Quantitative Analysis of Proton-Linked Transport Systems

THE LACTOSE PERMEASE OF *ESCHERICHIA COLI*

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Evidence is presented that lactose uptake into whole cells of *Escherichia coli* occurs by symport with a single proton over the range of external pH 6.5–7.7. The proton/lactose stoichiometry has been measured directly over this pH range by comparison of the initial rates of proton and lactose uptake into anaerobic resting cell suspensions of *E. coli* ML308. Further, the relationship between the protonmotive force and lactose accumulation has been studied in *E. coli* ML308-225 over the range of external pH 5.9–8.7. At no point was the accumulation of the β -galactoside in thermodynamic equilibrium with the protonmotive force. It is concluded that the concentration of lactose within the cell is governed by kinetic factors rather than pH-dependent changes in the proton/substrate stoichiometry. The relevance of these findings to the model of pH-dependent proton/substrate stoichiometries derived from studies with *E. coli* membrane vesicles is discussed.

The β -galactoside permease of *Escherichia coli* is probably the most studied of the proton-linked transport systems. Many of the predictions of the chemiosmotic model of transport have been tested and verified with this system. Thus the carrier has been identified as a membrane protein, the product of the *lac y* gene (Fox & Kennedy, 1965), and is functionally symmetrical in the absence of energy coupling (Lancaster & Hinkle, 1977a,b; Teather *et al.*, 1977). In metabolizing cells the permease operates in an asymmetric manner, leading to the accumulation of lactose against its concentration gradient (Winkler & Wilson, 1966; Lancaster *et al.*, 1975).

Although the exact molecular mechanism of energy coupling is still unknown, that it involves a transmembrane protonmotive force, inside-alkaline and negative, and carrier-mediated flow of protons down their electrochemical gradient is well established. Four lines of evidence lead to this conclusion. Firstly, the presence in metabolizing cells of a correctly orientated protonmotive force has been demonstrated in a variety of organisms (Harold, 1976; Hamilton, 1977). Agents that interfere with the maintenance of the protonmotive force also prevent active accumulation of β -galactosides and other solutes. Secondly, accumulative uptake of β -galactosides into non-metabolizing cells in response to artificially imposed pH gradients and membrane potentials has been demonstrated (Hirata *et al.*,

1973; Flagg & Wilson, 1976). Thirdly, in non-metabolizing cells, proton uptake coupled to the flow of β -galactosides down their concentration gradient has been observed (West, 1970). At pH 7.0 the stoichiometry of the coupled flow has been estimated as one proton per lactose molecule (West & Mitchell, 1973). Finally, mutants have been isolated that no longer demonstrate proton flux coupled to the uptake of β -galactosides. These mutants also fail to accumulate lactose, although the carrier still facilitates transmembrane equilibration of this compound (Wilson & Kusch, 1972; West & Wilson, 1973).

More recently it has been proposed that the stoichiometry of the lactose-transport system might be pH-dependent (Rottenberg, 1976); in vesicle preparations of *E. coli* ML308-225, the measured protonmotive force was observed to be inadequate as a driving force for lactose uptake above pH 6.5 (Ramos & Kaback, 1977a). Subsequent experiments in which the protonmotive force was modulated with antibiotics and a comparison was made between the steady-state internal lactose concentration and the driving force, showed an apparent increase in the proton/lactose stoichiometry to 2 at pH 7.5 (Ramos & Kaback, 1977b). The conceptual basis for these experiments lies in the equation derived from Mitchell (1973) to relate the accumulation of metabolites to the driving force through the proton/

substrate stoichiometry, n , and the charge on the metabolite, m :

$$Z \log \left(\frac{[S]_i}{[S]_o} \right) = (m+n)\Delta\Psi - nZ\Delta pH \quad (1)$$

where $[S]_i$ and $[S]_o$ are the internal and external concentrations, respectively, of metabolite, $\Delta\Psi$ is the membrane potential, ΔpH the transmembrane pH gradient, and Z the factor (equal to 59 at 25°C) that converts concentration ratios into mV. The assumption implicit in the use of this equation is that the substrate accumulation achieves thermodynamic equilibrium with the protonmotive force. Ramos & Kaback (1977*a,b*) have therefore made this assumption in their analysis of proton/substrate stoichiometries by substituting measured values of $[S]_i$, $\Delta\Psi$ and ΔpH into eqn. (1), and solving for n .

On the other hand, several excellent kinetic analyses of the lactose-transport system have claimed that the accumulation of the β -galactoside represents rather a kinetic steady state (Rickenberg *et al.*, 1956; Cohen & Monod, 1957; Maloney & Wilson, 1973). At the steady state, influx via the carrier is matched by β -galactoside exit by two routes, one the carrier itself and the second a non-specific leak (Cohen & Monod, 1957; Kepes, 1960; Maloney & Wilson, 1973).

The aims of the experiments presented here were: (i) to attempt to relate the steady-state lactose accumulation to the protonmotive force; (ii) to determine experimentally the proton/substrate stoichiometry over a range of pH; and (iii) to provide a basis for critical examination of the proposal that the proton/substrate stoichiometry is a variable function of the external pH.

The results are consistent with lactose accumulation representing a kinetic steady state up to 70 mV below the protonmotive force, and the stoichiometry of the transport system being one proton per lactose molecule, independent of the external pH.

Materials and Methods

Materials

Carbonic anhydrase, iodoacetic acid, valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone were obtained from Sigma. PPO (2,5-diphenyloxazole) was obtained from Koch-Light Laboratories and POPOP [1,4-bis-(5-phenyloxazol-2-yl)-benzene] was purchased from Nuclear Enterprises, Sighthill, Edinburgh 11, Scotland, U.K. Tetrachlorosalicylanilide was generously given by Unilever Research Laboratories, Colworth House, Sharnbrook, Bedford, U.K. All other chemicals were purchased from BDH, Poole, Dorset, U.K.

Bacterial strains

Two strains of *Escherichia coli* were used in these investigations: ML308-225 (*lac*⁻*z*⁻*y*⁺*a*⁺) and ML308 (*lac*⁻*z*⁺*y*⁺*a*⁺). Both strains were generously supplied by Dr. I. C. West, University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.

Growth conditions

Both strains of *E. coli* were stored at 4°C after overnight growth at 30°C on agar slopes of a nutrient medium that contained, per litre, peptone (20g), NaCl (5g) and agar (15g).

For experimental studies the organisms were grown in 100 ml volumes of media in 250 ml Erlenmeyer flasks. The cultures were shaken at 30°C on a New Brunswick gyrotary shaker at setting 9. The growth medium contained, per litre, K₂HPO₄ (7g), KH₂PO₄ (3g), trisodium citrate (0.5g), MgSO₄·7H₂O (0.1g), (NH₄)₂SO₄ (1g) and sodium succinate (10g) (Davis & Mingioli, 1950). The medium was adjusted to pH 7.0 with KOH before autoclaving.

Overnight cultures were diluted to an *A*₆₅₀ of approx. 0.5 (an *A*₆₅₀ of 2.4 is equivalent to 1 mg bacterial dry weight/ml) with fresh growth medium and the cells reincubated as above until the *A*₆₅₀ reached 1.0. The organisms were then harvested by centrifugation at 10400g for 10 min at 4°C. Washing was carried out by resuspension in an appropriate buffer, as detailed below, followed by re-centrifugation at 18000g for 5 min at 4°C.

Measurement of intracellular volume

The intracellular volume was measured by the method of Stock *et al.* (1977). The outer and inner membranes of *E. coli* present permeability barriers to compounds of different sizes. Hydroxymethylinulin and inulin cannot penetrate the outer membrane, whereas sucrose passes through the outer membrane but is excluded by the inner cell membrane. Water passes through both membranes and equilibrates with the water in the lumen of the cell. Thus cells were incubated with different combinations of non-permeating radioactively labelled compounds and ³H₂O.

Organisms were harvested, washed twice and resuspended in 50 mM-KH₂PO₄/K₂HPO₄ buffer, pH 7.0. Cell suspensions (3 mg bacterial dry weight/ml) were incubated with one of the following pairs of radioactively labelled compounds: [U-¹⁴C]sucrose/[³H]inulin; [U-¹⁴C]sucrose/³H₂O; [G-¹⁴C]hydroxymethylinulin/[³H]inulin; [G-¹⁴C]hydroxymethylinulin/³H₂O. All the compounds with the exception of water were present at low concentration (approx. 5 μM) to avoid osmotic effects, and at the following specific activities: sucrose, 8.6 Ci/mol; inulin, 1.8 Ci/mmol; hydroxymethylinulin, 10.3 Ci/mol; water, 30 μCi/ml. No evidence was found for the active

accumulation of radioactive label from sucrose, inulin or hydroxymethylinulin.

Stirred cell suspensions were incubated at 25°C with one of the pairs of compounds listed above. After 1 min incubation, triplicate 0.5 ml samples were transferred to centrifuge tubes (capacity 1 ml) and centrifuged at 10000g in an Eppendorf centrifuge (model no. 5412) for 30s. This time interval was sufficient to collect the cells into a firm pellet. Portions (100 µl) of the supernatant were removed by Hamilton microsyringe and placed into sealed Eppendorf tubes that contained an equivalent blank cell pellet that had not been exposed to the radioactively labelled compounds. The remaining supernatant was aspirated off by a vacuum line, taking care to remove all visible traces of liquid. The pellets from both tubes were resuspended in 50 mM-KH₂PO₄/K₂HPO₄ buffer pH 7.0 (final vol. 600 µl), and transferred to scintillation vials. Scintillant {10 ml of Triton/toluene (1:2, v/v) containing 4 g of PPO (2,5-diphenyloxazole) and 100 mg of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre} was then added and the capped vials placed in a Packard liquid-scintillation counter (model 3255) at 8°C. When the initial opacity of the scintillant had cleared, the vials were counted for radioactivity on a preset double-label program. The intracellular volume was calculated as described by Stock *et al.* (1977).

EDTA treatment of cells

E. coli is not normally sensitive to the ionophorous antibiotics nigericin and valinomycin. A technique was developed by Leive (1968) to increase the permeability of these cells to the antibiotics. Cells of *E. coli* ML308-225 were harvested, washed once and resuspended in 120 mM-Tris/HCl buffer, pH 8.0, at approx. 20 mg dry bacterial weight/ml, and incubated for 1 min at 37°C. EDTA/KOH, pH 7.0, was then added to a final concentration of 1 mM and incubation at 37°C continued for a further 2 min, accompanied by gentle agitation. The cell suspension was then diluted 5-fold with 120 mM-Tris/HCl buffer, pH 8.0, and centrifuged at 3000g for 5 min at room temperature. The cell pellet was washed by resuspension and centrifugation in 5 mM-Tris/5 mM-Mes (4-morpholine-ethanesulphonic acid/HCl buffer, pH 7.0, containing 150 mM-choline chloride and 0.2 mM-KCl. The washed cells were resuspended in the above buffer to a final concentration of approx. 40 mg dry bacterial weight/ml, immediately treated with valinomycin (final concn. 4 µg/mg dry bacterial weight) and stored at 4°C for up to 4 h.

Measurement of internal pH

The assay for measuring the transmembrane pH gradient, and hence the internal pH (pH_i), was based on the distribution of weak acids and bases (Rottenberg, 1975).

EDTA/valinomycin-treated cells of *E. coli* ML308-225 were incubated in the Tris/Mes/choline chloride/KCl buffer adjusted to pH values over the range pH 5.9–8.7. Radioactively labelled weak acids (dimethyloxazolidinedione, 5 µM, 16 Ci/mol; acetic acid, 6 µM, 10 Ci/mol) or a weak base (methylamine, 3 µM, 22.4 Ci/mol) were added to the cells, along with [³H]inulin (0.1 µM, 1.8 Ci/mmol), which was present as a marker of extracellular water. After 5 min incubation, triplicate 500 µl samples were withdrawn and filtered under vacuum through dry Oxoid filters (2.5 cm diameter, 0.45 µm pore size). The filters were then placed in scintillation vials and dried in a warm oven (80°C). Two 50 µl reference samples were also withdrawn from the reaction vessels, placed on dry filters in scintillation vials and dried. When the vials had cooled to room temperature, 6 ml of scintillant (toluene containing 6 g of PPO and 75 mg of POPOP/litre) was added. The vials were then transferred to the Packard liquid-scintillation counter at 8°C and counted for radioactivity on the preset ¹⁴C/³H double-labelling program.

The concentration ratio of the weak acid or base was calculated after corrections were made for (a) ¹⁴C overlap into the ³H channel (determined with a standard of the ¹⁴C isotope in use), and (b) for the acid or base trapped on the filter outside the cells (determined from the 50 µl reference samples and the [³H]inulin trapped on the filter).

The internal pH was calculated by substituting the concentration ratio of the weak acids [A⁻] into the following equations:

$$\Delta\text{pH} = \log \left(\frac{[\text{A}^-]_i}{[\text{A}^-]_o} \right) (1 + 10^{\text{pK} - \text{pH}_o}) - 10^{\text{pK} - \text{pH}_o} \quad (2)$$

$$\text{pH}_i = \text{pH}_o + \Delta\text{pH} \quad (3)$$

where pH_o is the pH of the suspension medium.

The pK for dimethyloxazolidinedione was assumed to be 6.32 (Addanki *et al.*, 1968), and for acetate, 4.7.

No calculations of ΔpH were made by using the accumulation of methylamine, as the weak base was found not to equilibrate with ΔpH.

Determination of the membrane potential

The membrane potential was estimated by the transmembrane distribution of K⁺ in the presence of valinomycin.

EDTA/valinomycin-treated cells of *E. coli* ML308-225 were incubated in the Tris/Mes/choline chloride/KCl buffer adjusted to pH values over the range pH 5.9–8.7. After K⁺ uptake by the cells was complete (approx. 1 min) the external K⁺ concentration was 0.15–0.17 mM. After 5 min incubation, triplicate 1 ml samples were removed and filtered under vacuum through dry filters, as described above. Uncoupler (tetrachlorosalicylanilide, 10 µM, or carbonyl cyanide *m*-chorophenylhydrazone,

25 μM) was then added to the remaining cell suspension and after a further 5 min incubation triplicate 1 ml samples were taken and filtered as above. The filtrates from each sample were collected in test tubes placed below the filters.

The filters and cells were extracted in 1 ml of distilled water at 100°C for 10 min, with care being taken to prevent water losses due to evaporation. After cooling, the K^+ content of the cell extracts and that of filtrates was determined with a flame photometer (EEL model 150). Control experiments with [^3H]inulin were included to calculate the water trapped on the filter between the cells, and thus to correct for extracellular K^+ . The membrane potential was calculated by substitution of the transmembrane accumulation ratio for K^+ into the Nernst equation:

$$\Delta\Psi = \frac{RT}{F} \ln \frac{[\text{K}^+]_i}{[\text{K}^+]_o} \quad (4)$$

This method of measurement of the membrane potential involves quantification of the efflux of K^+ on de-energization of the cells with uncoupler. It therefore avoids the pitfalls normally associated with the use of K^+ as a probe of $\Delta\Psi$ such as binding of K^+ to the cell wall and internal cell components.

The total protonmotive force (Δp) was calculated from the measured values of the pH gradient (ΔpH) and membrane potential by using eqn. (5):

$$\Delta p = \Delta\Psi - Z\Delta\text{pH} \quad (5)$$

Measurement of lactose transport

Lactose transport was also determined in EDTA/valinomycin-treated cells of *E. coli* ML308-225 incubated at 25°C in the Tris/Mes/choline chloride/KCl buffer system. In control experiments it was ascertained that a steady-state accumulation of lactose was achieved after 18 min. In all subsequent experiments the cell suspensions were sampled only at 20 min after the addition of lactose.

Portions (500 μl) of cell suspension (0.1–0.25 mg of bacterial dry weight/ml) were incubated with 5 μM -lactose ([*gluco*-1- ^{14}C]lactose, 15 Ci/mol). The tubes were shaken at 2–3 min intervals to ensure aeration. This technique enabled several experiments to be carried out simultaneously and gave identical results to incubations of cells carried out in stirred vessels. After 20 min incubation, triplicate 100 μl samples were removed from each tube and filtered under vacuum. Prewetted filters were used in this case and the filtered cells were immediately washed with 3 ml of the incubation buffer. Portions (50 μl) of the cell suspension were transferred to scintillation vials containing dry filters. All the filters were then dried and were counted for radioactivity as described above.

The concentration ratio of lactose was calculated from the ratio of radioactivity inside the cells to

that in cell-suspension samples, with appropriate corrections for the relevant volume differences. This concentration ratio was converted into mV accumulation by substitution into the term $Z \log ([\text{S}]_i/[\text{S}]_o)$.

Determination of the proton/lactose stoichiometry

Cells of *E. coli* ML308, the β -galactosidase-positive strain, were grown and harvested as described above, and washed once with 150 mM-KCl and once with 1 mM-glycylglycine buffer, pH 6.5, containing 150 mM-KCl. The washed cells were then resuspended in the glycylglycine buffer at approx. 50 mg bacterial dry weight/ml and stored at 4°C for up to 4 h.

In order to detect proton uptake induced by lactose flux down its concentration gradient, it is necessary to prevent the subsequent ejection of protons by energy-dependent processes. Thus the cells were incubated anaerobically to prevent respiration and were treated with 1 mM-potassium iodoacetate to inhibit glycolysis. Incubation was carried out at 25°C in a closed glass vessel, the contents of which were bubbled with water-saturated O_2 -free N_2 and stirred magnetically. A single port was left open to allow gas to escape and to enable additions to be made. Carbonic anhydrase (20 $\mu\text{g}/\text{ml}$) was added to the cell suspension (4–8 mg bacterial dry wt. in 4 ml) to catalyse the equilibration of the $\text{CO}_2/\text{H}_2\text{CO}_3$ system (Scholes & Mitchell, 1970).

The pH of the reaction vessel was monitored continuously by using a screened glass micro-electrode (Procion model no. 103) coupled with a calomel electrode connected to the incubation system through a remote liquid junction in a three-way ground-glass tap. The pH-electrode was connected to a Beckman research pH-meter (model no. 101901) and the pH was recorded on a Rikadenki multichannel pen recorder (model no. B341). The buffer concentration in the incubation mixture was decreased over the range pH 6.2–7.6 such that addition of 10 nmol of anaerobic HCl gave a pH shift of approx. 0.03 pH units.

The cell suspension was allowed to equilibrate for 20–30 min, during which time the pH was kept constant by small additions of anaerobic 5 mM-HCl. When equilibration had been achieved (rate of change of pH less than 0.002 pH units/min) the buffering capacity of the cell suspension was calibrated by additions of 5 μl of anaerobic 1 mM-HCl. After a short period to allow re-equilibration, anaerobic [*gluco*-1- ^{14}C]lactose (final concn. 1 mM, 0.1 Ci/mol) was added. Lactose uptake and lactose-induced proton movements were measured simultaneously. Immediately after addition of lactose, five 250 μl samples were taken over a 50 s time course and filtered on wet membrane filters under vacuum. Each sample was immediately washed with 3 ml of

150 mM-KCl. The filters were dried and counted for radioactivity as described above.

All procedures of addition or removal of contents from the incubation vessel were carried out by using syringes washed with either anaerobic water or the anaerobic solution being added. The initial rate of lactose uptake was calculated from plots of lactose content of the cells against time.

The initial rate of proton uptake was measured from the pH trace obtained with the chart recorder. Tangents to the proton uptake induced by lactose addition were taken at approx. 10 s intervals. The logarithm (base 10) of the rate of proton uptake obtained from the tangent was plotted against time, and the relationship was found to be linear over the first 60 s of proton uptake. Extrapolation of the line to zero time gave the value of the initial rate of proton entry. The decay of the rate of proton uptake was found to be pH-dependent, showing marked increase at alkaline pH. Thus above pH 7.8 this method of estimation of the initial rate of proton uptake could not be used, and this pH represents the upper useful limit of this technique.

Values for n , the proton/lactose stoichiometry were determined by dividing the initial rate of proton uptake by the initial rate of lactose uptake from the same experiment.

Results

Internal volume of Escherichia coli

In most quantitative transport studies, *E. coli* cells have been considered to consist of a single compartment of approximate volume $2.7 \mu\text{l}/\text{mg}$ bacterial dry weight (Winkler & Wilson, 1966; West, 1970; Collins & Hamilton, 1976; Lagarde, 1977). This value is based on the exclusion of hydroxymethylinulin from cells, and does not take into consideration the division of the total volume into periplasm and cytoplasm. Measurements suggest that the periplasm may constitute as much as 40% of the total cell volume (Stock *et al.*, 1977). It was thus important for our quantitative investigations of the proton-motive force and lactose transport that we measure the cytoplasmic volume of *E. coli* strain being used in the study.

In order to compare our results with those of previous workers in the field we chose to investigate the cell volumes by using their markers for extra-cellular water. The results of a study using hydroxymethylinulin, inulin and sucrose to define the cell volumes are shown in Table 1.

By using hydroxymethylinulin we obtained good agreement ($2.68 \pm 0.22 \mu\text{l}/\text{mg}$ bacterial dry weight) with the value for total cell volume measured by Winkler & Wilson (1966). However, by using sucrose, which passes through the outer membrane but which does not permeate the inner membrane, a much

smaller value was obtained (1.03 ± 0.35). This represents the cytoplasmic volume as distinct from the total volume of the cell. It is noteworthy that the periplasm measured by inulin and sucrose is of a similar size to the cytoplasm (approx. $1.03 \pm 0.31 \mu\text{l}/\text{mg}$ bacterial dry weight). This value corresponds well to that demonstrated for *E. coli* ML308-225 by Stock *et al.* (1977). However, it was observed that the total cell volume measured with inulin was smaller than that estimated by hydroxymethylinulin (Table 1). This may be due to a small amount of inulin binding to the cells (R. J. Wallace, personal communication).

*When the effect of external pH on the intracellular volume was investigated, there was found to be no significant pH-dependent variation of cytoplasmic space. In all subsequent calculations an intracellular volume of $1 \mu\text{l}/\text{mg}$ bacterial dry weight was assumed. This value is slightly lower than that found in K12 strains of *E. coli* (Bakker, 1978; D. Strickland, I. R. Booth & W. A. Hamilton, unpublished work).

Measurement of the pH gradient

The pH gradient was measured in respiring *E. coli* cells by observing the distribution of a radioactively labelled weak acid across the cell membrane (Rottenberg, 1975). The weak acids used in the present study were dimethyloxazolidinedione and, less frequently, acetic acid. At pH values below the pK of the acid being used, the method is less sensitive, owing to the high proportion of the acid in the undissociated form. Thus at a pH_0 of 5.9, acetic acid ($pK 4.7$) was used to check the results obtained with dimethyloxazolidinedione ($pK 6.32$). Both weak acids gave similar results, internal pH values of $pH_i 7.75$ (dimethyloxazolidinedione) and $pH_i 7.87$ (acetic acid) being recorded. For the major portion of the work discussed below, dimethyloxazolidinedione was used to measure pH_i .

The pH gradient was measured both in EDTA/valinomycin-treated cells and in cells that had not been treated with either compound, and identical results were obtained. Only those results obtained

Table 1. *Internal volume of E. coli* ML308-225 as measured by the method of Stock *et al.* (1977)

Conditions were as described in the Materials and Methods section. The numbers of observations made are given in parentheses.

Isotope couple	Excluded volume	Space
Sucrose/water	1.03 ± 0.35 (44)	Cytoplasm
Sucrose/inulin	1.03 ± 0.31 (5)	Periplasm
Hydroxymethylinulin/ inulin	0.60 ± 0.17 (5)	—
Hydroxymethylinulin/ water	2.68 ± 0.22 (14)	Whole cell

with EDTA/valinomycin-treated cells will be presented here.

At an external concentration of $5\mu\text{M}$, dimethyl-oxazolidinedione was accumulated between 1.5- and 25-fold depending on the external pH (pH_0). Over the range pH_0 5.9–8.7 the pH gradient (ΔpH) fell from 1.85 units to 0.1 units, reflecting the tendency of the internal pH (pH_i) to remain within narrow limits (pH_i 7.75–8.8). Thus the cells maintain a relatively constant internal pH at the expense of the ΔpH component of the protonmotive force (Fig. 1).

In these experiments no external energy source was supplied and therefore the cells were utilizing endogenous energy reserves. To ascertain the effect of pH_0 on the respiration rate in cell suspensions under these conditions, the rate of oxygen uptake was monitored by an oxygen electrode. In *E. coli* ML308-225 over the range pH_0 5.9–8.7, the respiration rate was constant at approx. 15–20 ng-atoms of oxygen consumed/min per mg bacterial dry weight. When the effect of varying the cell density on the rate of oxygen consumption was tested, no effect on the respiration rate was found on increasing the cell concentration to 2 mg bacterial dry weight/ml. This eliminates the possibility that the protonmotive force measured at high cell densities was decreased due to oxygen starvation.

Other groups of workers have reported the effect of pH_0 on the internal pH of *E. coli* cells and vesicles (Padan *et al.*, 1976; Lagarde, 1977; Ramos & Kaback, 1977c). All the results are qualitatively

Fig. 1. Variation of ΔpH (●) and pH_i (○) with external pH. EDTA/valinomycin-treated *E. coli* ML308-225 cells were incubated in Tris/Mes/choline chloride/KCl buffer at the indicated pH. ΔpH was measured with dimethyl-oxazolidinedione at an external concentration of $5\mu\text{M}$.

similar, showing decreasing ΔpH with increasing external pH. However, there seems to be considerable variation in the internal pH measured in different *E. coli* strains. Thus with an *E. coli* K12 strain, Lagarde (1977) found that pH_i varied between 8.2 and 8.7 over the range pH_0 5.7–8.0; these results are very similar to our own. In another *E. coli* K12 strain, however, it was reported that the internal pH (pH_i) was approx. 7.8, and that, above a pH_0 of 7.8, the pH gradient inverted, becoming internal acid (Padan *et al.*, 1976). Under such conditions methylamine, a weak base, would be expected to accumulate, and this was observed by Padan and his co-workers (Padan *et al.*, 1976). However, it has been reported that methylamine is a substrate for an electrogenic NH_4^+ -transport system (Stevenson & Silver, 1977), and this would invalidate its use as a pH_i indicator. In *E. coli* ML308-225 we have indeed observed that methylamine is accumulated over the whole pH range studied. The concentration ratio of methylamine showed some pH-dependence, being 4–5-fold at pH_0 5.9, rising to 20-fold at pH_0 7.0, and being essentially constant above this value of pH_0 . Thus methylamine did not appear to be responding to the pH gradient alone.

In *E. coli* membrane vesicles prepared from strain ML308-225, pH_i was reported to be maintained constant. However, the value of pH_i appeared to be a function of the buffer used in preparation of the vesicles rather than an intrinsic property of the cell's metabolism (Ramos & Kaback, 1977c).

As a direct consequence of this maintenance of a relatively constant pH_i , the contribution of the pH gradient to the protonmotive force is therefore a function of the external pH. At pH_0 5.9 ΔpH constitutes approx. 50% of the total proton electro-

Fig. 2. Variation of ΔpH , $\Delta\Psi$ and Δp with pH_0 . For details, see Fig. 1. ●, ΔpH ; ▲, $\Delta\Psi$; ■, Δp .

chemical gradient (Fig. 2). However, at $pH_0 8.7$ the pH gradient makes no significant contribution to the protonmotive force.

Measurement of the membrane potential

The membrane potential was measured in EDTA/valinomycin-treated cells at an external K^+ concentration of 0.2 mM. A narrow pH-dependence was observed. Thus $\Delta\Psi$ was approx. 130 mV at $pH_0 5.9$, rose to 160 mV at $pH_0 7.0$ and was essentially constant at this value at pH values above $pH_0 7.0$ (Fig. 2). These values are largely in accord with those obtained by other groups of workers (Collins & Hamilton, 1976; Altendorf *et al.*, 1975; Griniuvienė *et al.*, 1974; Padan *et al.*, 1976). However, the values quoted in the present study must be considered as lower estimates of the membrane potential that is formed in untreated cells. Two effects may contribute to a lowering of the membrane potential as measured in these experiments. Firstly the effect of EDTA treatment may increase non-specifically the ionic permeability of the inner membrane as well as that of the outer membrane. Secondly, in valinomycin-treated cells the membrane potential is a function of the external K^+ concentration. Increasing K^+ causes a progressive lowering of the membrane potential in *E. coli* cells (Padan *et al.*, 1976). In our experiments, 0.2 mM was chosen as the external K^+ concentration because it was the lowest concentration that gave reproducible results in the flame photometer. Thus at a lower concentration, 50 μM - K^+ , the membrane potential at $pH_0 7.0$ was found to be 170 mV (results not shown). Consequently the measured values are lower than would be expected in untreated cells.

Fig. 3. Effect of pH_0 on lactose accumulation. For details, see Fig. 1. The external lactose concentration was 5 μM .

The membrane potential and pH gradient were measured simultaneously in parallel incubations of the same cell suspension. This enables the total protonmotive force to be calculated, by using eqn. (5), for each experiment, and this is shown in Fig. 2. The s.d. values are calculated for the total protonmotive force and thus do not always reflect those of the component parts. The proton electrochemical gradient has a magnitude of 240 mV at $pH_0 5.9$ and declines slowly to a value of 165 mV at $pH_0 8.7$. This slow decline reflects in part the rise of the membrane potential, which offsets the fall in ΔpH .

Measurement of lactose transport

The accumulation of lactose by *E. coli* ML308-225 was measured in EDTA/valinomycin-treated cells by using the same conditions as were used to estimate $\Delta\Psi$ and ΔpH .

Lactose accumulation was found to be markedly pH-dependent (Fig. 3). At $pH_0 5.9$ the β -galactoside was accumulated 700-fold and this ratio decreased in linear fashion as the external pH was raised. Above $pH_0 8.1$ there was a rapid decline in the lactose accumulation achieved, and at $pH_0 8.7$ the internal concentration of sugar was only 100-fold greater than that present outside the cells.

If the proton/substrate stoichiometry of the lactose-transport system were 1, then eqn. (1) would simplify to

$$Z \log \left(\frac{[S]_i}{[S]_o} \right) = \Delta p \quad (6)$$

and therefore the accumulation of lactose, expressed in mV, should be affected by pH_0 in an identical manner to Δp . However, when the lactose-accumulation ratio was converted into mV and compared with the protonmotive force, it was observed that at no point were the two in equilibrium (Fig. 4). The magnitude of the protonmotive force was 70 mV in excess of the lactose accumulation at $pH_0 5.9$.

Fig. 4. Comparison of Δp (●) with lactose accumulation, expressed in mV (○), at various values of pH_0 .

Fig. 5. Decay of the rate of proton uptake rate during experiments to measure n

E. coli ML308 cells were resuspended in 150 mM-KCl weakly buffered with glycylglycine and incubated anaerobically in the presence of iodoacetate. Lactose (1 mM) was added at zero time. ○, pH₀ 7.6; ●, pH₀ 6.5.

Fig. 6. Effect of pH₀ on proton/lactose stoichiometry in *E. coli* ML308
For details, see Fig. 5.

Only at pH₀ 8.1 do the driving force and the lactose concentration gradient approach equality, but even here the protonmotive force is in excess of that needed for the observed transport. Thus clearly the accumulation of lactose does not achieve thermodynamic equilibrium with the driving force.

It is therefore apparent from these findings that eqn. (1) cannot be used to predict the stoichiometry of the transport system. Since the concentration of lactose inside the cell appears to be regulated by some process other than the driving force, any value of n could apply. It was therefore important to attempt to measure directly the stoichiometry of the transport system over a range of pH.

Measurement of the proton/lactose stoichiometry

The proton/lactose stoichiometry was measured in *E. coli* ML308-225 at pH₀ 7.0 and found to be 1:1 (West & Mitchell, 1973). The method used was to compare the initial rates of uptake of lactose and of protons into resting cells in which the protonmotive force was zero. A major problem is that, in the absence of a driving force, lactose leaks out of the cell during the washing stage of the filtration assay. In this early work the sensitivity of the lactose carrier to *p*-chloromercuribenzoate was exploited to prevent significant efflux (West & Mitchell, 1973).

We were unable to reproduce this elegant technique and solved the problem in a different way. A β -galactosidase-positive strain of *E. coli*, ML308, was utilized. The principle is simple. Since β -galactosidase is present in excess inside the cell, as soon as [*glucono*-1-¹⁴C]lactose enters it is hydrolysed to [1-¹⁴C]glucose and galactose. The presence of iodoacetic acid prevents further metabolism of the glucose. Glucose is not a substrate for the lactose carrier and is trapped inside the cell during filtration and washing of the cells.

By using this method, lactose and proton uptake were measured simultaneously in the same anaerobic, resting-cell suspension. Lactose uptake was found to be linear over the time course of the assay (50 s). The initial rate of proton uptake was found to decay at an exponential rate, and the $t_{1/2}$ of the decay was pH-dependent (Fig. 5). The half-time of the decay decreased as the chosen starting pH₀ was raised. Thus extrapolation to zero time became increasingly difficult above pH₀ 7.4, and at pH₀ 7.8 this method of determining the initial rate of proton uptake was not possible.

The difficulties associated with this extrapolation are reflected in an apparent slight rise in the proton/lactose stoichiometry as the external pH was raised (Fig. 6). At pH₀ 6.5 the value of n was found to be 0.85 ± 0.23 ng-ion of H⁺/nmol of lactose, and at pH₀ 7.7, n was 1.04 ± 0.44 ng-ion of H⁺/nmol of lactose. However, within the limits of error of the method, we conclude that stoichiometry of the transport system is pH-independent at a value of one proton per lactose molecule.

Discussion

For the first time a single transport system has been studied with respect to all the parameters present in eqn. (1). Thus $\Delta\Psi$, ΔpH , $[\text{S}]_i$ and n have been determined for the β -galactoside-transport system of *E. coli*. Since lactose is a neutral molecule, and by assuming $n = 1$ (West & Mitchell, 1973; Fig. 6), eqn. (1) simplifies to:

$$Z \log \frac{[\text{S}]_i}{[\text{S}]_o} = \Delta p \quad (6)$$

However, it is obvious from Fig. 4 that eqn. (6) does not describe lactose uptake in *E. coli* whole cells. The protonmotive force was found always to exceed the steady-state lactose accumulation. It is therefore concluded that the intracellular concentration of lactose represents a kinetic steady state rather than a thermodynamic equilibrium with the driving force. Uptake is energized by the protonmotive force, but the size of the internal pool is governed by 'leak' pathways. This conclusion, arrived at from a wholly energetic analysis, clearly supports the previous kinetic studies of this transport system (Rickenberg *et al.*, 1956; Kepes, 1960; Maloney & Wilson, 1973).

At the steady state the rates of entry and exit of the non-metabolized lactose are equal. The exit rate is a composite of carrier-mediated and leak-mediated efflux. Thus

$$v_i = v_e + D[S]_i \quad (7)$$

where v_i and v_e are the rates of carrier-mediated β -galactoside influx and efflux respectively, D is the diffusion coefficient of β -galactoside through the membrane, and $[S]_i$ is the internal concentration of sugar (Maloney & Wilson, 1973). The magnitude of the leak pathway governs how closely the kinetic steady state approaches thermodynamic equilibrium with the protonmotive force. Only at low values of $[S]_i$, when $D[S]_i$ becomes negligible, can the contribution of the leak pathway to the steady state be ignored and will the accumulation of β -galactoside approach equilibrium with the protonmotive force.

Clearly the effects on $[S]_i$ of ionophores that decrease ΔpH or $\Delta \Psi$ will reflect the relationship between v_i , v_e and $D[S]_i$ and their response to the components of the protonmotive force. If D were to be zero, then thermodynamic equilibrium would be achieved and manipulation of Δp with ionophores would be reflected directly in $[S]_i$. However, since $D > 0$, then $D[S]_i$ becomes significant in regulating $[S]_i$. Thus although Δp may change and with it the values of v_i and v_e , the effect on $[S]_i$ will depend largely on the value of D . Consequently it is impossible to predict quantitatively the effects of ionophores on $[S]_i$, but it is certain that they will not be the same as their effects on indicators of $\Delta \Psi$ and ΔpH .

It has been proposed that the stoichiometry of the lactose-transport system in *E. coli* is pH-dependent. From studies with membrane vesicles in which the protonmotive force was modulated with ionophores and compared with $[S]_i$, it was concluded that $n = 1$ at $pH_0 5.5$ and $n = 2$ at $pH_0 7.5$ (Ramos & Kaback, 1977b). However, it is clear from the foregoing discussion that this type of experimentation is inadequate, because the underlying assumption that the transported substrate achieves thermo-

dynamic equilibrium with the driving force is incorrect.

In experiments designed to measure n directly we were unable to detect a significant increase in the stoichiometry over the range $pH_0 6.4-7.7$. Furthermore, from our studies a value of $n = 1$ was adequate to explain the magnitude of the lactose accumulation relative to the protonmotive force. One caveat is that the values of $\Delta \Psi$ measured in vesicles are very much lower than those found in cells. It is not yet certain whether this represents a limitation of the technique for estimating $\Delta \Psi$, or a genuine decrease in the membrane potential in these experimental systems. The magnitude of $\Delta \Psi$ in our cells is similar to that found in a range of bacteria (Padan *et al.*, 1976; Michel & Oesterhelt, 1976; Bakker *et al.*, 1976; Collins & Hamilton, 1976; Mitchell *et al.*, 1979; Bakker, 1978). Thus it seems unlikely that it is a significant overestimate.

The involvement of kinetic steady state in the accumulation of other metabolites could also explain the apparently different contributions of $\Delta \Psi$ and ΔpH to their driving force over a range of pH_0 values. For example, uptake of acid anions by protonmotive-force-dependent transport systems has been suggested to be electroneutral at low pH and electrogenic at alkaline pH (Ramos & Kaback, 1977a,b). A theoretical treatment of such systems has also been presented (Rottenberg, 1976). However, it is evident that these systems do not achieve thermodynamic equilibrium with their suggested driving force (see Fig. 4 of Ramos & Kaback, 1977a). If uptake of an anion ($m = -1$) were electrogenic ($n = 2$) at all pH values, then from eqn. (1) the putative driving force would be $\Delta \Psi - 2Z\Delta pH$. At lower values of pH_0 the ΔpH component contributes more significantly to the driving force than does $\Delta \Psi$; at $pH_0 5.9$, 220 mV of a total of 350 mV driving force comes from the pH gradient. Furthermore, when $\Delta \Psi$ is decreased by a valinomycin/ K^+ couple, ΔpH has been shown to increase (Padan *et al.*, 1976; Ramos & Kaback, 1977c; I. R. Booth & W. A. Hamilton, unpublished work). If the accumulation of the anion is maintained around 110 mV by the operation of a leak pathway, then decrease of $\Delta \Psi$ would be expected to have little or no effect on $[S^-]_i$. Thus, because of the operation of a kinetic steady state, $\Delta \Psi$ would appear not to have a role in the transport system at $pH_0 5.9$. At higher pH values, however, where $\Delta \Psi$ is the most significant component of the driving force, decrease of the membrane potential would immediately reveal the electrogenic nature of the transport system through a significant decrease in the observed accumulation of the solute.

Evidence to support this argument is at present scarce. The transport system for 3-deoxy-2-oxo-D-gluconate has been investigated in detail by Lagarde

and co-workers in *E. coli* K12 cells and vesicles (Lagarde & Stoeber, 1974; Lagarde & Haddock, 1977; Lagarde, 1977). The substrates for this transport system achieve a kinetic steady state that, assuming a membrane potential of 160 mV, is always below the driving force. The stoichiometry has been demonstrated to be two protons per acid anion translocated, and transport is electrogenic at all pH values tested (Lagarde, 1977). This transport system is one that was proposed to have a pH-dependent stoichiometry change (Ramos & Kaback, 1977a). As discussed above, however, such a proposal is unnecessary and is based on the false premise of the attainment of a thermodynamic equilibrium.

Glutamate transport in *Staphylococcus aureus* has also been shown to be electrogenic at all pH values tested. The stoichiometry has been deduced to be two protons per glutamate anion (Mitchell *et al.*, 1979).

It is therefore important to recognize the functioning of kinetic steady states and the limitations this imposes on interpretation of certain types of experimentation. Further it is necessary to stress that where accumulation of a substrate is limited by kinetic factors, eqn. (1) cannot be used to predict the stoichiometry and to propose models for the mechanism of energy coupling.

Since this work was completed, we became aware of similar work on the lactose-transport system in *E. coli* (Zilberstein *et al.*, 1979). The work of those authors also leads to the conclusion that the lactose-transport system has a proton/lactose stoichiometry of 1 at both pH₀ 6.4 and pH₀ 7.8.

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