨ and ΔpH affect the limiting step in the overall reaction.

Although the rate of efflux is demonstrably altered by imposed ΔΨ and ΔpH, it is apparent that these parameters exert no effect whatsoever on the loss of intravesicular lactose under exchange conditions (Figure 7). In addition to providing further support for some of the conclusions presented in the previous paper (Kaczorowski & Kaback, 1979), the findings taken together have important implications with regard to the translocation mechanism. It has been suggested (Schuldiner et al., 1975; Rottenberg, 1976) that an unloaded symporter carries a net negative charge while the loaded porter (i.e., the ternary complex between the carrier, substrate, and protons) is neutral, and evidence has been presented (Ramos & Kaback, 1977b,c) that is most easily interpreted in light of this notion. On a simplistic level, the effects of imposed ΔΨ and ΔpH on efflux and exchange documented here are also consistent with this hypothesis. Given the observation that lactose translocation under exchange conditions is unaffected by the imposition of ΔΨ, ΔpH, or $\Delta\bar{\mu}_{H^+}$, it seems unlikely that any of the species formed during this reaction carries a net charge. Moreover, since ΔΨ, in particular, decreases the rate of efflux when the polarity is interior negative and enhances the rate of efflux when interior positive, it is reasonable to suggest that the unloaded carrier may carry a net negative charge. If the unloaded carrier were positively charged, the opposite effects would be expected. On the other hand, it should be apparent that these considerations shed little light on the mechanism by which ΔpH alters the rate of efflux, which further highlights the importance of determining whether ΔΨ and ΔpH affect the same or different steps in the translocation process.

Although kinetic analyses cannot be used to make positive inferences regarding the translocation mechanism, the results presented in Figures 8 and 9 are important because they provide a limit for speculation. When various diffusion gradients are imposed during efflux, it is apparent that ΔΨ and ΔpH alter the $V_{max}$ of the process without producing a significant change in the apparent $K_m$. Moreover, although data were not presented, a kinetic analysis of efflux under the conditions described in Figure 5 (i.e., with ΔΨ, interior negative, and ΔpH, interior alkaline, imposed simultaneously) demonstrates that the $V_{max}$ is reduced even further [to 10 nmol/(min mg of protein)], while the apparent $K_m$ remains unchanged (2.1 mM). It is also interesting that the apparent $K_m$ obtained here is in agreement with the value reported by Lancaster & Hinkle (1977a,b) for facilitated diffusion into inverted vesicles where the cytoplasmic side of the membrane is exposed to the medium (i.e., influx studies with inverted vesicles are analogous to efflux studies with right-side-out vesicles). Strikingly, however, the translocation reactions catalyzed by the *lac* carrier appear to be kinetically asymmetric as evidenced by studies of influx kinetics under homologous conditions (Figure 9). When facilitated diffusion is monitored in the absence of $\Delta\bar{\mu}_{H^+}$, the $V_{max}$ is identical with that observed for efflux under the same conditions [53 nmol/(min mg of protein)], while the apparent $K_m$ is relatively high (18.9 mM). In the presence of $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) the apparent $K_m$ is decreased about 100-fold (0.17 mM) and $V_{max}$ increases [115 nmol/(min mg of protein)], and selective dissipation of either ΔΨ or ΔpH lowers the $V_{max}$ without significantly altering the apparent high affinity $K_m$. Thus, it appears that either ΔΨ (interior negative) or ΔpH (interior alkaline) can decrease the apparent $K_m$ of the carrier externally, as well as increase the $V_{max}$ for influx.

Finally, it must be stated that some of the kinetic results presented here are not in complete agreement with certain other studies carried out with intact *E. coli* ML 308-225

(Winkler & Wilson, 1966; Lancaster et al., 1975). Winkler and Wilson's study (1966) indicated that energy coupling did not affect the apparent $K_m$ for entrance, but raised the apparent $K_m$ for efflux by two orders of magnitude, and it was suggested that the steady-state level of lactose accumulation is related to the ratio of these kinetic constants. The data of Lancaster et al. (1975), on the other hand, were interpreted to indicate that both influx and efflux are modified by energization. In this case, actively metabolizing cells were shown to manifest an inifinite $K_m$ for efflux that became finite upon partial deenergization. In addition, the authors indicated that there was a corresponding decrease in the $V_{max}$ for influx when the cells were deenergized without a discerible effect on the apparent $K_m$ for influx, and they suggested that solute accumulation results from a combined effect of energization on both the influx and efflux reactions. The apparent contradictions between studies with intact cells and those with vesicles may be partially explained by the following considerations. With regard to the effect of energization on the apparent $K_m$ for influx, it is not surprising that changes dependent on diminution of $\Delta\bar{\mu}_{H^+}$ were not detected because of the difficulty inherent in deenergizing intact cells (Koch, 1971). This point becomes considerably more relevant if the change in apparent $K_m$ is not a linear function of $\Delta\bar{\mu}_{H^+}$ (i.e., if the decrease in apparent $K_m$ can occur at low values of $\Delta\bar{\mu}_{H^+}$). Also, the discrepancy between the efflux kinetic data presented here and those mentioned above may reflect technical difficulties associated with efflux measurements in whole cells. For example, significant recapture of substrate from the periplasmic space of the cell may be a problem, especially since the outer membrane can act as a diffusion barrier (Stock et al., 1977). Furthermore, the internal lactose concentration used in some cases (Lancaster et al., 1975) was as high as 250 mM, and under these conditions passive diffusion may make such a large contribution to the overall rate of efflux (Maloney & Wilson, 1973) that the carrier-mediated component is obscured.

## Added in Proof

Given some of the conclusions discussed in this and the preceding paper (Kaczorowski & Kaback, 1979), it is apparent that one means by which to further investigate the suggested mechanism is to search for a solvent deuterium isotope effect. Preliminary experiments (G. J. Kaczorowski & H. R. Kaback, unpublished experiments) have yielded promising results in this regard. At equivalent pH and pD (i.e., pD = pH + 0.4), the rate of lactose efflux is approximately 2–2.5 times slower in deuterated media (with >95% of the protium replaced with deuterium) relative to control conditions in protium, while the rate of exchange is identical in the presence of deuterium and protium. Furthermore, during counterflow with external lactose concentrations below the apparent high-affinity $K_m$ of the carrier (cf. Figure 6 in the preceding paper), the magnitude of the overshoot is greater in deuterium relative to protium.

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