Quantitative Analysis of Proton-Linked Transport Systems

β -GALACTOSIDE EXIT IN *ESCHERICHIA COLI*

Ian R. BOOTH and W. Allan HAMILTON

Microbiology Department, Aberdeen University, Marischal College, Aberdeen AB9 1AS, Scotland, U.K.

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The exit of lactose and thiomethyl- β -D-galactoside from *Escherichia coli* ML308-225 has been studied to determine the role of carrier-dependent (zero-trans efflux) and carrier-independent (leak) processes. On the basis of its sensitivity to *p*-chloro-mercuribenzenesulphonate the exit of lactose was found to be almost wholly mediated by the carrier. Consistent with this conclusion was the finding that the rate of exit of this sugar was dependent on the external pH, being considerably slower at acid pH. On the other hand exit of thiomethyl- β -D-galactoside was found to be composed of both carrier-dependent and carrier-independent processes. Both processes exhibited first-order kinetics with the rate constants for zero-trans efflux and leak being 0.137 min^{-1} and 0.079 min^{-1} , respectively. The relevance of these findings for our earlier proposal for the method of attenuation of solute accumulation is discussed [Booth, Mitchell & Hamilton (1979) *Biochem. J.* 182, 687–696].

The β -galactoside permease of *Escherichia coli* is the most extensively studied of the bacterial proton-linked secondary transport systems (Mitchell, 1973). It has always provided a vehicle for testing the many postulates regarding the coupling of energy in active transport (Cohen & Monod, 1957; Kepes, 1971; West & Mitchell, 1973; West, 1980). Thus the growing understanding of the mechanism of energy coupling via the proton-motive force ($\Delta\bar{\mu}_{\text{H}^+}$) and the role of its two components, the membrane potential ($\Delta\Psi$) and the pH gradient (ΔpH), may be attributed directly to studies on this system (West, 1970; West & Wilson, 1973; Lancaster *et al.*, 1975; Lancaster & Hinkle, 1977a,b; Schuldiner *et al.*, 1975; Kaczarowski & Kaback, 1979; Kaczarowski *et al.*, 1979; Overath *et al.*, 1979; Wright *et al.*, 1979).

Generally workers in this field would agree that the apparent asymmetry of carrier function induced by energy coupling (Winkler & Wilson, 1966; Lancaster *et al.*, 1975) is a result of changes in the kinetic properties of the permease due to the size and polarity of $\Delta\bar{\mu}_{\text{H}^+}$. Most recently, different groups have viewed the mechanism of energy coupling in terms of both modulation of the rate of translocation of the β -galactoside binding site across the membrane (Schuldiner *et al.*, 1975; Lancaster & Hinkle, 1977a,b), and control of the affinity of the carrier for its two substrates, the proton and the β -galactoside (Kaczarowski & Kaback, 1979; Kaczarowski *et al.*, 1979; Wright *et al.*, 1979). Further discussion of this aspect of carrier function and

a review of progress towards understanding the fine details of the molecular mechanism of transport can be found in a forthcoming review (West, 1980).

Over a number of years we have been interested in the efficacy of the proton-motive force in bacterial cells to explain the observed accumulation of a variety of solutes (Niven *et al.*, 1973; Niven & Hamilton, 1974; Hamilton, 1975, 1977; Mitchell *et al.*, 1979; Booth & Hamilton, 1980). We have recently been studying β -galactoside transport in *E. coli* and have demonstrated that the $\Delta\bar{\mu}_{\text{H}^+}$ is always in excess of the accumulation of the chemical potential gradient of lactose (Booth *et al.*, 1978, 1979). In order to explain this disparity we invoked the presence of a carrier-independent leak such as had already been proposed for β -galactosides by earlier workers (Cohen & Monod, 1957; Kepes, 1960; Maloney & Wilson, 1973). The present report is the result of attempts to characterize the leak of lactose from *E. coli* ML308-225 and to compare it with the similar process for thiomethyl- β -D-galactoside.

Zero-trans exit of a solute from either cells or vesicles is a term applied to the rate of efflux from preloaded cells into medium containing no solute. In most cases investigated to date the process exhibits first-order kinetics and has been considered to be independent of carrier function (Novotny & Engesberg, 1966; Kepes, 1970, 1971; Maloney & Wilson, 1973; Pouyssegur *et al.*, 1974; Lagarde & Stoeber, 1974). The exit is often termed the leak and

is generally thought not to involve a membrane protein, although a cautionary note has been made on this point (Kepes, 1970). With regard to β -galactoside exit in *E. coli* there has been some confusion regarding the extent to which it is mediated by the lactose transport system and what proportion of exit is due to a carrier-independent leak process (Kepes, 1970, 1971; Maloney & Wilson, 1973). Thus, the present study of lactose and thiomethyl- β -D-galactoside exit was undertaken to ascertain whether the leak of β -galactosides was sufficient to explain the reduced accumulations of these sugars (Booth *et al.*, 1978, 1979; I. R. Booth & W. A. Hamilton, unpublished data).

At the outset it is necessary to define certain terms in order to clarify the ensuing discussion. 'Exit' refers to the sum total of all the various pathways by which non-metabolized β -galactosides may leave the cell; 'zero-trans efflux' refers to that portion of exit from the cell which is dependent on the lactose carrier protein; and 'leak' is that exit which occurs independently of the β -galactoside transport system.

Materials and Methods

Materials

All materials were as previously specified (Booth *et al.*, 1979) with the following additions: thio[^{14}C]-methyl- β -D-galactoside was obtained from Nuclear Enterprises, Sighthill, Edinburgh 11, Scotland, U.K.; *p*-chloromercuribenzenesulphonate was obtained from Sigma.

Bacterial strain

All experiments were carried out using *E. coli* ML308-225 (*lac* i^- z^- y^+ a^+) supplied by Dr. I. C. West, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne, U.K.

Growth conditions

The organism was stored and grown as described previously (Booth *et al.*, 1979).

Transport assays

Cell suspensions for transport experiments were prepared from mid-logarithmic phase cultures (Booth *et al.*, 1979) by centrifugation at 10400 *g* for 10 min at 4°C. Washing was carried out by resuspension in Tris/Mes/choline chloride buffer, pH 7.0, containing 5 mM-Tris, 5 mM-2-(*N*-morpholino)-ethanesulphonic acid (Mes), 150 mM-choline chloride, and 0.2 mM-KCl. After two washes by resuspension and centrifugation at 18000 *g* for 5 min at 4°C, the cells were stored on ice at a density of 20 mg of bacterial dry weight/ml. For some

experiments EDTA/valinomycin-treated cells were used and these were prepared as described previously (Booth *et al.*, 1979).

The initial rate of lactose uptake was measured using dilute suspensions of EDTA/valinomycin-treated cells in Tris/Mes/choline chloride/KCl buffers over the range pH 5.9–8.7. Cell suspensions, containing 0.25 mg of dry bacterial mass/ml, were incubated at 25°C with 5 μM -[glucono-1- ^{14}C]lactose (specific activity 57.6 Ci/mol). Samples (100 μl) were removed at intervals over the first 50 s of incubation and harvested by membrane filtration (Oxoid 0.45 μm -pore-size filters). The harvested cells were immediately washed with 3 ml of the appropriate buffer. The filters were then dried and prepared for scintillation counting as described previously (Booth *et al.*, 1979).

Exit of β -galactosides from *E. coli* was measured by the method of Lagarde & Stoeber, (1974). *E. coli* ML308-225 cells at a density of 1.0–1.5 mg of dry bacterial mass/ml were incubated at 25°C in Tris/Mes/choline chloride/KCl buffer, pH 5.9, with either [glucono-1- ^{14}C]lactose or thio[^{14}C]methyl- β -D-galactoside at a range of concentrations (5–500 μM ; specific activity range 3.5–20 Ci/mol). At the end of the incubation (20 min with lactose, 15 min with thiomethyl- β -D-galactoside) 1 ml volumes of cells were removed into Eppendorf centrifuge tubes and centrifuged at 10000 *g* for 30 s. The supernatants were aspirated with a fine-tipped Pasteur pipette attached to a vacuum line and the pellets were resuspended in 100 μl of Tris/Mes/choline chloride/KCl buffer, pH 5.9, and stored on ice for up to 2 h. No significant loss of radioactivity occurred during storage on ice. However, during the centrifugation step approximately 30% of the radioactivity was lost from the cells, as had been noted previously (Lagarde & Stoeber, 1974).

The exit rate of β -galactoside was measured by diluting 25 μl of the concentrated loaded cell suspension into 3 ml of Tris/Mes/choline chloride/KCl buffer at the relevant pH. Portions (500 μl) were removed at intervals and the cells harvested and washed by membrane filtration as above. Liquid scintillation counting of the filters was carried out as described previously (Booth *et al.*, 1979).

The rate of exit of β -galactosides was measured both in the presence and absence of the uncoupler, carbonylcyanide-*m*-chlorophenylhydrazone at a concentration of 5 μM . Leak of lactose and thiomethyl- β -D-galactoside was measured by diluting cell suspensions into the usual buffer containing 200 μM -*p*-chloromercuribenzenesulphonate. In control experiments this concentration of *p*-chloromercuribenzenesulphonate was shown to be sufficient to inhibit the β -galactoside carrier within 10 s of the addition of cells.

The steady state accumulation ratio of β-galactoside was measured in the above incubations prior to the centrifugation step as described previously (Booth *et al.*, 1979). In this way the internal concentration of lactose and of thiomethyl-β-D-galactoside prior to centrifugation could be calculated and compared with our earlier studies (Booth *et al.*, 1979).

Throughout this study an internal volume of 1 μl/mg of dry bacterial mass was used in calculations (Booth *et al.*, 1979). No allowance was made for the expansion of the cytoplasm that may have occurred upon uptake of high concentrations of β-galactoside; this aspect merits investigation.

Results

In order to explain the disparity between $\Delta\bar{\mu}_{H^+}$ and the lactose accumulation in *E. coli* ML308-225 we have previously invoked leakage of this sugar from the cell (Booth *et al.*, 1979). Thus, at the steady state the inward and outward fluxes of β-galactoside across the membrane would be equal and take the form of eqn. (1):

$$V_{in} = V_{exit} + D[S]_{in} \quad (1)$$

where V_{in} and V_{exit} are the carrier-mediated rates of entry and exit of solute, $[S]_{in}$ is the internal concentration of solute and D is the diffusion coefficient of the solute through the membrane. The term $D[S]_{in}$ therefore constitutes the leak.

In order to characterize the exit of β-galactoside it was necessary first to estimate the expected magnitude of this process. Thus, we attempted to measure V_{in} at the steady state. However, because of

the low concentration of lactose (5 μM) used in our earlier experiments (Booth *et al.*, 1979) it was not possible to measure V_{in} directly at the steady state by tracer studies. Earlier workers had demonstrated that the initial rate of uptake of β-galactoside is the same as the steady state rate under conditions of unchanged external lactose concentration (Cohen & Monod, 1957). We, therefore, proceeded on this assumption to measure the initial rate of lactose uptake, v , in dilute cell suspensions of *E. coli* ML308-225 and to equate it with the steady state rate (Fig. 1).

It was observed that over the range of external pH (pH_o) 5.9–7.4 the rate of uptake was relatively constant at 0.91 nmol/min per mg of bacterial cells. Above pH_o 7.4 there was a significant decline in v until at pH_o 8.7 the rate was only 0.45 nmol/min per mg of bacterial cells. The shape of this graph is in general accordance with the relationship of lactose accumulation to pH_o (Booth *et al.*, 1979).

Calculation of the value of D

Using these data and our earlier measurements of both the lactose concentration and the proton-motive force, it is possible to calculate the leak that would give rise to the observed accumulation. The calculation rests on the assumption that in the absence of a leak the carrier would cause lactose to equilibrate with $\Delta\bar{\mu}_{H^+}$, and on the observation that lactose exit is a first-order process (Cohen & Monod, 1957). Thus, assigning the first order rate constants k_c to zero-trans efflux, D to the leak and K_{exit} to overall exit, we can say:

$$k_{exit} = k_c + D$$

at steady state:

$$V_{in} = k_c[S]_{in} + D[S]_{in} \quad (2)$$

assuming $v = V_{in}$

$$v = (k_c + D)[S]_{in} \quad (3)$$

Taking experimentally determined values at pH_o 5.9 of $v = 0.91$ nmol/min per mg of cells and $[S]_{in} = 3.5$ mM (Booth *et al.*, 1979), substitution in eqn. (3) yields a value of $(k_c + D)$ of 0.26 min^{-1} . If, however, there were no leak and thus $\Delta\mu_{Lac} = \Delta\bar{\mu}_{H^+}$, then at pH_o 5.9 $\Delta\bar{\mu}_{H^+} = 239$ mV (Booth *et al.*, 1979):

$$\Delta\mu_{Lac} = 59 \log_{10} \frac{[S]_{in}}{[S]_{out}} = 239 \quad (4)$$

at $[S]_{out} = 5 \mu\text{M}$ the theoretical maximum figure for $[S]_{in}$ can be calculated from eqn. (4) as 56 mM. From eqn. (3) above, when $D = 0$, k_c has a value of 0.0163 min^{-1} , and, substituting for k_c in the relationship $(k_c + D) = 0.26 \text{ min}^{-1}$, a value for D of 0.244 min^{-1} can be calculated.

That is to say, a calculated value of 0.244 min^{-1} for the carrier-independent leak is required to explain

Fig. 1. Lactose uptake rate versus external pH. The external concentration of lactose was 5 μM and the cell density was 0.25 mg of dry bacterial weight/ml.

the difference between the theoretical and observed accumulation of lactose on the assumption that this difference results only from the leak process.

Similar calculations with data obtained at pH_o 8.1 gave a value of $D = 0.184 \text{ min}^{-1}$. These values of D are very similar to the exit constants for thio-methyl- β -D-galactoside (Maloney & Wilson, 1973) and arabinose (Novotny & Englesberg, 1966), but are an order of magnitude higher than those for the sugar acids gluconate (Pouyssegur *et al.*, 1974) and 2-oxo-3-deoxygluconate (Lagarde & Stoeber, 1974). Thus it seemed not unreasonable that we should be able to characterize experimentally the actual leak of lactose from *E. coli*.

Exit of lactose from E. coli

Previous workers studying the uptake of β -galactosides and of sugar acids have reported that exit of the appropriate solute from cells into medium lacking solute occurs by a carrier-independent leak (Kepes, 1970, 1971; Lagarde & Stoeber, 1974). The rate of exit is first order with respect to internal substrate concentration and the value of D could be measured from this relationship.

In the present study lactose efflux was measured from *E. coli* cells preloaded with lactose to give a final post-centrifugation internal concentration of 5–6 mM. It was observed that the rate of lactose transport out of the cells was markedly dependent upon the pH of the external medium (Table 1). The values of the exit constants, k_{exit} were variable from day to day but the results of a typical experiment are given in Table 1. At pH_o 5.9, k_{exit} was 0.109 min^{-1} rising to a value of 0.416 min^{-1} at pH_o 8.1. This result was surprising if the carrier-independent leak constituted the whole or even a major part of the exit process, but became more understandable if the lactose was leaving the cells via the permease. Thus

we examined the relative contributions of leak and zero-trans efflux to the exit of lactose.

It has been claimed that zero-trans efflux of thio-methyl- β -D-galactoside is insensitive to sulphhydryl group reagents, such as *p*-chloromercuribenzenesulphonate, which rapidly inactivate the carrier (Kepes, 1970, 1971). It was on the basis of this evidence that the exit of thiomethyl- β -D-galactoside was proposed to be entirely due to leakage (Kepes, 1970, 1971). In *E. coli* ML308-225 cells $200 \mu\text{M}$ -*p*-chloromercuribenzenesulphonate was sufficient to inactivate rapidly the carrier, as indicated by the inhibition of uptake and exchange of β -galactosides within 10s of addition of the inhibitor (data not shown). Thus this compound was used to assay the relative contributions of leak and zero-trans efflux to exit.

In the presence of $200 \mu\text{M}$ -*p*-chloromercuribenzenesulphonate exit of lactose from cells preloaded to an internal concentration of 5 mM-sugar was completely blocked (Fig. 2a). At pH_o 5.9 very little efflux of the β -galactoside was detected over a 20 min incubation compared with loss of 50% of the radioactivity in approximately 6 min in the absence of *p*-chloromercuribenzenesulphonate (Fig. 2a). This result indicates that the major route of lactose exit is in fact zero-trans efflux, the carrier-mediated process. The presence of a leak pathway for lactose cannot be ruled out, however, since the internal concentration of lactose was quite low, only 5 mM, and also because of the unlikely possibility that leakage of lactose occurs by a *p*-chloromercuribenzenesulphonate-sensitive channel.

The pH_o-dependence of lactose exit is also consistent with the principal route of sugar efflux being via the carrier. Exit of β -galactoside by this route would require that the sugar-binding site of the carrier be available at the inner face of the membrane in order to bind a lactose molecule. It is probable that the observed pH-dependence of exit reflects the degree of protonation of the carrier at the outside since this would be expected to increase as pH_o decreases. It is widely assumed that the protonated carrier cannot traverse the membrane (West, 1980) in the absence of bound β -galactoside. Therefore the degree of protonation of the carrier at the outside of the membrane will be a significant factor in determining the rate of zero-trans efflux. The increasing rate constant for exit at alkaline pH would reflect the greater availability of the unprotonated carrier at this pH and thus its availability to cross the membrane and participate in efflux.

The exit constant for lactose was markedly increased by the addition of the uncoupler carbonylcyanide-*m*-chlorophenylhydrazine to the incubation medium (Table 1). A 20-fold increase in k_{exit} was observed at pH_o 5.9 while at pH_o 8.1 a smaller, 5-fold, increase was observed (Table 1). This

Table 1. Values of k_{exit} under various conditions
Exit of lactose was measured as described in the Materials and Methods section in the presence and absence of the uncoupler, carbonylcyanide *m*-chlorophenylhydrazine (CCCP). Half-times of efflux were variable from day to day and typical data are shown. The $t_{\frac{1}{2}}$ was calculated from a plot of $\log_{10}$ radioactivity remaining in cells versus time and was converted to give the k_{exit} value. ($k_{\text{exit}} = (\ln 2)/t_{\frac{1}{2}}$)

pH _o	[CCCP] (μM)	k_{exit} (min^{-1})
5.9	0	0.109
5.9	5	2.45
6.4	0	0.109
7.0	0	0.185
7.6	0	0.185
8.1	0	0.378
8.1	5	2.31
8.4	0	0.416

Fig. 2. Effect of *p*-chloromercuribenzenesulphonate on exit of β -galactosides from *E. coli* ML308-225

Cell suspensions preloaded with galactoside were diluted into buffer in the absence (■) or presence (△) of 200 μ M *p*-chloromercuribenzenesulphonate at pH_o 5.9. Final cell density was 0.1 mg of dry bacterial weight/ml. (a), lactose exit; (b), thiomethyl- β -D-galactoside exit.

difference in the effect on k_{exit} means that, in the presence of uncoupler, exit is changed from being pH_o-dependent to being pH_o-independent. However, recent results obtained with membrane vesicles from *E. coli* ML308-225 indicate that in the absence of energy coupling lactose exit is still pH_o-dependent. Thus at pH_o 5.5 k_{exit} was 0.924 min⁻¹ and at pH_o 7.5 (k_{exit} was 2.77 min⁻¹) (Kaczarowski & Kaback, 1979). The reason for this difference is not known.

Is there a lactose leak pathway?

In the experiment (Fig. 2a) at low internal concentration of lactose (5 mM), a slow leak might possibly not be detected. In order to test rigorously for the presence of a lactose leak pathway, as defined above, the rate of loss of this β -galactoside from *p*-chloromercuribenzenesulphonate-treated cells was measured as a function of intracellular sugar concentration. Cells of *E. coli* ML308-225 were preloaded with up to 170 nmol of radioactive lactose/mg of dry bacterial mass (approximately 170 mM) and diluted into *p*-chloromercuribenzenesulphonate-containing buffer at pH_o 5.9. Over a 20 min period of observation there was no significant net loss of radioactivity from the cells. Thus if there is a leak pathway for lactose the value of D cannot be greater than 0.003 min⁻¹, the considered limit of sensitivity of our assay. This value is considerably smaller than that calculated from measurements of $\Delta\bar{\mu}_{\text{H}^+}$, lactose accumulation and v , and the significance of this will be discussed below.

Exit of thiomethyl- β -D-galactoside from *E. coli*

In view of the apparent absence of lactose leak it was decided to investigate further the characteristics of thiomethyl- β -D-galactoside exit from *E. coli* cells. At an internal concentration of 6–7 mM-thiomethyl-

β -D-galactoside, 200 μ M *p*-chloromercuribenzenesulphonate reduced the rate of sugar efflux approximately 3-fold (Fig. 2b). Thus it appears that thiomethyl- β -D-galactoside exit is composed of two parts, one of which is *p*-chloromercuribenzenesulphonate-sensitive and is thus zero-trans efflux, and the other is *p*-chloromercuribenzenesulphonate-insensitive and represents leakage. The two routes of thiomethyl- β -D-galactoside exit were further characterized by measurement of the rate of loss of thiomethyl- β -D-galactoside at different internal concentrations of this β -galactoside in the presence and absence of *p*-chloromercuribenzenesulphonate (Fig. 3). At pH_o 5.9 both processes had first-order kinetics with rate constants of D (thiomethyl- β -D-galactoside) = 0.079 min⁻¹ and k_c (thiomethyl- β -D-galactoside) = 0.137 min⁻¹. The observations of first-order kinetics for the leak of thiomethyl- β -D-galactoside confirms the findings of Koch (1964) and Kepes (1970, 1971), while the similar observation for zero-trans efflux (k_c) is consistent with the prediction of Cohen & Monod (1957). However, it is apparent that the carrier-independent leak is significantly different for thiomethyl- β -D-galactoside and lactose. In the former case it is readily demonstrable but for lactose no similar exit process was found.

Discussion

The results presented here illustrate two facets of β -galactoside transport in *E. coli*: firstly, the presence or absence of a carrier-independent leak dependent upon the nature of the β -galactoside, and secondly, the nature of the zero-trans efflux of β -galactosides.

Fig. 3. The relationship between rate of leak (●) or zero-trans efflux (□) and internal concentration of thiomethyl- β -D-galactoside

The rate of zero-trans efflux is the total exit minus the *p*-chloromercuribenzenesulphonate-insensitive rate (i.e. leak) under the same conditions.

We propose that zero-trans efflux of lactose is mediated solely by the β -galactoside permease on the basis of its inhibition by *p*-chloromercuribenzenesulphonate (Fig. 2a) and its sensitivity to external pH (Table 1). However, thiomethyl- β -D-galactoside exit is composed of at least two transport processes; firstly zero-trans efflux as for lactose, and secondly a leak pathway. Of the two routes, we have found that zero-trans efflux constitutes the major route of exit. As to the nature of the leak pathway we can conclude little except that if a protein is involved its interaction with lactose must be significantly different from that with thiomethyl- β -D-galactoside. Such an occurrence would not be impossible as is suggested by the affinity characteristics of the thiomethyl- β -D-galactoside II permease (Rotman *et al.*, 1968). However, this permease is absent from the organism used in the present study and cannot itself account for the leak characteristics.

The technique used here to discriminate between zero-trans efflux and leak is indirect. It would be desirable to confirm these results using a strain of *E. coli* which lacked β -galactoside permease and thus avoiding the use of sulphhydryl group reagents which also interact with other proteins. Such strains are

available (Overath *et al.*, 1979) and this aspect requires investigation.

If, as at present seems likely, lactose leak is either very slow or absent, our earlier suggestion that such a process was responsible for the decrease in lactose accumulation must be considered invalid (Booth *et al.*, 1978, 1979). In this situation a new model must be formulated and tested. If zero-trans efflux represents the only exit process occurring at the steady state, then it is obvious that the measured rate constant is significantly greater than that predicted for the carrier catalysing equilibrium between the proton-motive force and lactose accumulation. Thus there is a 6–10-fold difference between the calculated value of k_c (0.0163 min^{-1}) and the measured value of k_{exit} ($0.11\text{--}0.15 \text{ min}^{-1}$; Table 1 and unpublished data) at pH_0 5.9. Two possibilities are being considered: firstly, that there is a *cis*-inhibition of accumulation analogous to *trans*-inhibition of uptake (Ring *et al.*, 1970; Kadner, 1975), and secondly that there is inefficient coupling of $\Delta\mu_{\text{H}^+}$ to carrier activity.

The phenomenon of *trans*-inhibition entails the inhibition of uptake of a solute brought about by internal solute either of the same kind or a related compound (Ring *et al.*, 1970; Kadner, 1975). It is well established that the rate of uptake of β -galactosides at the steady state is identical to that at the initiation of uptake (Cohen & Monod, 1957). Furthermore, preliminary experiments have revealed that internal lactose up to 150 nmol/mg of dry bacterial mass has no significant effect on the rate of uptake of external β -galactoside (I. R. Booth & W. A. Hamilton, unpublished observations). Thus any *trans*-inhibition effect would have to be mediated by stimulation of exit by internal β -galactoside, i.e. *cis*-inhibition of accumulation; this would have the effect of raising k_c and limiting $[S]_{\text{in}}$.

Secondly, there is the possibility of thermodynamic inefficiency in energy coupling to carrier-mediated transport. It seems possible that modification of the transport carrier's kinetic properties, by imposition of either $\Delta\Psi$, ΔpH , or both, may not result in a stoichiometric increase in solute accumulation. Recently evidence has been presented from a different transport system which supports this concept. Using *Halobacterium halobium*, Lanyi (1978) has shown that above certain values of a chemical gradient of sodium ions ($\Delta\mu_{\text{Na}^+}$) no further increase in amino-acid accumulation occurs. On the other hand increasing $\Delta\Psi$ led to apparently stoichiometric increases in internal solute accumulation. Similarly, it has been demonstrated in *E. coli* that at pH_0 8.1, where ΔpH is zero, $\Delta\Psi$ couples to lactose transport with near 100% efficiency (Zilberstein *et al.*, 1979). Thus it may be that ΔpH in *E. coli*, by analogy with $\Delta\mu_{\text{Na}^+}$ in *H. halobium*, is not coupled to transport with the same efficiency as $\Delta\Psi$. This

inefficient coupling could affect either entry or exit since the calculated value of k_c is dependent on the measured value of the initial rate of influx of lactose. If energy were inefficiently coupled to this step an artificially lowered value of k_c would be obtained. Thus, at present it is not possible to say which steps in the uptake and exit cycle are poorly coupled to the electrochemical gradient of H^+ .

Both of these proposals would explain the disparity between the proton-motive force and the lactose accumulation and further inquiries along these lines should be pursued.

Finally, the absence of a leak for lactose does not invalidate our criticisms of the indirect approach to proton solute stoichiometries (Ramos & Kaback, 1977a,b; Booth *et al.*, 1979). Our analysis of the experimental system used in such studies stands, that unless thermodynamic equilibrium between solute accumulation and the proton-motive force is demonstrated, the results of indirect approaches are invalid. On the basis of the published data (Ramos & Kaback, 1977a,b) it is clear that this condition is not met. Thus, the disparity between the direct (Booth *et al.*, 1979; Zilberstein *et al.*, 1979) and the indirect approaches (Ramos & Kaback, 1977a,b) only stimulates the formulation of new models that recognize and can explain the differences. Furthermore, examination of the predictions of such models with regard to the indirect approaches will lead to a greater understanding of the mechanism of energy coupling to nutrient transport.

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