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The role of protons in the mechanism of galactoside transport via the lactose permease of *Escherichia coli*

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The kinetic mechanism of lactose transport across the cytoplasmic membrane has been investigated and the results related to standard models for the lactose-H^+ symport reaction using computer simulation. It is shown that the biphasic kinetics reported for lactose uptake (Kaczorowski, G.J. and Kaback, H.R. (1979) Biochemistry 18, 3691–3697) are consistent with random binding of lactose and protons and rapid subsequent translocation of the ternary lactose-H^+-permease complex. Such a model is also shown to explain the observed dependence of the kinetic parameters on the magnitude of the protonmotive force. Both sugar and protons are shown to cause product inhibition of lactose flux and the ability of standard models to account for the pattern of inhibition is discussed. Three apparent dissociation constants have been determined for the protonation reactions in the external medium: two (pK_a 6.3 and 9.6) control the activity of the permease, whilst the third (pK_a 8.3) controls the affinity of the permease for galactosides. A similar set of dissociation constants has been determined for the internal reactions. Again two (pK_a 6 and 9.8) control activity and a third (pK_a 8.8) controls the affinity for galactosides. The dissociation reactions characterised by pK_a 8.3, 8.8, 9.6 and 9.8 are attributed to the dissociation of the substrate (symported) proton from the binary proton-permease complexes (pK_a 8.3 and 8.8) and the ternary proton-galactoside-permease complexes (pK_a 9.6 and 9.8). The third pair (pK_a 6.3 and 6.0) must be interpreted as describing a separate protonation reaction which may have a regulatory or auxiliary role in transport.

Introduction

The lactose permease catalyses the coupled flux of galactosides and protons across the cytoplasmic membrane of *Escherichia coli* [1]. Thermodynamic and kinetic determinations of the stoichiometry of transport give values around one proton transported per galactoside over a wide range of conditions [1–3] and genetic and kinetic studies suggest that there is one galactoside binding site in each molecule of permease [4–7]. Binding of the sugar to this site promotes a conformational change that appears to occlude the galactoside [8] and alters the reactivity of a number of amino acid residues [5,9–11]. Net turnover, during the influx of sugar, promotes the accumulation of an intermediate of the permease reaction cycle that has a conformation which is different from that adopted in either the resting state or when the galactoside is bound [10]. Studies using genetics [12,13], kinetic analysis [14] and affinity labelling with reactive galactosides [15,16] all suggest that the galactoside binding site is formed by parts of the central two-third of the polypeptide chain while the parts of the molecule formed from the first 150 and last 100 residues appear to be involved, directly or indi-

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rectly, in the conformational changes that occur during galactoside binding and transport. Very little is known about the mechanism of proton-binding, its effects on the conformation of the protein and the residues involved. However, recent work with site-directed mutagenesis suggests that residues around His-322 and Glu-325 may be important to the release of protons during efflux [17].

The steady-state kinetic mechanism is consistent with the formation of a ternary complex between permease, galactoside and proton [18]. It has been suggested that binding of substrates occurs in an ordered mechanism where the proton binds first and leaves last [20] or by a random mechanism, where the ternary complex can be formed through either of the two possible binary complexes [18,21,22]. It has been shown that, for the random mechanism, there must be an asymmetry between the kinetic constants describing the reactions in the two branches leading to the ternary complex [18]. This results in effective ordering of the substrate binding over the physiological range of pH (6–8) and at usual galactoside concentrations (millimolar or less). It is, however, still not certain whether any of the suggested models can properly describe the kinetic mechanism of galactoside-proton symport, particularly because there is an uncertainty in the proton dissociation constants [18], and one of the aims of the experiments reported here was to investigate this problem.

Previous kinetic studies [14,18,23] have led to the prediction that internal protons should act as inhibitors of influx and, similarly, that external protons should act as inhibitors of efflux. Evidence consistent with these expectations has been obtained [18,23] but a detailed study has not yet been reported. In the present study the results of a more complete investigation of the effects of varying the pH on both sides of the membrane are described. The inhibition produced by the opposing (or *trans*) proton concentration was found to contain both a competitive and a non-competitive element. Although this pattern of inhibition is that expected for the random mechanism, it became apparent that there is a second ionisation that affects activity and this complicates the precise interpretation of the non-competitive element. Thus it appears that, in addition to the two pairs of proton dissociation reactions expected for a non-symmetrical random mechanism, there is a pair of proton dissociation reactions (pK_a 6.0 and 6.3) that appear to have a regulatory role in transport.

Materials and Methods

Bacteria

Escherichia coli ML308-225 (i^-, z^-, y^+, a^+) was grown in minimal medium M9 [24] containing 0.5% glycerol and harvested in late exponential phase. The bacteria were washed once by resuspension in 150 mM KCl containing 100 μg/ml chloramphenicol then treated with EDTA [43] and washed once by resuspension in the medium of the experiment.

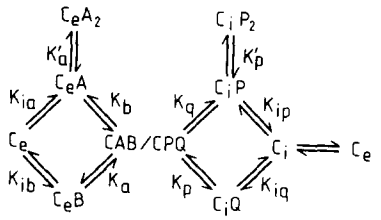
Measurement of transport

Uptake measurements were performed as described previously [14]; in some experiments [^{3}H]methyltriphenylphosphonium iodide was included to measure $\Delta\psi$ and in others radioactive [^{14}C]methylamine or 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione were included to measure ΔpH. Measurements were made before initiating the lactose flux, during the time-course of the experiment and at the end-point of the reaction. When a significant change occurred during uptake of lactose, the initial values (before initiation and during the first 10 s) were used for subsequent analysis. The internal volume was found to be between 0.8 and 1.5 (mean 1.07 ± 0.13) μl/mg of dry bacteria.

Data analysis

Initial rates of flux were estimated using the appropriate integrated rate equations [14]. The dependence of the initial rates on lactose concentration and the pH dependence of the derived kinetic parameters (see Eqn. 1 and Definitions) were analysed using appropriate weighted linear or non-linear regression [18,25] based on Eqns. 2–9 in the text below. Simpler and more complicated equations were also tested and rejected either because they did not fit the data so well or because the additional terms did not improve the fit to the data (see Refs. 18 and 25 for a discus-

sion). Thus each expression represents the simplest equation that made a close fit to the experimental points. A qualitative discussion of the models considered and the reasons for their rejection is given in the appendix.



(1)

Definitions

[A] = external proton concentration
[B] = external lactose concentration
[P] = internal proton concentration
[Q] = internal lactose concentration
Δp = protonmotive force
ΔpH = chemical potential gradient for protons
$\Delta\psi$ = electrical potential difference across the membrane
K_a = lactose-independent Michaelis constant for external protons
K'_a = inhibition constant describing the non-competitive effect of external proton concentration on flux
K_b = pH-independent Michaelis constant for external lactose
K_b^A = pH-dependent Michaelis constant for external lactose
K_{ia} = dissociation constant for external protons
K'_{ia} = inhibition constant describing the effect of external protons on efflux reactions
K_{ip} = dissociation constant for internal protons
K'_{ip} = inhibition constant describing the competitive effect of internal protons on influx reactions
K_{Ib} = pH-independent inhibition constant describing the mixed inhibition of efflux by external lactose
K_{Ib}^A = pH-dependent inhibition constant
K_{Iq} = pH-independent inhibition constant describing the mixed inhibition of influx by internal lactose
K_{Iq}^A = pH-dependent inhibition constant
K_p = lactose-independent Michaelis constant for internal protons
K'_p = inhibition constant describing the non-competitive effect of internal proton concentration on flux
K''_p = theoretical inhibition constant describing non-competitive inhibition of influx by internal protons
K_q = pH-independent Michaelis constant for internal lactose
K_q^A = pH-dependent Michaelis constant for internal lactose
V_{ab} = pH-independent maximum velocity of influx
V_{ab}^A = pH-dependent maximum velocity of influx
v_{obs} = initial rate of flux
V_{pq} = pH-independent maximum velocity of efflux
V_{pq}^A = pH-dependent maximum velocity of efflux

Results

(i) The time-course of influx at low lactose concentration

The uptake of lactose by endogenously respiring cell suspensions was rapid during the first few seconds and then it continued more slowly for some minutes until a steady-state was reached, where no further net flux occurred (Fig. 1a). When the external lactose concentration was below 5 mM, the first 80% of the net flux reaction could be described by an integrated form of Eqn. 2 (Fig. 1b).

$$v_{obs} = \frac{V_{ab}^A[B]}{(K_b^A + [B])(1 + [Q]/K_{Iq}^A)} \quad (2)$$

The influx of lactose was inhibited by the accumulated sugar ([Q]) which is one of the products of the uptake reaction. The product inhibition was of mixed type (containing both competitive and non-competitive elements) with $K_{Iq}^A = 31 \pm 2$ mM (Fig. 1b). The three kinetic parameters could be determined from a series of experiments at different lactose concentrations, as shown in Fig. 1. However, these are only apparent kinetic parameters because they do not take into account the effect of the other substrate, the proton.

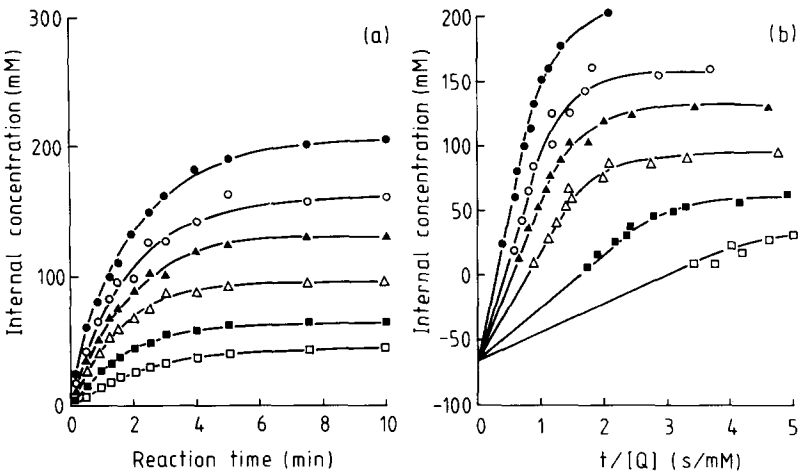


Fig. 1. The time-course of lactose uptake. (a) Concentration dependence of the progress-curve of lactose uptake. Assays were performed using endogenously respiring cell suspensions in 0.1 M potassium phosphate (pH 7.0). (b) Product inhibition plot of the data presented in (a). The analysis is described in Methods. The intercept on the concentration axis gives the inhibition constant and the intercept on the $t/[P]$ axis gives the extrapolated initial rate of uptake.

(ii) The pH dependence of the kinetic parameters describing influx

The value of V_{ab}^{A} was constant over fairly wide ranges of internal and external pH. It decreased at acid internal or external pH (below pH 6.5) and at alkaline pH (pH 9.5) as shown in Fig. 2(a). The pH dependence of V_{ab}^{A} is described by Eqn. 3

$$V_{ab}^{A} = \frac{V_{ab}[A]}{(K_a + [A])(1 + [A]/K'_a + [P]/K'_p)} \quad (3)$$

and the values of the kinetic constants are given in Table I. The form of Eqn. 3 is most readily explained by decrease in the turnover number caused by protonation at low pH on either side of the membrane (pK'_a and pK'_p 6.3) and by deprotonation at alkaline pH (pK_a 9.6). The theoretical pH dependence for such a system would be described by Eqn. 4

$$V_{ab}^{A} = \frac{V_{ab}[A]}{(K_a + [A](1 + [P]/K''_p))(1 + [A]/K'_a + [P]/K'_p)} \quad (4)$$

and thus the non-competitive terms in $[P]/K''_p$ appear to be missing from Eqn. 3. There are a number of reasons why these terms might not be detected; it is possible either that they are merged with the inhibition ascribed to terms in $[P]/K'_p$ by the fitting procedure or that they are genuinely absent from the rate expression. The former possibility cannot be completely excluded because the quality of the data at low activity is poor. The latter possibility is discussed in more detail below. The dependence of V_{ab}^{A} on external pH, described by Eqn. 2, is similar to that reported for lactose uptake in membrane vesicles [26], thiomethylgalactoside and 2-nitrophenyl-β-galactoside uptake to intact cells [35], and thiolactose and melibiose uptake into intact cells [14].

The value of K_b^A was constant at acid and neutral external pH but increased at alkaline pH, which is consistent with previous reports [14,26,25]. Lowering the internal pH caused the value of K_b^A to increase through competitive inhibition by the *trans*-protons. Because K_b^A is a complex function, involving some of the terms that also appear in V_{ab}^{A}, the effect of pH on apparent affinity is more conveniently analysed using the specificity constant V/K_m (Fig. 2b). This parameter is also particularly relevant because it describes the behaviour of the permease at infinitely low lactose concentrations and hence provides an estimate of, among other things, the dissociation constant for the substrate-proton from the binary proton-permease complex. The pH dependence of the specificity constant is described by Eqn. 5

TABLE I

KINETIC PARAMETERS DESCRIBING LACTOSE-PROTON SYMPORT

PROTON DISSOCIATION REACTIONS

Constant	Source	Value
K_a	Eqn. 3	$2.1 \pm 0.1 \cdot 10^{-10}$ M
K'_a	Eqns. 3, 5, 6, 9	$4.3 \pm 0.3 \cdot 10^{-7}$ M
K_{ia}	Eqn. 5	$4.8 \pm 0.3 \cdot 10^{-9}$ M
K'_{ia}	Eqns. 6, 10	$3.0 \pm 0.2 \cdot 10^{-9}$ M
K'_{ip}	Eqn. 5	$1.5 \pm 0.6 \cdot 10^{-9}$ M
K_{ip}	Eqn. 6	$2.7 \pm 0.4 \cdot 10^{-9}$ M
	Eqn. 10	$5.0 \pm 0.9 \cdot 10^{-9}$ M
K_p	Eqns. 6, 9	$1.9 \pm 0.1 \cdot 10^{-10}$ M
K'_p	Eqns. 3, 5, 6, 9	$2.8 \pm 1.2 \cdot 10^{-7}$ M

Minimum rate constant for association of external protons with the permease-lactose complex [a] $= 1.9 \cdot 10^{12}$ $M^{-1} \cdot s^{-1}$.

Minimum rate constant for association of internal protons with the permease-lactose complex [a] $= 1.1 \cdot 10^{12}$ $M^{-1} \cdot s^{-1}$.

Minimum rate constant for dissociation of the external proton [b] $= 9.1 \cdot 10^3$ s^{-1} (370 s^{-1}).

Minimum rate constant for dissociation of internal proton [b] $= 3.3 \cdot 10^3$ s^{-1} (470 s^{-1}).

GALACTOSIDE DISSOCIATION REACTIONS

Constant	Source	Value
K_b	Eqns. 5, 6	$5.0 \cdot 10^{-4}$ M
K_{Ib}	Eqn. 8	$1.8 \cdot 10^{-3}$ M
K_{Iq}	Eqns. 2, 6	$1.0 \cdot 10^{-3}$ M
K_q	Eqn. 10	$2.9 \cdot 10^{-3}$ M

Minimum association rate of external lactose [a] $= 7.4 \cdot 10^6$ $M^{-1} \cdot s^{-1}$

Minimum association rate of internal lactose [a] $= 4.7 \cdot 10^5$ $M^{-1} \cdot s^{-1}$

Minimum dissociation rate for internal lactose [b] $= 8.4 \cdot 10^4$ s^{-1} (370 s^{-1})

Minimum dissociation rate for external lactose [b] $= 2.2 \cdot 10^4$ s^{-1} (470 s^{-1})

MAXIMUM VELOCITIES

Influx 3.7 ± 0.2 mM $\cdot s^{-1}$, Efflux 4.7 ± 0.5 mM $\cdot s^{-1}$

EXCHANGE

Maximum velocity 7.8 ± 1.9 mM $\cdot s^{-1}$, K_b $2.31 \cdot 10^{-2}$ M, K_{ia} $1.7 + 0.5 \cdot 10^{-9}$ M.

[a] Calculated by assuming that the system obeys simple Michaelis-Menten kinetics and that there is 0.01 nmol permease per mg dry wt.

[b] Calculated with the same assumptions as (a) and also assuming that the association with the binary complex occurs at the same rate as it does with the unloaded permease. The first figure is for dissociation from the binary complex and the second figure is for dissociation from the ternary complex. By the nature of the assumptions made this latter figure is identical to the turnover number of the permease.

$$\frac{V^A_{ab}}{K^A_b} = \frac{V_{ab}[A]}{K_b(K_{ia}(1+[P]/K'_{ip})+[A])(1+[A]/K'_a+[P]/K'_p)} \quad (5)$$

and the values of the kinetic constants are given in Table I. The form of the Eqn. 5 is explained by the decrease in the turnover number at acid pH discussed above (described by K'_a and K'_p), by competitive inhibition of influx by the *trans*-protons (described by K'_{ip}) and by a decrease in affinity for external galactoside when the *cis*-proton dissociates (described by K_{ia}). The form of this equation is close to that predicted by the simple kinetic models [18].

The galactoside-product inhibition constant, K^A_{Iq}, had a value of 2–5 mM when internal and external proton concentrations were low (pH 8.0–8.5) but its value increased considerably in respiring cells when the external proton concentration was increased (pH 6.0–7.0) (Fig. 3). This can be attributed to greater competitive inhibition from the increased external proton concentration while the internal proton concentration remains low due to proton pumping by the electron transport chain. Increasing the internal proton concentration by the addition of, for example, nigericin resulted in a decrease in the value of K^A_{Iq} (Fig. 3). This occurs partly because increasing the internal proton concentration decreases the significance of the competition from external protons and partly because protonation of an internal site increases the apparent affinity for galactoside. Very low internal and external proton concentrations (pH 8.5–10) resulted in an increase in the value of K^A_{Iq}, which parallels the increase in K^A_b seen under the same conditions and is caused by a decrease in affinity when the *cis*-proton dissociates. The dependence on internal and external pH is described by Eqn. 6

$$K^A_{Iq} = \frac{K_{Iq}(K_{ip}(1+[A]/K'_{ia})+[P])}{(K_p+[P])(1+[A]/K'_a+[P]/K'_p)} \quad (6)$$

and the values of the kinetic constants are given in

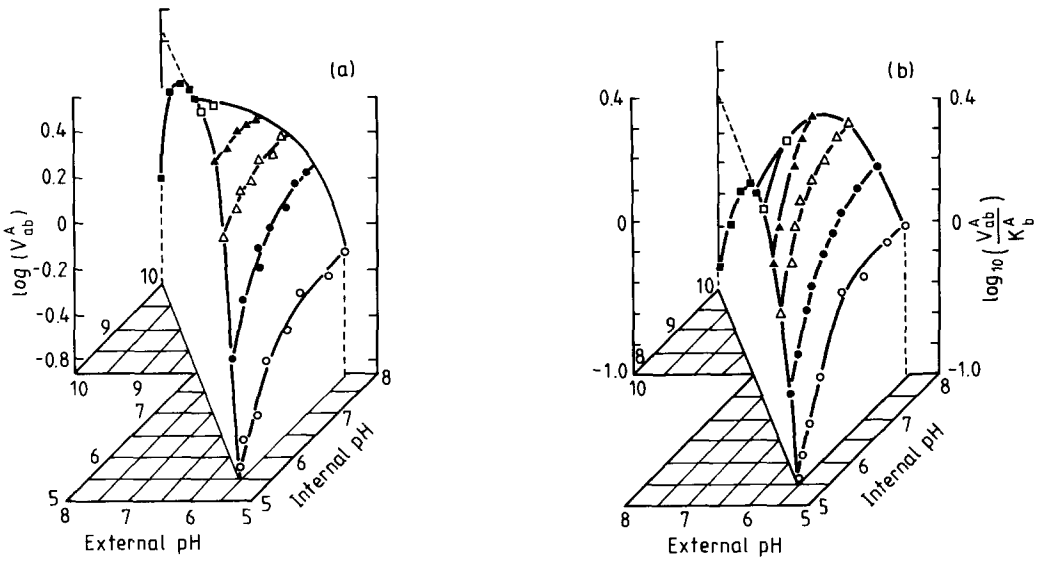


Fig. 2. The pH dependence of lactose influx. (a) The logarithm of the maximum velocity of influx, determined from experiments such as these shown in Fig. 1, is plotted as a function of internal and external pH. External pH was fixed by the choice of buffer for the experiment (potassium phosphate for the range 5.5–8.0 and potassium borate for the range 8.0–10.0) and internal pH was manipulated by incubating with different amounts of nigericin; all suspensions contained valinomycin (10 μg/ml) which kept $\Delta\psi$ at values between 0 and −20 mV. The external pH values were 5.5 (○), 6.0 (●), 6.5 (△), 7.0 (▲), 7.5 (□), 8.0, 8.5, 9.0, 9.5 and 10.0 (all shown as ■). (b) The logarithm of V/K_m for influx is plotted as a function of internal and external pH. The symbols are as described for (a).

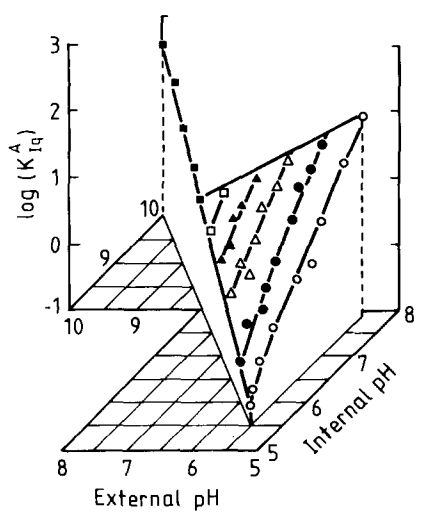


Fig. 3. The pH dependence of the product-inhibition constant describing the effect of internal lactose on lactose influx. The logarithm of the inhibition constant, determined from experiments such as those shown in Fig. 1, is plotted as function of internal and external pH. The experimental detials and symbols are as described in Fig. 2a.

Table I. K_{Iq}^{A} is a complex kinetic parameter describing the effect of internal and external protons on the apparent affinity for internal galactosides. One should note that (i) external protons acted as competitive inhibitors for the efflux reaction by raising the value of K_{Iq}^{A} (through the $[A]/K'_{ia}$ term), (ii) the ionisations that affected the rate of turnover (described by K'_a and K'_p) here appeared to increase the affinity for internal galactoside, which has been reported before [14], (iii) deprotonation of the permease decreased the apparent affinity for galactoside (described by K_{ip}).

(iii) The effect of a membrane potential on the kinetics of influx

So far only the substrate dependence of the initial rate of influx has been examined but it is now well-established that the membrane potential also influences uptake [26,27]. Only the value of V_{ab}^{A} was found to be significantly affected by variation of the membrane potential (Fig. 4); there was no significant effect on the proton-binding constants and K_b appeared to be lower at small

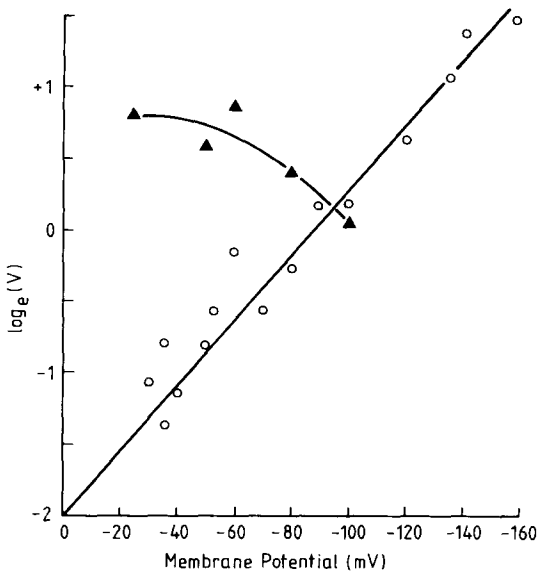


Fig. 4. The effect of a membrane potential on the rates of influx and efflux. The natural logarithm of the maximum velocity of influx (○) and efflux (▲) is plotted against the magnitude of the membrane potential. The medium was 20 mM potassium phosphate, 150 mM KCl (pH 7.0) containing 10 μg/ml nigericin (ΔpH ≈ 27–40 mV); $\Delta\psi$ was manipulated by varying the amount of valinomycin and by replacing some of the KCl by NaCl.

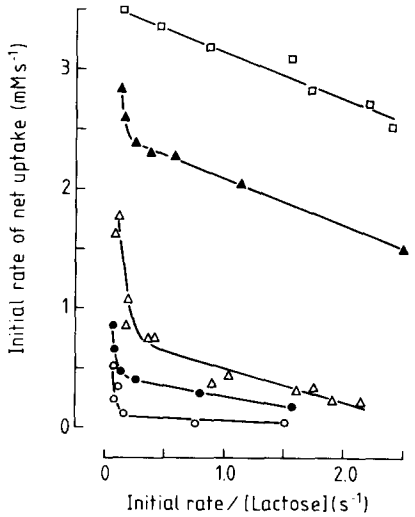


Fig. 5. Non-Michaelis-Menten kinetics of lactose flux. Uptake assays were performed as described but with concentrations of lactose up to 50 mM in the external medium (20 mM potassium phosphate/150 mM KCl). Nigericin was present in all buffers (ΔpH was less than 20 mV) and $\Delta\psi$ was manipulated by varying valinomycin and KCl concentrations. The observed values of $\Delta\psi$, measured before addition lactose, were -31 mV (○), −50 mV (●), −80 mV (△), −120 mV (▲) and −138 mV (□) and these did not change significantly during the first 10–20% of the reaction.

values of $\Delta\psi$ (0.1 mM at $\Delta\psi = -40$ mV) than it was at larger values (0.7 mM at $\Delta\psi = -120$ mV) (Fig. 5). An effect of this nature is predicted by some models of symport, including the random mechanism (see section (vi) below). The dependence of V_{ab}^{A} on was quite simple and close to that expected from simple models of uptake [28] which predict that the maximum velocity should have the dependence on $\Delta\psi$ described by Eqn. 7.

$$\log_e(V_{ab}^{A})_{\Delta\psi} = \log_e(V_{ab}^{A})_{\Delta\psi=0} - nF\Delta\psi/2RT \qquad (7)$$

Thus a plot of $\log_e(V_{ab}^{A})$ against $-\Delta\psi$ should give a straight line of slope $= nF/2RT$. The actual slope is 0.0203 ± 0.0021 so, since $F/2RT$ has a value of 0.0196 at the temperature of the experiment, the value of n, the formal charge translocated by the permease, is 1.07 ± 0.11. Previous quantitative analyses of the effect of membrane potential on kinetics have also established that V is more affected than K_m [25]. In one study it was suggested that the velocity depended on $(\Delta\psi)^2$ [26] and in another [27] it was suggested that the formal charge carried was 2 not 1. However, reanalysis of the published data according to Eqn. 6 gives estimates for n that lie between 1 and 1.3, so there is no conflict with the present results.

(iv) Influx at high lactose concentration

At high lactose concentrations the substrate-dependence of the initial rate of uptake no longer obeys Michaelis-Menten kinetics (Fig. 5 and Ref. 20). A second component with a higher apparent K_m (20–25 mM instead of 0.1–0.8 mM) was detected at pH 6.5–7.5 and may exist over the whole range of pH. This component of uptake was difficult to analyse satisfactorily because of the high lactose concentrations required for saturation. The biphasicity was most marked when $\Delta\psi$ and ΔpH were small (Fig. 5) and the maximum velocity of the high-K_m component under these conditions was 10–20% of the maximum velocity under energised conditions, which is consistent with previous observations [27,29,30].

(v) The efflux reaction

Efflux could be described by an expression analogous to Eqn. 2 (Eqn. 8 below).

$$v_{obs} = \frac{V^A_{pq}[Q]}{(K^A_q + [Q])(1 + [B]/K^A_{Ib})} \quad (8)$$

The integrated form of this equation described approximately 80%–90% of the time-course of the reaction. It should be noted that [B] had the same isotopic composition as [Q]; when [B] was unlabelled the familiar acceleration of efflux through exchange of labelled and unlabelled pools [31] was observed. Although the pH dependence of V^A_{pq} (Fig. 6a and Eqn. 9) was similar to that of V^A_{ab}, the value of V^A_{pq}/K^A_q (Fig. 6b and Eqn. 10) had a dependence on pH that was rather different from that of V^A_{ab}/K^A_b in that the terms in $[A]/K'_a$ and $[P]/K'_p$ were not detected.

$$V^A_{pq} = \frac{V_{pq}[P]}{(K_p + [P])(1 + [A]/K'_a + [P]/K'_p)} \quad (9)$$

$$\frac{V^A_{pq}}{K^A_q} = \frac{V_{pq}[P]}{K_q(K_{ip}(1 + [A]/K'_{ia}) + [P])} \quad (10)$$

The values for the kinetic constants are given in Table I. As with influx, the maximum velocity of efflux was decreased by protonation on either side of the membrane with apparent pK close to 6.3 (described by K'_a and K'_p) and by a deprotonation with pK 9.6 (described by K_p). Again, it was not possible to detect a separate non-competitive inhibition from the substrate-proton (in this case terms in $[A]/K'_{ia}$). In contrast to the influx reaction, the protonation at 6.3 did not affect the magnitude of the specificity constant, V^A_{pq}/K^A_q (Eqn. 10) and the apparent affinity (K^A_q) increased in parallel with the decrease in turnover number (V^A_{pq}). Such an increase in apparent affinity for internal galactoside was also noted for the product inhibition constant (K^A_{Iq}, Fig. 3) and suggests that the protonation is affecting the properties of the substrate bound-permease complexes. External protons acted as competitive inhibitors of efflux, decreasing the apparent affinity nearly 10-fold as their concentration increased 10-fold between pH 8 and pH 7 (described by the $[A]/K'_{ia}$ term in Eqn. 10).

The effect of imposing a membrane potential of normal polarity was to slow down efflux (Fig. 4). The dependence of V^A_{pq} is opposite to that of V^A_{ab} in this respect, as has been observed in membrane vesicles [32]. The slope of the plot of $\log_e(V^A_{pq})$

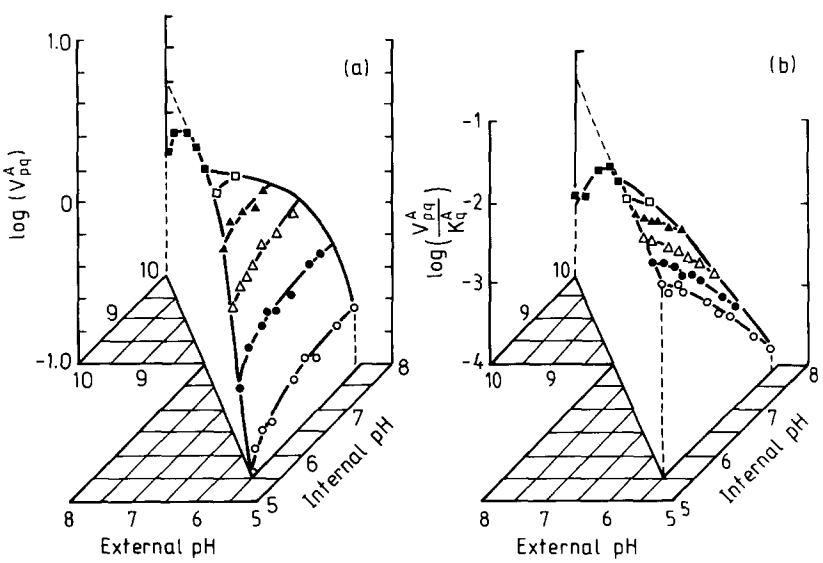


Fig. 6. The pH dependence of the kinetic parameters describing efflux. The logarithm of the maximum velocity (a) and of the specificity constant, V/K_m (b) are plotted as functions of internal and external pH. The experimental details and symbols are described in Fig. 2.

against $-\Delta\psi$ (Fig. 4) -0.0090 ± 0.0018, which gives a vale of 0.5 for the formal charge translocated outwards by the permease. This low value (compared to influx) may be due to the difficulty in estimating the initial value of efflux when $\Delta\psi$ is very low, as well as the uncertainty in determining $\Delta\psi$. Using the data collected above $-\Delta\psi = 50$ mV (10 experiments) gave a value of 0.0165 for the slope and hence a value of 0.84 for the formal charge translocated by the permease.

(v) Isotope exchange at equilibrium

The lactose permease catalysed rapid exchange between internal and external pools of lactose (Fig. 7a). The rate of exchange was proportional to lactose concentration up to 10 mM (Fig. 7b) and extrapolation suggests that the apparent K_m is 20–25 mM and that V was 6–10 mM · s^{-1} (at pH 7.0 the parameters were $K_m = 22.3 \pm 1.8$ mM, $V = 6.3 \pm 0.8$ mM · s^{-1}), which makes exchange significantly faster than net influx under the same, uncoupled, conditions (V for influx = 0.9 mM · s^{-1}). Thus rate limitation of influx seems to occur at a step in the transport cycle after release of galactoside. The rate of exchange at low lactose concentrations showed a rather flat pH dependence (Fig. 7c) which appeared to be due to a pH-independent component that contributed about one-tenth of the total flux at pH 7.0 but contributed the major part of the flux at extremes of pH and a pH-dependent component that contributed the major part of the flux at pH 7.0. By deducting the limiting value of exchange, determined by extrapolation to extreme acid and alkaline pH, from the total flux at each intermediate value of pH, the pH-dependent contribution could be determined (Fig. 7d). This showed an ionisation with pK 5.9 ± 0.2 and a second one at alkaline pH (pK_a 8.7 ± 0.3). The pH-independent component could arise through uncorrected background flux of lactose through external routes or through the permease along minor internal routes that could lead to slip [22]. Wright [33] examined the pH dependence of exchange of thiodigalactoside and found a pH profile rather like that shown in Fig. 7c.

Decreasing the internal proton concentration at constant external proton concentration in the range pH 7–8 decreased the rate of exchange

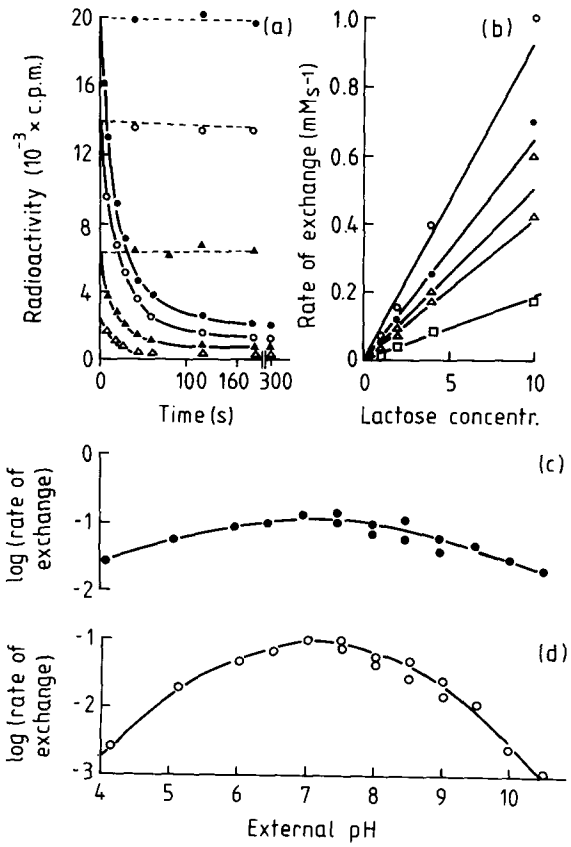


Fig. 7. The kinetics of isotope exchange at equilibrium. (a) The time-course of lactose exchange in cells treated with 30 mM NaN_3 and 10 μg/ml valinomycin at pH 7.0. There is no detectable protonmotive force. The different curves represent cells incubated with 0.2 (△), 0.5 (▲), 1.0 (○) and 5.0 (●) mM lactose. The solid lines represent exchange in all suspensions free of inhibitor and the broken lines represent exchange in cells treated with 0.1 mM $HgCl_2$.

(b) The substrate dependence of lactose exchange. The rate of exchange, determined from experiments like those shown in (a) is plotted against lactose concentration. The rate of isotope exchange is given by

$$v = \frac{[B][Q]}{[B]+[Q]} \cdot \frac{\log_e(1-F)}{t}$$

where $F = ([Q^*]-[Q_0^*])/([Q_e^*]-[Q_0^*])$, $[Q^*]$ is the reactivity in [Q] at time t, $[Q_0^*]$ is the radioactivity in [Q] at time zero and $[Q_e^*]$ is the radioactivity in [Q] at equilibrium (see, for example, Ref. 53). The symbols represent pH 7.0 (○), 7.5 (●), 8.0 (△), 9.0 (▲) and 10.0 (□).

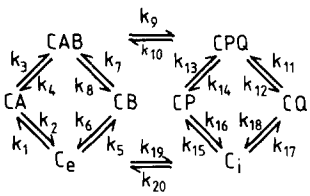
(c) The pH dependence of the exchange rate. The logarithm of the initial slope of the curves shown in (b) (equivalent to V/K_m for exchange) is plotted as a function of pH.

(d) The pH dependence of the pH-dependent component of exchange. The pH-independent component (see text) has been deducted from the total rate of exchange and the logarithm of the net rate of exchange is plotted as a function of pH.

slightly but this was not a very significant effect (a factor of 1.5 ± 0.2) and is in accordance with expectation since decreasing the proton concentration will decrease the abundance of the binary proton-permease complex that is necessary for exchange. Imposing a membrane potential was also without significant effect and this is in agreement with previous findings reported by Kaback and colleagues for membrane vesicles [20,26,32]. Wright [33] reported an acceleration of the rate of exchange by Δp but this was in counterflux experiments, where an acceleration of the initial rate of tracer uptake is expected because of an increase in trapping at the internal face of the membrane [14]. This is due to the effect of Δp on efflux (see above) and is not indicative of a direct effect on a component of Δp on one of the steps involved in exchange (galactoside dissociation or translocation of the ternary complex). A similar effect of Δp on counterflux has been reported before [34].

(vi) Simulation of transport kinetics

Substrate-binding was assumed to occur by a random mechanism (Eqn. 11)



(11)

and the protein isomerisations involved in translocation were assumed to occur with a rate constant comparable to galactoside dissociation (10^3–10^5 s^{-1}). This is much less rapid than the isomerisation of soluble enzymes which have rate constants in the range 10^6–10^7 s^{-1} [46,47]. It is, however, considerably faster than the isomerisation of the free state of the erythrocyte glucose carrier which has been suggested to have a rate constant of 147 s^{-1} [48]. The turnover number of the lactose permease is, at 200–500 s^{-1} [18,21,29 and herein], rather higher than that of the glucose carrier (90 s^{-1}, [48] and references therein) so a higher rate constant for the isomerisation could be expected.

Transfer of the protein from the permease to

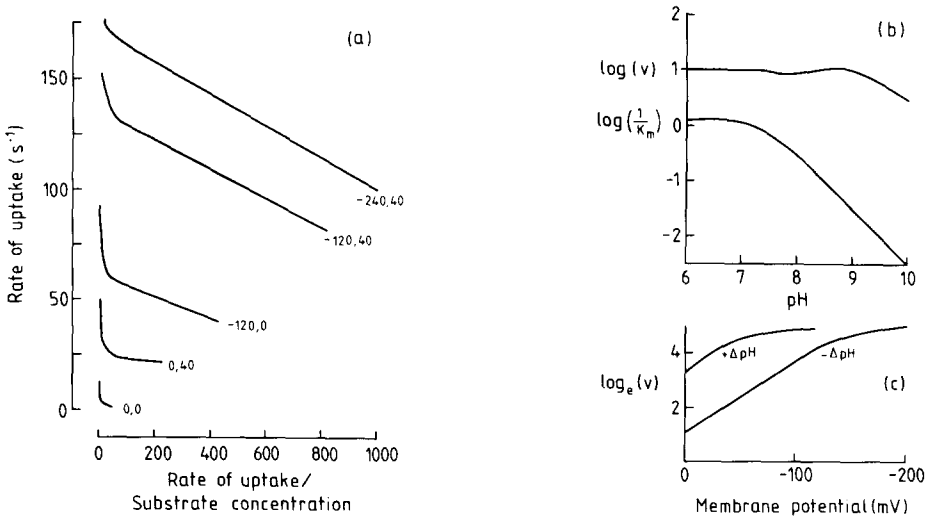


Fig. 8. Simulation of the kinetics of lactose transport using a random binding model. The steady-state rate-equation is used [17] with the following values for the rate-constants shown in Fig. 8; $k_1 = k_{15} = 10^{11}$ $M^{-1} \cdot s^{-1}$, $k_2 = k_{16} = 200$ s^{-1}, $k_3 = k_{13} = 10^7$ $M^{-1} \cdot s^{-1}$, $k_4 = k_{14} = 10^4$ s^{-1}, $k_5 = k_{17} = 10^7$ $M^{-1} \cdot s^{-1}$, $k_6 = k_{18} = 10^5$ s^{-1}, $k_7 = k_{11} = 10^{11}$ $M^{-1} \cdot s^{-1}$, $k_8 = k_{12} = 20$ s^{-1}, $k_9 = k_{10} = 5 \cdot 10^3$ s^{-1}, $k_{19} = k_{20} = 10^3$ s^{-1}. In addition k_{19} is assumed to depend on the magnitude of the membrane potential using a factor $\exp(nF\Delta\psi/2RT)$ and k_{20} is assumed to depend on the magnitude of the membrane potential using a factor $\exp -(nF\Delta\psi/2RT)$.

(a) Lactose concentration dependence of the rate of influx. The magnitude of the membrane potential and of the pH gradient are shown for each curve. [A] $= 10^{-7}$ M.

(b) The pH dependence of influx in the presence of a pH gradient, [A] $= 10^{-7}$ M.

(c) The dependence on membrane potential, [A] $= 10^{-7}$ M, [P] $= 10^{-7}$ M ($-\Delta$pH) or 10^{-8} M ($+\Delta$pH).

TABLE II

MODEL KINETIC PARAMETERS DERIVED FROM THE SIMULATION

The kinetic parameters are derived from the simulation described in Fig. 9. K_b' refers to the 'high K_m' component shown in Figs. 7 and 9. The parameters for efflux are the same as those for influx provided $\Delta\psi = 0$ and [A] = [P].

INFLUX	
Turnover number: 200 s^{-1}	
K_b $5.0 \cdot 10^{-4}$ M	K_b' $7.5 \cdot 10^{-3}$ M
K_{ia} $1.5 \cdot 10^{-8}$ M	K_a $3.0 \cdot 10^{-10}$ M
EXCHANGE	
Turnover number: 1250 s^{-1}	
K_b $2.0 \cdot 10^{-4}$ M	K_b' $9.8 \cdot 10^{-3}$ M
K_{ia} $7.0 \cdot 10^{-9}$ M	K_a $2.0 \cdot 10^{-10}$ M

water was assumed to be the rate-limiting step on the basis of the apparent pK_a for dissociation of the binary proton-permease complex and the known rates of proton transfer from model compounds to water [45]. The available evidence indicated that neither translocation of the ternary complex nor galactoside dissociation could be uniquely rate-limiting [10,19,20,26,33] but it has not been possible to discriminate between the proton-dissociation step and translocation of the unloaded permease. This might be possible once a detailed analysis of the reported deuterium isotope effect [52] has been performed. Anyway, for the analysis presented here, very similar results were obtained if the re-orientation of the unloaded permease was made rate-limiting instead of proton dissociation.

The dependence of V_{ab}^A on galactoside concentration was found to be biphasic with this model (Fig. 8a). The degree of biphasicity could be altered by changing either $\Delta\psi$ or ΔpH. Further K_b^A in the high affinity region was found to be low at very low values of $\Delta\psi$ and somewhat higher at high values of $\Delta\psi$, paralleling the behaviour reported in section (iv) above.

The pH dependence of V_{ab}^A showed a relatively flat region when [P] > [A] and a slope of -1 in the region above the apparent pK_a for the dissociation of the substrate proton from the ternary complex. When [P] = [A] the slope of -1 was retained but at p[P] less than pK_a the value of V_{ab}^A decreased with a slope of 1 (not shown).

The pH dependence of K_b^A also showed a relatively flat region when p[P] > pK_a for dissociation of the internal substrate proton from the proton-permease binary complex and slope of -1 when p[A] > pK_a for dissociation of the external substrate proton from the proton-permease binary complex.

Discussion

The maximum activity (V_{ab}, V_{pq}) of the permease has been found to be controlled by four apparent protonations. One pair (one internal and the other external) has an apparent pK close to 6.3 and results in a decrease in the turnover number of the permease and an increase the apparent affinity for internal substrate. This decrease in the rate of net turnover often appears as a decrease in the ability to accumulate substrates at low pH (for examples see [14,35,36]). This pair of ionisations appears as a non-competitive inhibition of flux by the *trans*-protons. The permease appears to be nearly symmetrical with respect to these ionisations, for there is less than a factor of two difference between the dissociation constants for internal and external protons (pK_a' = 6.5, pK_p' = 6.3). However, the inhibition by *cis*-protons is unexpected and this inhibition must be treated as a separate, regulatory protonation reaction, as shown schematically in Eqn. 1 and in the appendix. The similarity in values between K_a' and K_p' suggest that both *cis* and *trans* inhibitions described by these constants should be treated as related events, not connected with the primary interaction between permease and a substrate proton. The apparent pK is close to that expected for histidine residues and modification of these residues is known to affect activity by decreasing V_{max} [39–42]. The second pair of protonations (pK 9.6) appears to increase the turnover number of the permease [18] and may also increase its affinity for substrate in equilibrium binding [37] although here the evidence is equivocal [21,38]. this pair of ionisations is suggested to reflect the dissociation of the substrate (symported) proton from the ternary galactoside-proton-permease complex, as shown in Fig. 8b.

A third pair of apparent protonations, with

apparent pK 8.3, affects the apparent affinity (K_b, K_q) of the permease for galactosides, at least during turnover [18] and possibly also during equilibrium binding [38]. The decrease in affinity above pH 8, controlled by this apparent ionisation, is responsible for the decrease in the ability of the permease to accumulate substrates at alkaline pH that is frequently observed at low substrate concentration (for examples, see Refs. 35 and 36). It is also the cause of the decrease in efficiency of counterflux [34] and the rate of isotope exchange that occur at alkaline pH. This ionisation is suggested to represent binding of the substrate-proton to form the binary proton-permease complex because it affects the specificity constant (V/K_m) and contributes to competitive inhibition of flux by the *trans*-protons. The permease is also fairly symmetrical with respect to these ionisations, the value of the external proton-binding constant being slightly greater than that of the inner proton-binding constant (Table I).

Two kinetic models can explain the pH profiles observed at alkaline pH: one in which binding of the proton always occurs first, because the galactoside binding site does not exist (kinetically) until it has bound (the ordered mechanism [18,20]) and the other in which binding of the proton usually occurs first because the combination of the affinity of the unloaded permease for its substrates and their relative concentrations results in more rapid formation of the binary proton-permease complex than of the binary galactoside-permease complex. This is a random mechanism (see Fig. 8) with an asymmetry in the dissociation constants from the binary and ternary complex [18,22]. It has been noted that the available evidence is more consistent with the random mechanism than the ordered mechanism particularly because the former mechanism can account for non-Michaelis-Menten kinetics whereas the latter cannot [22]. However, not all random mechanisms give rise to the biphasic reciprocal plots typical of non-Michaelis-Menten kinetics and observed for uptake of lactose via the permease [19,20,27]; only those in which there is an asymmetry between the dissociation constants and in which the rate of translocation of the ternary complex is not negligible with respect to the rate of dissociation of galactoside from the ternary complex result in biphasic reciprocal plots.

It will be seen from Fig. 8 that such a model readily accounts for the kinetic behaviour of the permease during influx, including the biphasic reciprocal plots, the dependence on external pH and on the components of the protonmotive force. Adding a second, regulatory protonation reaction with pK'_a 6.3 improves the fit of the model to the data at low pH (see appendix). The degree of biphasicity was found to depend critically on the relative rates of translocation of the ternary complex and galactoside dissociation. Increasing one relative to the other could move the model out of the region where biphasic behaviour was observed. This provides an interpretation of the apparent division of galactosides into 'kinetically complex' substrates (which show a pronounced biphasic behaviour) and 'kinetically simple' substrates (which show very little biphasicity) [21]. In the former class the translocation and dissociation rates would lie in a range of values where biphasic kinetics are observed whereas in the latter class the translocation and dissociation rates are further apart and lie out of the range of values giving biphasic behaviour. In the model described in Fig. 8, and in most models (see appendix), the predicted effect of internal pH on V_{ab} is much greater than the observed effect (compare Eqns. 3 and 4). However, the non-competitive effect of internal protons in the model can be decreased in several ways, particularly by increasing the rate constant for reorientation of the unloaded carrier and by making the permease inherently asymmetrical such that the rate constants for all outward translocations are greater than those for all inward translocations. Provided that $k_9 \times k_{20} = k_{10} \times k_{19}$ this would not perturb the equilibrium constant or necessitate a non-identity in the substrate-binding reactions on either side of the membrane.

A more speculative solution (presented in the appendix) would be to allow the external proton to bind to a modified, low affinity, external site while the internal proton is still bound to the internal site (and vice versa). This bi-protonated complex could break down either to re-form the internally sequestered binary complex (C_iP in Eqn. 1) or to form the externally exposed complex (C_eA in Eqn. 1) allowing the transport cycle to continue. Such an auxiliary protonation displaces the predicted inhibition of influx to much lower val-

ues of pH, where the bi-protonated form dominates.

The results of steady-state kinetic analysis of some accumulation-defective mutants [36,44] already suggests that influx and efflux reactions are not symmetrical. In these mutants, zero-trans influx of galactoside is virtually unaltered but the efflux reaction is markedly different from that occurring in the parents [13,14,44]. Precise kinetics of efflux are difficult to establish but nevertheless, even in the wild-type, there seems to be some difference between V_{ab} and V_{pq} and the galactoside dissociation constants (Table I). Kinetic asymmetry has been discussed at great length for the erythrocyte glucose carrier (see Ref. 49 for a review) and might be expected in any system where binding sites interconvert through a conformational change such as has been established for the glucose carrier [48,50,51] and suggested for the lactose permease [10].

Appendix

Predicted pH dependence of kinetic parameters for various models of proton-galactoside symport

The models that need to be considered are the ordered reaction in which a proton always binds first and the random mechanism [18].

The ordered mechanism where substrates are in equilibrium with the permease because the rate of translocation is very low compared to the rates of dissociation can be rejected because V_{ab}^{A} is predicted to be independent of [A] (Fig. 9b).

The ordered mechanism where the rate of translocation is not negligible has the proper dependence on [A] at high pH but can be rejected because it is independent of [A] at low pH (Fig. 9c).

The same model, but with the addition of a second, regulatory protonation that decreases the amount of active carrier, gives the correct dependence of V_{ab}^{A} on [A]. This model fails to predict biphasic dependence of V_{ab}^{A} on galactoside concentration (see text) and predicts a larger effect of [P] on V_{ab}^{A} than is observed (Fig. 9d).

If the external substrate proton can combine with the external site while the internal proton is still bound to the initial site, and vice versa (Eqn. 12), even with a much lower affinity (pK 6 instead of 8.5), this auxiliary protonation reaction will overcome some of the inhibition expected from internal protons (Fig. 9e). This model still fails to predict the biphasicity of the galactoside dependence.

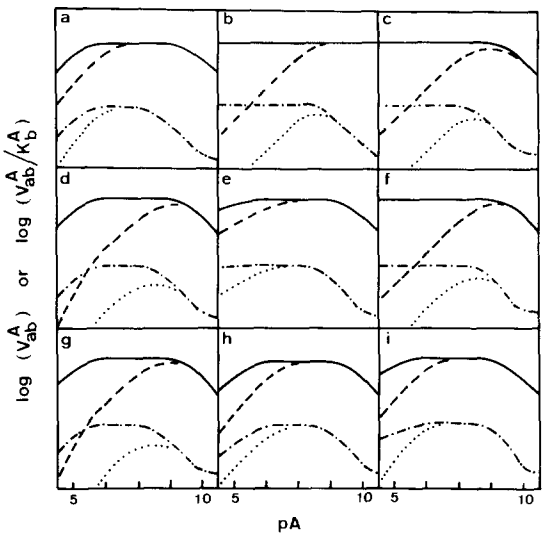
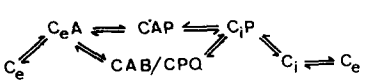


Fig. 9. pH profiles predicted for symport models. In each figure V_{ab}^{A} is shown as a function of [A] when [P] = 10^{-8} M (- - - - -) and [P] = [A] (— — —). Similarly V_{ab}^{A}/K_b^{A} is shown as a function of [A] when [P] = 10^{-8} M (–·–·–) and when [P] = [A] (· · · · · ·). (a) Extrapolated experimental results. The curves for [P] = [A] are taken directly from Fig. 2 and those for [P] = 10^{-8} M are calculated from the derived kinetic parameters (Table I).
In the remaining figures pH-profiles are shown for the following models: (b) ordered, rapid equilibrium (c) ordered, rapid translocation, (d) the same but with a regulatory protonation (pK_a 6) added, (e) the same but with an auxiliary protonation (pK_a 6) added, (f) random, rapid equilibrium, (g) the same with a regulatory protonation added, (h) random, rapid translocation, taken from the model described in Fig. 8 but with k_{10}, $k_{20} = 10 \cdot k_9$, k_{19} and with a regulatory protonation added, (i) random, rapid translocation, taken from the model in Fig. 8 but with an auxiliary protonation added where the proton on constants are 10^{11} $M^{-1} \cdot s^{-1}$ and the off constants are 10^{5} s^{-1}.



(12)

els with rapid translocation and fail on the same accounts.

The random models with rapid translocation are discussed in the main text. Here it is shown that the asymmetrical model with a regulatory proton (Fig. 9h) and the symmetrical model with an auxiliary protonation (Fig. 9e) can account for the observed pH profile.

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