The kinetics of the β -galactoside–proton symport of *Escherichia coli*

Malcolm G. P. PAGE and Ian C. WEST

Department of Biochemistry, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

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β -Galactoside transport by *Escherichia coli* occurs with the concomitant uptake of a proton. The kinetics of β -galactoside uptake at various values of external pH are interpreted in terms of a model in which both the galactoside and the proton are substrates of the transport reaction. The values of some of the kinetic constants for this two-substrate reaction were determined. The observed effects of the protonmotive force on the apparent Michaelis constant for galactoside can be explained in terms of the proton being a substrate of the transport reaction.

The uptake of exogenous galactosides by *Escherichia coli* occurs with the apparent concomitant uptake of a proton (West, 1970; West & Mitchell, 1972). The apparent reaction can be described by eqns. (1) and (2) below, where C denotes the carrier (lactose permease; Rickenberg *et al.*, 1956), H^+ denotes the proton, S denotes the galactoside and subscripts e and i indicate external and internal species respectively:

Eqn. (1) is analogous to a two-substrate enzymic reaction and can be analysed accordingly, bearing in mind that the 'products' and 'reactants' (the pools of H^+ and β -galactoside on either side of the membrane) exist in separate phases and that it is necessary for internal and external pools of unloaded carrier to interconvert (eqn. 2).

Previous kinetic analyses have concentrated on the behaviour of the carrier system with respect to various concentrations of the β -galactoside but without seriously considering the other putative substrate, the H^+ ion (Kepes, 1960; Winkler & Wilson, 1966; Lancaster *et al.*, 1975). In such analyses, the H^+ – β -galactoside symport has shown the hyperbolic relationship between rate and substrate concentration described by the Michaelis–Menten equation (Kepes, 1960; Winkler & Wilson, 1966). The metabolic state of the cell had proved to have a profound effect on the apparent kinetic constants of the reaction (Winkler & Wilson, 1966; Manno & Schachter, 1970). In the presence of metabolic substrate the apparent K_m for influx of galactosides has been found to be in the region of 0.1 mM (Kepes, 1960; Winkler & Wilson, 1966). In the absence of metabolic substrate the apparent K_m

does not change significantly unless the residual protonmotive force is adjusted to a very low value, whereupon the apparent K_m does become very much larger (Manno & Schachter, 1970; Page & West, 1980). Conversely, the K_m for efflux is very high in metabolizing cells, but considerably lowered when metabolism is inhibited (Winkler & Wilson, 1966).

Wright & Overath (1980) have observed that the apparent K_m for influx of lactose into membrane vesicles has a very much higher value when there is no protonmotive force. The high apparent K_m for influx in these conditions had been equated with the gross binding constant for lactose to membrane vesicles (Wright *et al.*, 1979; Wright & Overath, 1980). The change in apparent K_m produced by the presence of a protonmotive force has been attributed to one or both of two effects: either the presence of a protonmotive force might cause a change in the affinity of the permease for its substrates, or it might cause changes in the re-orientation rates of the loaded or unloaded forms of the permease. Either effect could, theoretically, cause the observed changes in the apparent K_m values.

In order to understand properly the effects of the protonmotive force on the apparent K_m values for transport of galactosides, it is necessary to determine the intrinsic kinetic constants describing the reaction. To this end, we have measured the initial rate of proton and galactoside influx at various external concentrations of both substrates and interpreted the data in terms of a two-substrate reaction. This analysis has enabled us to determine some of the kinetic constants of the reaction, and indicates that the reported effect of the protonmotive force on the apparent Michaelis constant for galactoside can be explained simply in terms of H^+ being a substrate of the symport reaction.

Materials and methods

Measurement of proton uptake

Escherichia coli ML308-225 was grown in baffled conical flasks to late exponential phase (approx. 1 mg dry wt. of bacteria/ml) in minimal medium M9 (Anderson, 1946) containing 0.5% (v/v) glycerol. The cells were harvested by centrifugation and resuspended in minimal medium lacking glycerol. After 1 h of shaking at 37°C the suspension was again centrifuged to harvest the bacteria. After two washes in 150 mM-KCl/50 mM-Tris/HCl buffer, pH 7.0, the bacteria were suspended at a cell density of 10–20 mg dry wt./ml in 100 mM-KCl/50 mM-KSCN/3 mM-Tris/HCl at the appropriate pH. The suspension was introduced into a large syringe, where it was allowed to become anaerobic, and where it was stored for 30 min for equilibration. During that time the pH was maintained at the desired value by additions of 20 mM-KOH.

For experiments, 3 ml of the anaerobic stock suspension was carefully transferred to a closed N₂-flushed chamber containing a pH electrode (Russell Instruments) and a magnetic follower. When the pH reached a steady value (drift less than 0.01 pH unit/50 s), a 100 µl addition of de-gassed N₂-flushed lactose solution (0.005 to 0.8 M in 100 mM-KCl/50 mM-KSCN, at the pH of the experiment) was made as rapidly as possible. Complete mixing occurred within 2 s, 90% within the first 0.5 s.

Cells with a greatly diminished protonmotive force across the membrane were prepared by adding 30 mM-NaN₃ to the incubation medium for the starvation period and to the subsequent washes (Page & West, 1980). The residual protonmotive force across the cell membrane was assessed by making additions of degassed ethanolic carbonyl cyanide *m*-chlorophenylhydrazone (to give a final concentration of 10 µM). Addition of 2,4-dinitrophenol (final concentration 0.65 mM) gave similar results (Page & West, 1980).

Rate of entry of 2-nitrophenyl β-galactoside

E. coli strain ML308 was grown to late exponential phase (approx. 0.5 mg dry wt. of bacteria/ml) in minimal medium M9 containing 17 mM-DL-lactate. The cells were harvested by centrifugation, washed once in 150 mM-NaCl containing 50 µg of chloramphenicol/ml and resuspended in the same medium at 10 times the cell density at harvest.

Two procedures were used to determine the rate of entry of 2-nitrophenyl β-galactoside, both based on the observation that the rate of hydrolysis of this galactoside *in vivo* by intracellular β-galactosidase is determined by its rate of entry and on the fact that the 2-nitrophenate anion produced by hydrolysis absorbs light strongly at 420 nm (Koch, 1964). In the first procedure the absorption at 420 nm of a

suspension was monitored continuously, whereas in the second procedure the hydrolysis *in vivo* was stopped after 1, 5, 10 or 15 min incubation by the addition of 2 vol. of 0.6 M-Na₂CO₃ and the absorbance was read after 5 min.

In both procedures the incubation medium contained 1 ml of one of 100 mM-sodium phosphate buffer, 200 mM-Tris/HCl buffer or 200 mM-glycine/NaOH buffer, depending on the pH range, 80–100 µg of cell protein, various amounts of 30 mM-2-nitrophenyl β-galactoside made up in 150 mM-NaCl and sufficient 150 mM-NaCl to make up the volume to 2.0 ml. To control for the presence of extracellular β-galactosidase (e.g. from broken cells) and for the carrier-independent entry of the 2-nitrophenyl galactoside by simple diffusion, assays were repeated (a) in the presence of 5 mM-thiodigalactoside (a competitive inhibitor; Kennedy, 1970) or (b) with cells pretreated at pH 7.0 with 1 mM-*N*-ethylmaleimide for 15 min (an irreversible inhibitor; Fox & Kennedy, 1965). Absorbance readings were converted into mol of galactoside hydrolysed by using a molar absorption coefficient (420 nm) of $2.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7.3 (equivalent to $3.93 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 10) and a pK_a of 7.18. These values were determined with 2-nitrophenol twice recrystallized from 1 mM-HCl.

(a) Ordered mechanism

King-Altman patterns:

(b) Random mechanism

Fig. 1. Kinetic mechanisms for galactoside-proton symport in *Escherichia coli*

A and B represent the external substrates, C the permease and P and Q the internal products. (a) Ordered mechanisms, in which the reaction passes through only one kind of binary complex in either phase. The six possible King-Altman patterns are also shown. (b) The random mechanism, in which the reaction can pass through either binary complex.

Theoretical

Generalized kinetic analysis of two-substrate transport

Reaction mechanism. A number of mechanisms of substrate binding are possible when the carrier

shown in Fig. 1(a). Each pattern gives rise to one term in the rate equation for each carrier species. Thus there are six terms in the numerator and 36 terms in the denominator. Since some of the terms have the same concentration-dependence, the rate expression can be simplified to:

$$v = \frac{[C_T](a[A][B][Q] + b[A][B] + c[B][P][Q] + d[P][Q] + e[A][B][P][Q])}{f + g[P] + h[Q] + i[A] + j[B] + k[A][Q] + l[B][P] + m[A][B] + n[P][Q] + o[A][P][Q] + p[B][P][Q] + q[A][B][P] + r[A][B][Q] + s[A][B][P][Q]} \quad (3)$$

(designated C) catalyses the translocation of two substrates. In this Theoretical section the two substrates are designated A and B, and the two products P and Q. In an ordered mechanism A binds first and P leaves first. It is not prejudged at this stage whether A or B represents the proton. For symport to occur in the manner observed the proton

where v is the reaction velocity, $[C_T]$ is the total carrier concentration and a, b etc. are clusters of rate constants. The initial-rate determinations can be made in the absence of internal β -galactoside, but the proton is always present on both sides of the membrane, so, although either $[P]$ or $[Q]$ can be zero, both cannot be zero. Thus eqn. (3) becomes:

$$v = \frac{\frac{b}{m}[A][B][C_T]}{\frac{f}{m} + \frac{h}{m}[Q] + \frac{i}{m}[A] + \frac{j}{m}[B] + \frac{k}{m}[A][Q] + [A][B]\left(1 + \frac{r}{m}[Q]\right)} \quad (4)$$

when $[P] = 0$, and:

$$v = \frac{\frac{b}{m}[A][B][C_T]}{\frac{f}{m} + \frac{g}{m}[P] + \frac{i}{m}[A] + \frac{j}{m}[B] + \frac{l}{m}[B][P] + [A][B]\left(1 + \frac{q}{m}[P]\right)} \quad (5)$$

when $[Q] = 0$.

and galactoside must combine with the carrier to form a ternary complex (CAB). If the ternary complex is formed exclusively from one of the possible binary complexes (CA or CB) and not the other, the mechanism is said to be ordered, whereas if the complex CAB is formed readily from either CA or CB it is said to be random (Cleland, 1963). The various possibilities are summarized in Fig. 1.

The forms of the rate equations describing ordered and random mechanisms are different. An ordered mechanism can be shown to be described by a relatively simple expression. For example, by using the graphic technique of King & Altman (1956), six patterns are possible for an ordered reaction such as

The random mechanism is described by a much more complex rate equation. There are 64 patterns in the King-Altman analysis, giving rise to an expression having 64 terms in the numerator and 512 terms in the denominator (23 and 85 in numerator and denominator respectively when terms with identical concentration-dependence are combined). In the absence of either $[P]$ or $[Q]$ the expression has six terms in the numerator and 23 terms in the denominator.

The number, and the complexity of the concentration-dependence, of these terms make it difficult to fit experimental findings to the complete equation. Haldane (1930) made the simplifying

assumption that the substrate-binding steps are in equilibrium, which has been shown to be valid if the rates of interconversion of the ternary complex are low relative to the rates of dissociation of the complexes (Laidler, 1956). With the reaction being discussed it is also necessary to assume that the equilibrium $C_e = C_i$ is not displaced greatly during the determination of the initial rate. The following equilibria can then be defined:

$$K_{ia} = \frac{[C_e][A]}{[CA]}, \quad K_b = \frac{[CA][B]}{[CAB]}, \quad K_a = \frac{[CB][A]}{[CAB]}, \quad K_{ip} = \frac{[C_i][P]}{[CP]},$$

$$K_T = \frac{[C_i]}{[C_e]}$$

and the rate expression takes the form shown in eqn. (6) (Haldane, 1930; Laidler, 1956):

$$v = \frac{V_{\max.}[A][B]}{K_{ia}K_b \left[1 + K_T \left(1 + \frac{[P]}{K_{ip}} \right) \right] + K_b[A] + K_a[B] + [A][B]} \quad (6)$$

Dalziel (1958) and Cleland & Wratten (1969) have investigated a number of relationships between the rate constants for the individual steps that will reduce the complete equation to one of the same form as eqn. (6), whereas Schwert (1954) has shown, from calculations using assumed values of the rate constants, that the curvature of the Lineweaver-Burk plots predicted by the complete equation is, in general, too small to be detected experimentally and that similar results can be obtained by using either the full or the simplified equation.

Eqn. (6) resembles eqns. (4) and (5) where $V_{\max.} = b/m$, $K_{ia}K_b(1 + K_T) = f/m$, $K_b = i/m$ and $K_a = j/m$. In fact, the constants i/m and j/m are not equal to the Michaelis constants K_a and K_b unless the translocation rates are negligible. In the ordered mechanism, where A binds first, K_a as defined should be zero (Segal *et al.*, 1952).

In eqns. (4), (5) and (6) terms in [P] and [Q] could be ignored (as in eqn. 7 below) if it were possible to maintain the intracellular product concentrations at zero. However, if inhibition due to internal acidity is observed it would become necessary to re-introduce these product terms. With the above simplifications being borne in mind, experimental results can be fitted to the expression below:

$$v = \frac{V_{\max.}[A][B]}{K_{ia}K_b(1 + K_T) + K_b[A] + K_a[B] + [A][B]} \quad (7)$$

Effect of the protonmotive force. It is essential to distinguish between the kinetic effects of external pH on reactions at the external surface of the cell membrane (likewise the internal pH on internal reactions) from the thermodynamic effects of having a pH gradient across the membrane. Further, the kinetic effects of external pH can be of two fundamentally different types: (a) where the H^+ acts as a catalyst and (b) where it acts as a substrate.

These effects, of course, are not unrelated.

Metabolism results in the expulsion of protons

from the cytoplasm, which creates an electrochemical potential gradient for protons across the membrane. There is therefore a driving force for the diffusion of protons through the membrane that can be formulated as:

$$\Delta\bar{\mu}_{H^+} = \Delta\mu_{H^+} + F\Delta\psi$$

where $\Delta\mu_{H^+}$ is the chemical potential gradient, F is Faraday's constant and $\Delta\psi$ is the membrane potential (Mitchell, 1961, 1966). The lactose permease catalyses the diffusion of protons through the membrane, and the driving force for proton diffusion will therefore also apply to it. Even when the activities of protons on each side of the membrane are equal, there will still be a driving force for proton uptake equivalent to $F\Delta\psi$. Similarly, the driving force for proton efflux will be diminished in the presence of a membrane potential.

These thermodynamic effects of the protonmotive force will appear in the kinetic analysis in either of two ways: (a) as changes in $[H^+]$ on the outside or the inside of the membrane or (b) as a modification of the rate constants of vectorial steps operating in the direction normal to the membrane, in which case changes might be observed in $V_{\max.}$ or K_T . (If the translocation rates are not negligible with respect to the other rate constants, then K_a and K_b would also change.)

If the external binding site for the proton is buried some distance within the membrane, but is still able

to be influenced by the electric field of the membrane potential, the local activity of the proton will be enhanced by a difference in electrochemical potential between the active site and the bulk phase (Mitchell, 1968; Mitchell & Moyle, 1974). Similarly, if the internal proton-binding site is buried in the membrane the local activity of the protons will be decreased by the electrochemical potential difference. These effects (the 'proton well') will appear as changes in $[H_0^+]$ for the externally exposed site and $[H_i^+]$ for the internally directed site.

Independently of the thermodynamic effects of the membrane potential, such an electric field could cause a conformational change resulting in an increase of affinity for the external substrate(s), and then changes in the rate expression will be observed. If it is the affinity for A that is changed the values of K_{ia} and K_a will be altered, and if it is the affinity for B that changes the value of K_b will be altered. Such changes could equally affect an electroneutral sugar uniport and are not a feature of the 'energy coupling'.

Calculation and statistical analysis

Eqn. (7) above reduces to the Michaelis-Menten equation if the concentration of one substrate is held constant. For example, with constant $[A]$ it becomes:

$$v = \frac{V^A[B]}{K^A + [B]} \quad (8)$$

Experimental data were fitted to this equation in its reciprocal form:

$$\frac{[B]}{v_{\text{obs.}}} = \frac{K^A}{V^A} + \frac{[B]}{V^A} + e \quad (9)$$

where $v_{\text{obs.}}$ is the observed initial rate of influx, V^A is the apparent maximum velocity at a particular pH, K^A is the apparent K_m at a particular pH, $[B]$ is taken to be the galactoside concentration and e is the error in $[B]/v_{\text{obs.}}$. The values of the kinetic constants in eqn. (7) were then determined from the equations:

$$\frac{K^A[A]}{V^A} = \frac{K_{ia}K_b}{V_{\text{max.}}} + \frac{K_b[A]}{V_{\text{max.}}} + e_1 \quad (10)$$

$$\frac{[A]}{V^A} = \frac{K_a}{V_{\text{max.}}} + \frac{[A]}{V_{\text{max.}}} + e_2 \quad (11)$$

where $[A]$ is the H^+ concentration and e_1 and e_2 are error terms. Eqns. (10) and (11) are the usual slope and intercept replots and are simply obtained by comparing eqns. (7) and (8).

Estimates of the constants in eqns. (9), (10) and (11) were made by weighted least-squares analysis by using five cycles of reiteration with progressively refined weights (Wilkinson, 1961).

Results

Variation of the apparent kinetic parameters for galactosides with external pH

The initial rate of lactose-induced proton uptake and of *o*-nitrophenyl galactoside uptake and hydrolysis both gave linear plots when fitted to eqn. (9) (Fig. 2). Substrate ranges of 0.05–8 mM-lactose and pH 7–10 were used for assays of proton uptake (Fig. 2) and of 0.3–5.4 mM and pH 5.7–9.2 for *o*-nitrophenyl galactoside uptake and hydrolysis.

The apparent maximum velocity of uptake in both assays showed very little dependence on the external pH, but the apparent K_m increased when the pH was raised above 8.0 (Figs. 3 and 4).

Variation of the apparent kinetic parameters with the magnitude of the protonmotive force

At pH 7.0 the starved cells used for the proton-uptake experiments have a residual protonmotive force of about one-third of that found in metabolizing cells (M. G. P. Page, unpublished work).

Fig. 2. Reciprocal of the initial rate of lactose-induced proton uptake plotted against the reciprocal of the external lactose concentration

Triplicates were performed for each concentration point in the individual experiments. Results from all experiments were pooled for calculation of the regression lines by weighted least-squares analysis. Bars represent the range of values in the pooled results.

Fig. 3. Variation of the apparent kinetic constants for the initial rate of lactose-induced proton uptake and of the residual protonmotive force with external pH

(a) For starved cells. (b) For azide-treated cells. Assays were performed as described in the Materials and methods section. Points represent values determined in individual experiments. Δ , $\log$ (apparent K_m); $\bullet$, apparent $V_{\max}$; $\blacksquare$, CCCP (carbonyl cyanide *m*-chlorophenylhydrazine)-induced pH excursion. K^A is expressed in mM.

Fig. 4. Variation of the apparent kinetic parameters for *o*-nitrophenyl galactoside uptake and hydrolysis with external pH. Assays were performed as described in the Materials and methods section. Points represent values determined in individual experiments. Δ , $\log$ (apparent K_m); O , apparent $V_{\max}$. K^A is expressed in mM.

Fig. 5. Secondary plots of the initial rate of proton uptake induced by lactose

Initial-rate data from the experiments depicted in Fig. 2 are plotted according to (a) eqn. (10) and (b) eqn. (11).

Raising the external pH to 8.0 diminishes the residual protonmotive force by about 80% (Fig. 3a) but produces no change in the apparent kinetic constants. When the external pH is raised still further, the residual protonmotive becomes zero.

As the protonmotive force becomes zero, there is no change in the apparent maximum velocity, although the apparent K_m starts to increase (Fig. 3a). However, the increase in apparent K_m continues with rising pH even when the protonmotive force is zero (Fig. 3a). This suggests that it is the external pH that is causing the increase in apparent K_m rather than the collapse of the protonmotive force.

Azide-treated cells at pH 7.0 have a very small residual protonmotive force (20%, or less, of that of starved cells) and a high apparent K_m (Fig. 3b), although once again there is no significant change in the apparent maximum velocity (Page & West,

Fig. 6. Secondary plots of the initial rate of *o*-nitrophenyl galactoside uptake and hydrolysis

Initial-rate data from the experiments described in the text are plotted according to (a) eqn. (10) and (b) eqn. (11).

Table 1. Kinetic parameters for lactose-proton symport

K_{ia}	$4.29 \times 10^{-9} \pm 0.89 \times 10^{-9} \text{ M}$
K_a	$0.006 \times 10^{-9} \pm 0.029 \times 10^{-9} \text{ M}$
K_b	$0.70 \times 10^{-3} \pm 0.27 \times 10^{-3} \text{ M}$
$V_{\max.}$	$0.645 \pm 0.071 \text{ ng-ion of H}^+ \cdot \text{s}^{-1} \cdot (\text{mg dry wt.})^{-1}$

Table 2. Kinetic parameters for *o*-nitrophenyl galactoside uptake

K_{ia}	$4.03 \times 10^{-9} \pm 1.02 \times 10^{-9} \text{ M}$
K_a	$0.29 \times 10^{-9} \pm 0.27 \times 10^{-9} \text{ M}$
K_b	$0.98 \times 10^{-3} \pm 0.23 \times 10^{-3} \text{ M}$
$V_{\max.}$	$6.9 \pm 0.6 \text{ nmol} \cdot \text{s}^{-1} \cdot (\text{mg dry wt.})^{-1}$

1980). At pH 8.0 and above, the residual protonmotive force is zero, but the K_m is indistinguishable from that of untreated cells at the same external pH. Although these observations can be explained in terms of an effect of membrane potential on the carrier, they are also consistent with product inhibition by the internal H^+ concentration as described in the Theoretical section and as shown in Fig. 7.

Fig. 7. Secondary plots of the initial rate of proton uptake by azide-treated cells

Initial-rate data from the experiments depicted in Fig. 3(b) are plotted according to (a) eqn. (10) and (b) eqn. (11). In (a) the upper continuous line (A) was calculated assuming that the inhibition is caused by a term of the nature $K_{ia}K_b[P]/V_{max}K_{ip}$, and the lower continuous line (B) was calculated assuming that the inhibition is caused by a term of the nature $K_b[P][A]/V_{max}K_p$, where [A] and [P] are the external and internal H^+ concentrations respectively and K_{ip} , K_{ia} , K_b and V_{max} are as defined in the Theoretical section. K_p is a product inhibition constant. The broken line is the regression line determined from the corresponding plot of data for starved cells.

Reaction mechanism

It was possible to fit the initial-rate values from both assays to eqns. (10) and (11) to give linear plots (Figs. 5 and 6). The data are therefore consistent with a two-substrate model to which Laidler's simplifications can be applied, as outlined in the Theoretical section (eqn. 7). The variation in apparent K_m in these experiments can thus be seen to be due entirely to the pH-dependence of the terms in the overall rate expression and not to the variation in the membrane potential. The values of the kinetic constants in eqns. (10) and (11) determined from each assay are summarized in Tables 1 and 2.

The initial-rate values for azide-treated cells gave non-linear plots when fitted to eqn. (10) (Fig. 7). It has been remarked that the non-linearity of this plot

could be due to changes in the magnitude of an internal-pH-dependent term in the rate expression. The two lines shown in Fig. 7(a) are calculated by assuming that the inhibition is coming from a term in either the $K_{ia}K_b/v$ or the $K_b[H^+]/v$ terms in eqn. (10). It can be seen that the best fit is obtained if the term is assumed to be in the $K_b[H^+]/v$ term.

Discussion

Wright and colleagues (Wright *et al.*, 1979; Wright & Overath, 1980) have suggested that the different apparent affinities of the carrier system for galactosides in metabolizing and non-metabolizing vesicles might be due to one or both of two effects. The first is an effect of the protonmotive force on the (external) binding step, so that the carrier has greater intrinsic affinities for extracellular substrate in the presence of a protonmotive force. The second effect arises if the loaded carrier translocates faster than the unloaded carrier in the presence of a protonmotive force.

We have previously shown (Page & West, 1980) that, when the protonmotive force in intact cells is adjusted to a very low value by prior incubation with NaN_3 , the apparent K_m for galactosides at pH 7.0 is raised to a much higher value. This effect was originally interpreted as being consistent with either of the two models proposed by Wright *et al.* (1979).

We have now shown that, although the protonmotive force is diminished by raising the external pH, the concurrent increase in K_m is wholly consistent with the requirement for a proton in the two-substrate models described in the Theoretical section. Although the apparent K_m is dependent on the external pH, there is no change in the intrinsic kinetic constants when the protonmotive force is diminished to zero by raising the external pH.

The inhibition of uptake in azide-treated cells cannot now be attributed to either of the two effects of the membrane potential proposed by Wright and colleagues. It can, however, be interpreted in terms of the effect of raising the internal acidity on the rate expression. As has been described in the Theoretical section, the internal H^+ ions are expected to inhibit the influx reaction. The extent of inhibition will depend on the internal H^+ concentration and on the affinity of the carrier for the internal H^+ ions. In azide-treated cells at pH 7.0 the effective internal H^+ concentration is assumed to be greater than that in starved cells at the same pH (since the protonmotive force is less), and so the inhibition is expected to be greater. Conversely, when the internal pH is raised the magnitude of the inhibition term will decrease and can become less significant in the rate expression. Thus in starved cells with a residual protonmotive force there seems to be no significant effect of internal pH on the initial rate of influx, and

in azide-treated cells the inhibition dwindles as the external pH is raised (Fig. 7). This may be the mechanism underlying the observations made by Wright & Overath (1980).

The values of the kinetic constants determined in both assays are similar (Tables 1 and 2): minor differences can be attributed to the different strains and substrates used for each assay. It should be noted that the value of the K_{ia} in the Tables is not the dissociation constant for the proton, but differs from it by a factor of $1 + K_T$, where K_T is the equilibrium constant for the interconversion of internal and external pools of the unloaded carrier. The values of the Michaelis constant for the proton (K_a) determined in both assays lack significance (Cleland, 1979), and this term may be absent from the rate expressions. The absence of this term would have considerable significance, since it would indicate that the reaction is of the ordered type, with the proton binding first, and that the interconversion between the ternary complexes (translocation) is slow relative to the binding steps (Segal *et al.*, 1952). However, it will be impossible to prove K_a absent, because a random mechanism could give very low though finite values for K_a . For example, by taking the Haldane relationship:

$$K_{ia}K_b = K_{ib}K_a \quad (12)$$

where $K_{ib} = [C_e][S_e]/[CS_e]$ and using the values in Tables 1 and 2, $K_{ib} = 500$ mM for lactose. Similarly, for *o*-nitrophenyl galactoside the dissociation constant (K_{ib}) would have to be 14 mM to account for the observed value of K_a . As these values are not impossible, one cannot rule out the random mechanism. To distinguish between random and ordered mechanisms it would be necessary to perform inhibition or isotope-exchange studies. It is difficult to control, or even to obtain precise knowledge of, the internal concentrations of either product of influx, so the potentially very useful technique of product-inhibition analysis is not yet feasible for quantitative studies of this transport system. Some data have been published describing isotope exchange in membrane vesicles that suggest an ordered mechanism with the proton binding first and leaving last (Kaczorowski & Kaback, 1979), but a wider range of pH and lactose concentrations needs to be

The very high K_m values reported for galactoside efflux (Winkler & Wilson, 1966; Lancaster *et al.*, 1975) can now be interpreted in terms of the two-substrate model. Even without a modification of the kinetic constants (i.e. assuming a kinetically symmetrical carrier) the apparent K_m for galactoside efflux from metabolizing cells would be expected to be higher than that for influx (or that for efflux in the absence of metabolism), on two accounts. Firstly, metabolism results in a high internal pH (Padan *et al.*, 1976; Booth *et al.*, 1979), which, because of the requirement for protons, results in a slightly higher apparent K_m for efflux of galactoside. Secondly, the efflux experiments are usually performed with low external pH (e.g. pH 7.0), so that efflux is occurring against a high effective concentration of the H^+ product. This will lead to product inhibition of efflux, just as occurs for influx into azide-treated cells at low pH. It is then necessary to argue that when metabolism is inhibited the effective concentration of the external H^+ is lowered, so that the inhibition will be less and the apparent K_m will be smaller.

Several attempts have been made to measure gross dissociation constants for the binding of galactosides to membrane vesicles (Reeves *et al.*, 1973; Bentaboulet & Kepes, 1973; Kennedy *et al.*, 1974; Rudnick *et al.*, 1976; Belaich *et al.*, 1976; Wright *et al.*, 1979; Wright & Overath, 1980; Belaich & Belaich, 1980).

The nature of these dissociation constants is obscure, since (i) it is not known to which species of the carrier the galactoside is binding and (ii) the vesicles are closed structures so that the internal face is in a different environment from the external face. The dissociation constant measured in the absence of metabolism is, for some galactosides, higher than either the apparent K_m for active transport (Wright *et al.*, 1979; Wright & Overath, 1980) or the dissociation constant measured in the presence of metabolism (Belaich & Belaich, 1980). For at least one galactoside (thiodigalactoside) there is no difference between the apparent constants. The relationship between the dissociation constant and the kinetic constants depends on the conditions of the assay. By taking first the ordered mechanism in which the galactoside dissociates first:

examined to confirm this proposal. The magnitude of the Michaelis constants for proton (K_a) and galactoside (K_b) exclude the possible ordered mechanism in which galactoside binds before the proton.

and defining the gross dissociation constant (K_D):

$$K_D = \frac{[\text{Free carrier}][\text{Free ligand}]}{[\text{Bound ligand}]} \quad (13)$$

it can be shown that:

$$K_D = \frac{K_2 \left(1 + \frac{K_1}{[H_e^+]} + \frac{K_1 K_6}{[H_e^+]} \right) + \frac{K_3 K_4 [S_e]}{[S_i]}}{1 + K_3} \quad (14)$$

If the proton dissociates before the galactoside on the internal surface of the membrane, the dissociation constant can be shown to be:

$$K_D = \frac{K_2 \left(1 + \frac{1}{[H_e^+]} + \frac{K_1 K_6}{[H_e^+]} \right)}{1 + K_3 + \frac{K_7}{[H_i^+]}} \quad (15)$$

where $K_7 = [CS_i][H_i^+]/[CHS_i]$.

Similarly, for the random mechanism:

the dissociation constant can be shown to be related to the individual equilibrium constants by the expression:

$$K_D = \frac{K_2 \left(1 + \frac{K_1}{[H_e^+]} + \frac{K_1 K_6}{[H_e^+]} \right) + \frac{K_3 K_4 [S_e]}{[S_i]}}{1 + K_3 + \frac{K_1 K_2}{K_9 [H_e^+]} + \frac{K_3 K_7}{[H_i^+]}} \quad (16)$$

Therefore, in each case, the gross dissociation constant will be a function, not only of the individual equilibrium constants, but also of the substrate and product concentrations. Our findings suggest that, in the range of pH studied by Wright & Overath (1980), nearly all of the free carrier will be in the protonated form, which is consistent with their finding that the dissociation constant is independent of pH. Under these circumstances eqns. (14) and (16) reduce to the form given below (eqn. 17):

$$K_D = \frac{K_2 + \frac{K_3 K_4 [S_e]}{[S_i]}}{1 + K_3} \quad (17)$$

which is still dependent on several equilibrium steps and on the galactoside concentration on each side of the membrane. Belaich & Belaich (1980) have demonstrated that the ratio $[S_e]/[S_i]$ is not necessarily the same for all substrates, and, in

addition, K_3 and K_4 might reasonably be expected to vary from substrate to substrate, so that K_D could differ from K_2 by different amounts with different substrates. It has already been pointed out that the kinetic constant K_b is not necessarily equal to K_2 and will only approach it if the translocation rates are negligible relative to the binding steps. Thus equality between K_b and K_D is not to be expected except in the most fortuitous combination of circumstances.

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