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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 pK_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.

0006-2960/83/0422-2531$01.50/0

may exhibit a solvent isotope effect when studied in deuterium oxide (D_2O)[1] [see Jencks (1969) and Schowen (1977) for reviews]. In brief, such reactions proceed slower in D_2O because of differences in the zero-point energies of the stretching vibrations of bonds to protium relative to deuterium.

A preliminary study (Kaczorowski et al., 1979) suggested that lactose efflux from right-side-out bacterial membrane vesicles is retarded in deuterated solvent, while exchange and counterflow (with saturating external lactose concentrations) remain unaffected. Moreover, it was observed that D_2O stimulates counterflow when the external lactose concentration is below the apparent K_m for the process. Although incomplete, the results are consistent with the proposed model. More importantly, however, they indicate that this general approach might be useful, particularly when applied to a reconstituted system containing purified *lac* carrier.

In this paper, lactose transport by purified *lac* carrier reconstituted into proteoliposomes is studied in H_2O and D_2O under a variety of conditions. The results confirm and extend previous observations (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Garcia et al., 1983) and provide stronger support for certain aspects of the proposed model.

Experimental Procedures

Materials

Deuterium oxide (D_2O; >99% pure) was purchased from Aldrich Chemical Co. All other materials were obtained as described (Garcia et al., 1983).

Methods

Purification and Reconstitution of lac Carrier Protein. The *lac* carrier protein was purified and reconstituted into proteoliposomes as described (Newman et al., 1981; Foster et al., 1982; Garcia et al., 1983). For experiments at different pHs and pDs, proteoliposomes were thawed at room temperature, sonicated for about 15 s by using a bath sonicator, and resuspended in a 100-fold excess of a given buffer at the desired pH. Samples were incubated at room temperature for 30 min and centrifuged for 1 h at 45 000 rpm ($175000g_{max}$) in a Beckman Type 50 Ti rotor. After the supernatant was discarded and the walls of the tube were wiped carefully with a cotton-tipped applicator, the proteoliposomes were resuspended in aqueous or deuterated buffers to a given protein concentration.

Preparation of Buffers in D_2O. Stock solutions of potassium or sodium phosphate, mono- and dibasic, were prepared in pure D_2O (>99%). The solutions were combined to prepare buffers such that the pH measured with a hydrogen ion selective glass electrode was 0.4 unit more acidic than the pD desired (*i.e.*, pD = pH + 0.4) (Jencks, 1969; Schowen, 1977). Alternatively, small aliquots of concentrated potassium or sodium deuteroxide were added to the corresponding dibasic phosphate to attain the desired pH (pD). When the solutions were not used immediately, they were stored under argon in tightly closed screw-top containers sealed with Teflon tape.

Transport Assays. Efflux, exchange, and counterflow measurements were performed as described (Garcia et al., 1983). For membrane potential ($\Delta\Psi$) driven lactose accumulation, proteoliposomes were resuspended in 50 mM potassium phosphate, pH or pD 7.5, and 1 mM dithiothreitol to a final protein concentration of 65 μg/mL, and valinomycin was added to a final concentration of 20 μM. An aliquot (1 μL) was then diluted 200-fold into 50 mM sodium phosphate, pH or pD 7.5, containing 0.3 mM [1-^{14}C]lactose (19.6 mCi/mmol) at 25 °C. At given times, 3 mL of ice-cold 50 mM sodium phosphate, pH 7.5, was added and the sample filtered immediately as described (Garcia et al., 1983). Radioactivity retained on the filter was assayed by liquid scintillation spectrometry.

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).

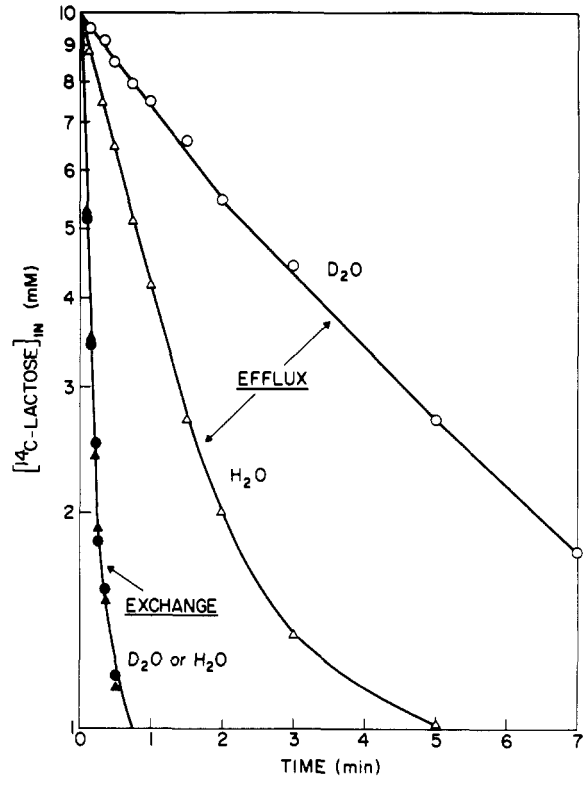

FIGURE 1: Effect of deuterium oxide on lactose efflux and exchange at equivalent pH and pD 7.5. Proteoliposomes containing purified *lac* carrier protein were resuspended in 50 mM potassium phosphate, pH or pD 7.5, and 1 mM dithiothreitol and equilibrated with 10 mM [1-^{14}C]lactose (11.8 mCi/mmol) in the presence of 20 μM valinomycin at room temperature for 1 h as described under Experimental Procedures. Aliquots (1 μL) were then rapidly diluted into 200 μL of a given medium (pH or pD 7.5) at 25 °C. At the times 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. (△) Proteoliposomes equilibrated in 50 mM potassium phosphate, pH or pD 7.5, were diluted into 50 mM potassium phosphate in H_2O, pH 7.5; (○) proteoliposomes equilibrated in 50 mM potassium phosphate, pH or pD 7.5, were diluted into 50 mM potassium phosphate in D_2O, pD 7.5; (▲) proteoliposomes equilibrated as described and diluted into 50 mM potassium phosphate in H_2O containing 10 mM lactose; (●) proteoliposomes equilibrated as described and diluted into 50 mM potassium phosphate in D_2O containing 10 mM lactose. Data are presented as the concentration of lactose retained within the proteoliposomes, calculated as described in the preceding paper (Garcia et al., 1983).

Results

Effect of D_2O on Efflux and Exchange. In order to test the possibility that proton abstraction may be rate determining for carrier-mediated lactose translocation down a concentration gradient, efflux was studied in H_2O and D_2O at an equivalent pH and pD of 7.5 (Figure 1). Valinomycin-treated proteoliposomes suspended in either aqueous or deuterated buffer were equilibrated with 10 mM [1-^{14}C]lactose and diluted 200-fold into appropriate media devoid of lactose. Clearly, the initial rate of efflux is retarded approximately 3-fold in D_2O relative to H_2O. When the same preparations are diluted

[1] Abbreviations: D_2O, deuterium oxide; $\Delta\Psi$, membrane potential.

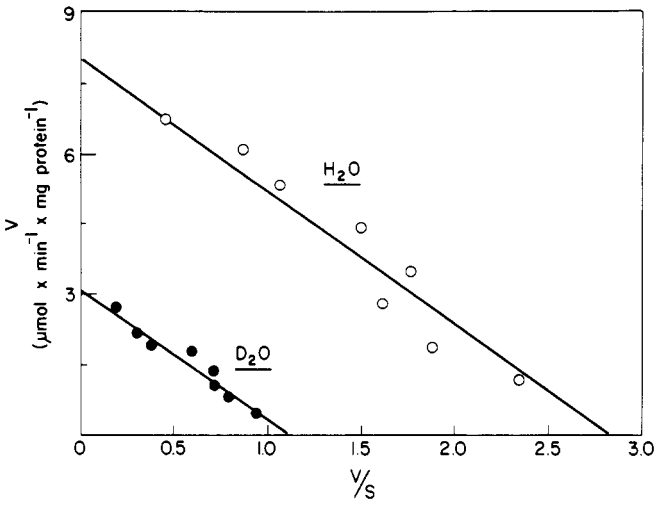

FIGURE 2: Kinetic analysis of deuterium solvent isotope effect on lactose efflux. Proteoliposomes containing purified *lac* carrier protein in 50 mM potassium phosphate, pH (○) or pD (●) 7.5, and 1 mM dithiothreitol were treated with 20 μM valinomycin and equilibrated at room temperature for 1 h with concentrations of [1-^{14}C]lactose ranging from 0.5 to 15 mM. Efflux was measured by diluting 1-μL aliquots into 200 μL of 50 mM potassium phosphate, pH (○) or pD (●) 7.5 at 25 °C. Rates were determined from time points taken during the first 30 s (initial rate conditions) as described under Experimental Procedures. *S*, substrate concentration in mM; *V*, initial velocity in μmol min^{-1} (mg of protein)$^{-1}$.

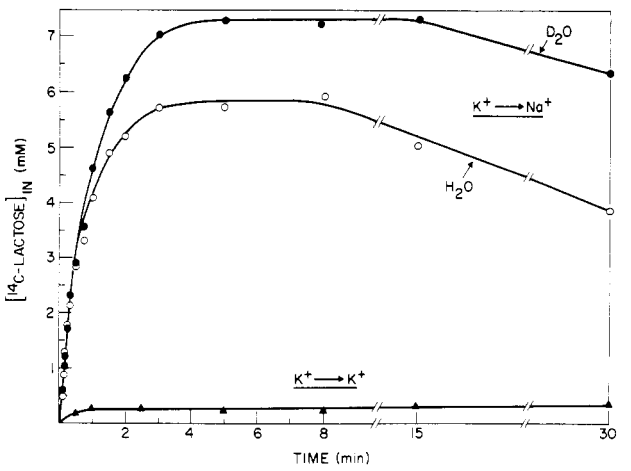

FIGURE 3: Effect of deuterium oxide on ΔΨ-driven lactose accumulation. Proteoliposomes reconstituted with purified *lac* carrier protein were resuspended in 50 mM potassium phosphate in H_2O (○) or D_2O (●), pH (pD) 7.5, containing 1 mM dithiothreitol to a final protein concentration of 65 μg/mL, and valinomycin was added to a final concentration of 20 μM. Aliquots (1 μL) were then diluted 200-fold into 50 mM sodium phosphate in H_2O (○) or D_2O (●), pH (pD) 7.5, containing 0.3 mM [1-^{14}C]lactose (19.6 mCi/mmol) at 25 °C. Alternatively, the proteoliposomes were diluted into 50 mM potassium phosphate in H_2O and D_2O at pH (pD) 7.5 (▲). At given times, 3 mL of ice-cold 50 mM sodium phosphate, pH 7.5, was added, and the samples were filtered immediately as described (Garcia et al., 1983).

into the same media containing equimolar lactose (i.e., exchange conditions), however, the rate of exchange is identical in H_2O and D_2O, an observation that is also consistent with the proposed model.

The data presented in Figure 2 demonstrate that the solvent isotope effect on efflux is due to a rate effect, rather than a change in apparent K_m. In the experiments shown, initial rates of lactose efflux were measured as a function of internal lactose concentration in H_2O and D_2O at pH (pD) 7.5 and the results plotted as indicated. It is apparent that D_2O causes a decrease in the V_{max} for efflux (cf. the intercepts with the *y* axis) with no effect on apparent K_m (i.e., the slopes yield an apparent K_m of about 3 mM in both cases).

In addition to these observations, a few other points are noteworthy: (1) a 3-fold deuterium solvent isotope effect on efflux is also observed at pH (pD) 5.5 and 6.6 (Figure 6, inset); (2) exchange rates are identical in H_2O and D_2O at these pHs (pDs) (Figure 6, inset); (3) identical results are obtained with or without prior equilibration of the proteoliposomes with deuterated buffer; (4) rates of ^{86}Rb efflux from valinomycin-treated proteoliposomes are identical in H_2O and D_2O (i.e., D_2O has no effect on the ability of valinomycin to collapse the ΔΨ generated during lactose efflux).

Effect of D_2O on ΔΨ-Driven Lactose Translocation. Since a proton electrochemical gradient is the immediate driving force for lactose accumulation against a concentration gradient, it was of obvious interest to determine whether or not uphill lactose transport is altered in deuterium relative to protium. As shown (Newman et al., 1981; Foster et al., 1982), proteoliposomes reconstituted with purified *lac* carrier accumulate lactose in response to an artificially imposed ΔΨ (interior negative). In the experiments presented in Figure 3, potassium-loaded proteoliposomes were treated with valinomycin and diluted into equiosmolar sodium, pH (pD) 7.5, containing [1-^{14}C]lactose. Notably, the initial rate of lactose uptake is identical in protium and deuterium. Subsequently, however, as the internal concentration approaches steady state and during the efflux phase, the samples incubated in D_2O retain a greater amount of lactose, a finding that is entirely consistent with solvent isotope effect on efflux. The results imply that when a driving force is applied (i.e., ΔΨ), those steps in the overall reaction that involve proton translocation are no longer rate determining.

Effect of D_2O on Lactose Counterflow. As demonstrated previously in right-side-out membrane vesicles (Kaczorowski & Kaback, 1979) and proteoliposomes containing *lac* carrier protein (Garcia et al., 1983), entrance counterflow can be used to assess the frequency with which the carrier returns from the outer to the inner surface of the membrane in the loaded vs. the unloaded form. With respect to the proposed mechanism [cf. Figure 9 (Garcia et al., 1983)], during counterflow, unlabeled internal lactose is translocated with a proton to the outer surface of the membrane where it is released prior to the symported proton. Depending on the external concentration of labeled lactose, the resulting C–H (protonated) form of the carrier can partition between two pathways: (1) binding of labeled external lactose and return of the loaded carrier, resulting in counterflow (i.e., exchange of internal unlabeled lactose for external labeled lactose); (2) deprotonation and return of the unloaded carrier, resulting in efflux.

With saturating external [1-^{14}C]lactose concentrations, the magnitude of the overshoot observed during counterflow is independent of pH, and the coupling efficiency is essentially 1:1 (Kaczorowski & Kaback, 1979; Garcia et al., 1983). Therefore, when external lactose is saturating, the vast majority of the carrier is presumably turning over in the protonated state (i.e., pathway 1 is favored), and D_2O should have no effect. The prediction is borne out by the experiments described in Figure 4, where entrance counterflow was measured in H_2O and D_2O at values of pH (pD) ranging from 5.5 to 7.5 with the external [1-^{14}C]lactose concentration approaching saturation.[2] Regardless of pH (pD), both the rate of counterflow

[2] The apparent K_m for entrance counterflow in proteoliposomes reconstituted with purified *lac* carrier protein is about 0.65 mM (unpublished observations). In the experiments shown in Figure 4, the external lactose concentration was 0.8 mM, a concentration that is above the apparent K_m but insufficient to produce maximum rates of counterflow.

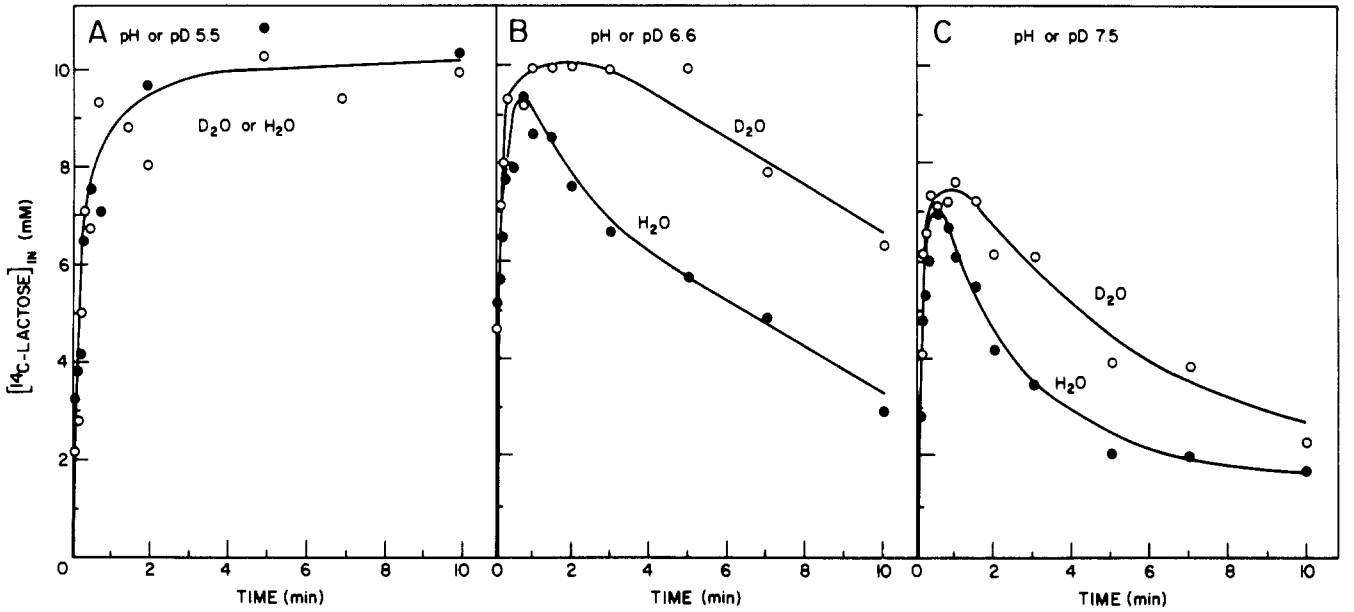

FIGURE 4: Deuterium solvent isotope effect on lactose counterflow at high external lactose concentrations. Proteoliposomes containing purified *lac* carrier protein in 50 mM potassium phosphate containing 1 mM dithiothreitol were equilibrated with 10 mM lactose as described under Experimental Procedures at the pH (●) or pD (○) values given, and valinomycin was added to a final concentration of 20 μM. Aliquots (1 μL) were then diluted 200-fold into 50 mM potassium phosphate at the corresponding pH (●) or pD (○) containing 0.8 mM [1-^{14}C]lactose (7.38 mCi/mmol) at 25 °C (cf. footnote 2). Samples were assayed at given times as described, and the internal concentration of [1-^{14}C]lactose was calculated as described (Garcia et al., 1983). Experiments were performed at pH (●) and pD (○) 5.5, 6.6, and 7.5, as indicated.

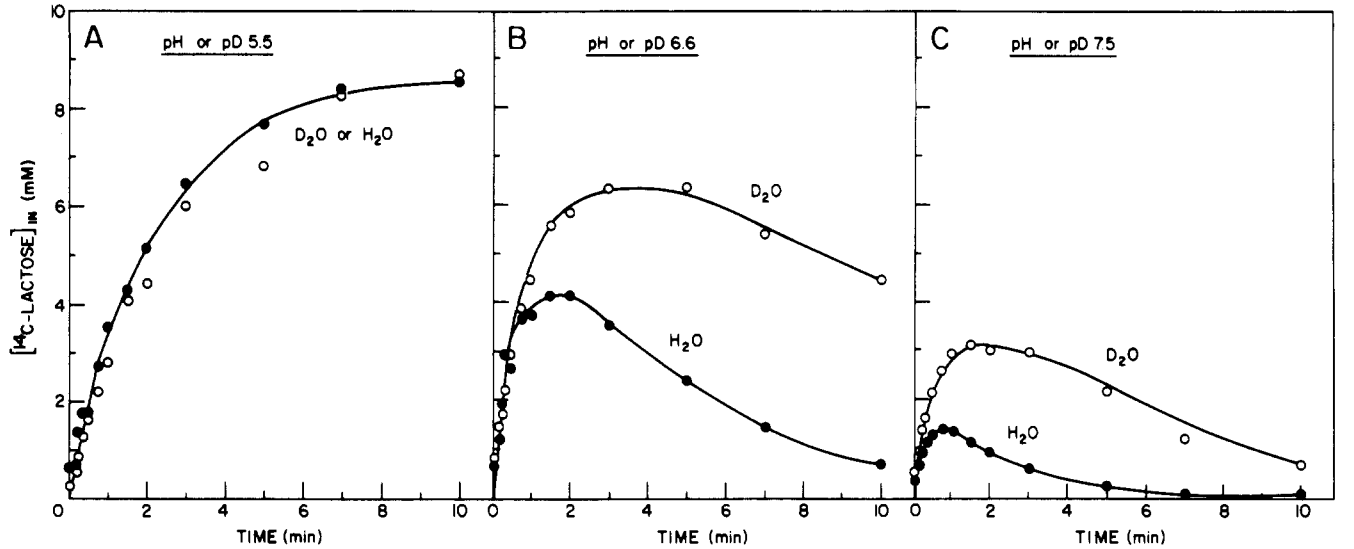

FIGURE 5: Deuterium solvent isotope effect on lactose counterflow at subsaturating external lactose concentrations. The experiments shown were performed exactly as described in Figure 4 except that the proteoliposomes were diluted into media containing 0.075 mM [1-^{14}C]lactose (19.5 mCi/mmol) (cf. footnote 2). Experiments were carried out in H_2O (●) and D_2O (○) at pH (pD) 5.5, 6.6, and 7.5, as indicated.

and the extent of the overshoot are similar in protium and deuterium. On the other hand, as expected, there is a significant pH (pD) dependence and an isotope effect on the rate at which the overshoot decays at pH (pD) 6.6 and 7.5.

Importantly, when counterflow is monitored with the external [1-^{14}C]lactose concentration below saturation, the magnitude of the overshoot is inversely proportional to pH, and the coupling efficiency is diminished (Kaczorowski & Kaback, 1979; Garcia et al., 1983). Thus, under these circumstances, lactose is released on the surface of the membrane, but binding of labeled substrate occurs relatively infrequently, allowing deprotonation and return of the unloaded carrier which is reflected by a decrease in counterflow (i.e., pathway 2 is favored). Furthermore, the process is accentuated as pH is increased, causing further diminution of counterflow. Since these effects are presumably dependent on deprotonation, a solvent deuterium isotope effect might be expected (Figure 5). The experiments shown were performed exactly as described in the previous figure, except that the external [1-^{14}C]lactose concentration was 8-fold lower than the apparent K_m for counterflow. As shown previously, the magnitude of the overshoot is now sensitive to pH, decreasing as pH is increased from pH 5.5 to 7.5. Furthermore, it is clear that the overshoot is markedly enhanced by deuterium at pH (pD) 7.5, somewhat less so at pH (pD) 6.6, and not at all at pH (pD) 5.5.

pH and pD Profiles for Efflux and Exchange. Thus far, the data are consistent with the proposal that proton removal from the *lac* carrier is rate determining for lactose efflux. However, it is still possible that D_2O acts at another level to retard efflux. That is, since it is well established that deuterium shifts the pK_as of acids to more alkaline values by 0.4–0.7 pH unit (Jencks, 1969; Schowen, 1977), the observed effects of D_2O might represent a pK_a effect rather than a true

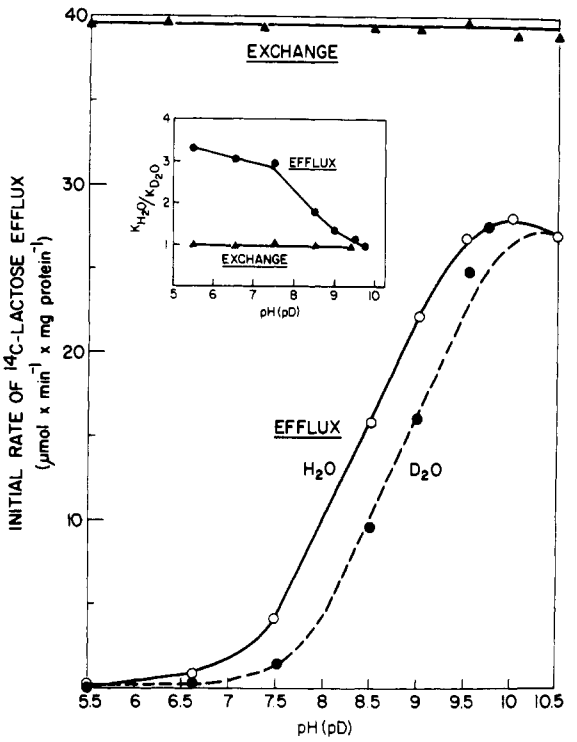

FIGURE 6: pH and pD profiles for lactose efflux and exchange. Proteoliposomes reconstituted with purified *lac* carrier protein were resuspended in 50 mM potassium phosphate containing 2 mM dithiothreitol at given pH or pD values as described under Experimental Procedures. Valinomycin and nigericin were added to final concentrations of 20 and 2 μM, respectively. A small aliquot of [1-^{14}C]lactose (11.8 mCi/mmol) was added to each suspension to a final concentration of 10 mM. After equilibration at room temperature, 1-μL aliquots were diluted into media without (EFFLUX) or with 10 mM unlabeled lactose (EXCHANGE) at a given pH or pD at 25 °C, and the reactions were terminated and assayed as described (Garcia et al., 1983). Rates were determined from time points taken during the linear portion of the reactions. (○) Proteoliposomes in 50 mM potassium phosphate at given pH values were diluted into the same medium devoid of unlabeled lactose; (●) proteoliposomes in 50 mM potassium phosphate at given pD values were diluted into the same medium devoid of unlabeled lactose; (▲) proteoliposomes in 50 mM potassium phosphate at given pH and pD values were diluted into the same medium containing 10 mM lactose [experimental values obtained in H_2O and D_2O were essentially identical at each pH (pD) tested]. (Inset) Relative rates of efflux (●) and exchange (▲) in H_2O vs. D_2O as a function of pH (pD).

kinetic isotope effect. Differentiation between these possibilities can be accomplished in some instances by rigorous comparison of relative velocities in protium and deuterium over an extended range of pH (pD) values (Figure 6). As shown, the pH profile for efflux is markedly sigmoidal, increasing slowly from pH 5.5 to pH 7.5 and very sharply between pH 7.5 and pH 9.5 and remaining essentially constant from pH 9.5 to pH 10.5. Superficially, at least, the profile resembles a classical titration curve with a pK_a at pH 8.3. In contrast, the rate of exchange remains constant over the same extended pH range. Moreover, while exchange is about 100 times more rapid than efflux at pH 5.5, it is only 25–30% more rapid at pH 9.5 and above.

The rate of efflux in deuterium also increases with pH (pD) in a sigmoidal manner, but the profile is shifted about 0.4 unit toward alkaline pH (pD). It is also apparent that the solvent isotope effect disappears at pH (pD) 9.5, where the rates of efflux are identical in H_2O and D_2O. The effect of pH (pD) on the magnitude of the solvent isotope effect is shown more clearly in the inset of Figure 6. From pH (pD) 5.5 to 7.5, the rate of efflux is about 3 times slower in deuterium relative to protium; above pH (pD) 7.5, the magnitude of the effect decreases, and it disappears at pH (pD) 9.5. Under the assumption that the rate-determining step for efflux does not change at alkaline pH, the results suggest that the solvent isotope effect is due to an alteration in the pK_a of a functional group that is critical for the efflux reaction. The data also demonstrate clearly that exchange exhibits no deuterium solvent isotope effect whatsoever over the entire pH (pD) range studied. Thus, it is evident that a functional group with a pK_a at pH 8.3 must be deprotonated for the *lac* carrier protein to catalyze efflux at maximum rates, while the state of protonation of this functional group is not critical for the exchange reaction.

Discussion

The experiments presented in this paper were designed to test more rigorously a proposed kinetic model for carrier-mediated lactose efflux down a concentration gradient (Kaczorowski & Kaback, 1979; Garcia et al., 1983); specifically, whether or not proton removal from the carrier on the outer surface of the membrane is rate determining. Since many reactions involving proton transfer in the rate-limiting step proceed at slower rates in deuterium relative to protium (Jencks, 1969; Schowen, 1977), it was felt that this approach might provide mechanistic insight. For example, according to the model (1) efflux, but not exchange, should proceed at a slower rate in deuterated solvent, (2) in the presence of saturating external lactose concentrations, counterflow should be unaffected by D_2O, (3) when external lactose is subsaturating, on the other hand, the coupling efficiency for counterflow should increase in D_2O, and (4) conditions that change the rate-determining step for efflux should abolish the solvent isotope effect.

The results presented in Figures 1 and 2 confirm the first prediction. Thus, the rate of lactose efflux in the pH (pD) range from 5.5 to 7.5 is about 3-fold slower in D_2O relative to H_2O, and the apparent K_m of the reaction is unaltered. In contrast, the velocity of the exchange reaction is not altered to any degree whatsoever in D_2O, presumably because deprotonation does not occur during this mode of translocation.

The data presented in Figures 4 and 5 bear out the second and third predictions. When counterflow is measured with external [1-^{14}C]lactose concentrations approaching saturation (Figure 4), D_2O has little or no effect on the rate of counterflow or the magnitude of the overshoot; regardless of pH (pD), the coupling efficiency is close to 1:1. With respect to the model [cf. Figure 9 (Garcia et al., 1983)], high external lactose concentrations prevent deprotonation of the carrier (the C–H form), and it recycles across the membrane in the fully loaded state, catalyzing 1:1 exchange of internal unlabeled lactose with external [1-^{14}C]lactose. When the external lactose concentration is well below the apparent K_m, however, D_2O increases the coupling efficiency for counterflow, particularly at higher pH (pD) values (Figure 5). Under these conditions, the C–H or C–D form of the carrier partitions between two pathways, one involving loss of protium or deuterium which results in net efflux and the other involving rebinding of lactose prior to loss of protium or deuterium which results in exchange (i.e., counterflow). The former pathway is favored at high pH (pD) values, but the C–D form of carrier is deprotonated at a slower rate than the C–H form, favoring binding of external [1-^{14}C]lactose. Consequently, the frequency with which the carrier returns to the inner surface of the membrane in the loaded vs. the unloaded form is enhanced in the presence of D_2O, and the effect is most marked at alkaline pH. At relatively acid pH (pD), where the coupling efficiency is already

essentially 1:1, the D_2O effect is masked.

Taken together, these results and those presented in the preceding paper (Garcia et al., 1983) are consistent with the interpretation that proton abstraction is rate determining for efflux. However, since D_2O is known to perturb the pK_as of acids, causing an alkaline shift of 0.4–0.7 pH unit (Jencks, 1969; Schowen, 1977), it is important to make the distinction between a rate effect and a pK_a effect which could alter the equilibrium between two forms of the *lac* carrier (e.g., C^- and C–H). The pH profile for efflux is markedly sigmoidal with an apparent pK_a at 8.3 in H_2O (Figure 6). The rate increases nearly 100-fold from pH 5.5 to pH 9.5 and remains constant from pH 9.5 to pH 10.5. Given the assumption that the proton translocated in symport with lactose must be associated with a functional group(s) in the *lac* carrier protein, it is reasonable to expect this group(s) to exhibit an apparent pK_a. Thus, it was anticipated that the pD profile for efflux in D_2O would be shifted to more alkaline pH (pD) values. Additionally, however, if the solvent isotope effect is due to a rate effect, the rate of efflux in D_2O should always be less than that observed in H_2O, even at the respective pH and pD optima. Clearly, this is not the case. That is, although the pD profile is shifted alkaline by about 0.4 pH (pD) unit, the rates are the same at pH 9.5. Although this finding lends credence to the contention that the solvent isotope effects observed here are secondary to a pK_a effect rather than a true kinetic isotope effect, one further consideration must be taken into account. Importantly, the rate of the exchange reaction is constant over the entire pH (pD) range tested and exhibits no solvent deuterium isotope effect. Furthermore, although the rate of exchange is more than 100 times faster than the rate of efflux at pH 5.5, the exchange process is only 25–30% faster than efflux at pH 9.5 and above. Therefore, the possibility exists that the rate-determining step for efflux changes at high pH, in which case a kinetic isotope effect would also be expected to disappear (i.e., conditions that change the rate-determining step for efflux should abolish the D_2O effect).

As discussed earlier (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979), the rate-determining step for efflux must involve deprotonation of the carrier on the outer surface of the membrane per se or the subsequent step, a reaction corresponding to the return of the unloaded carrier to the inner surface of the membrane. If the model is correct, the pH profile and the loss of the solvent isotope effect at alkaline pH, in particular, suggest that return of the unloaded carrier is the rate-determining step and that ambient pH affects the rate of efflux by altering the equilibrium between protonated and unprotonated forms of the carrier.

In any event, regardless of the precise nature of the solvent deuterium isotope effects described here, the experiments highlight certain remarkable differences between the translocation reactions catalyzed by the *lac* carrier protein. Exchange, a reaction that does not involve net proton movements, is unaffected by pH or by the imposition of $\Delta\Psi$ and/or ΔpH (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Garcia et al., 1983) and is completely unaffected by D_2O under all conditions tested. On the other hand, lactose efflux down a concentration gradient, a reaction that occurs in symport with protons, is markedly altered by ambient pH and by imposition of $\Delta\Psi$ and/or ΔpH and exhibits a solvent deuterium isotope effect at acid and neutral pH. Finally, the rate of $\Delta\Psi$-driven lactose/proton symport is essentially identical in protium and deuterium at pH (pD) 7.5, indicating that protonation or deprotonation reactions, whether or not they involve proton transfer per se, pH-dependent equilibria, or pK_as of specific functional groups, are not rate determining when the electrochemical proton gradient is the driving force for active transport.

Registry No. Lactose, 63-42-3.

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