Biochimica et Biophysica Acta 855 (1986) 391–416
Elsevier

BBA 72908

The kinetic mechanism of galactoside / H^+ cotransport in *Escherichia coli*

J. Keith Wright

Max-Planck-Institut für Biologie, Corrensstrasse 38, D-7400 Tübingen (F.R.G.)

(Received August 5th, 1985)

Key words: Galactoside/H^+ cotransport; Kinetics; Facilitated diffusion; Diffusion; (*E. coli*)

To determine the kinetic mechanism of galactoside active transport by the lactose / H^+ cotransporter of *Escherichia coli*, galactoside binding and transport are studied in the absence and presence of $\Delta\tilde{\mu}_{H^+}$. For several reasons, the substrate β-D-galactosyl-1-thio-β-D-galactoside (GalSGal) is preferred over lactose. In the absence of $\Delta\tilde{\mu}_{H^+}$, the cotransporter retains high affinity for GalSGal, and the affinity is the same on both sides of the membrane. At physiological pH, the cotransporter is protonated and the dissociation constant for H^+ may be 50 pM. The cosubstrates bind in a random fashion. An isomerization of the cotransporter corresponding to reorientation of the binding sites is rate-determining. When $\Delta\tilde{\mu}_{H^+}$ is imposed, two reorientations become faster, and one becomes slower. The affinity of the cotransporter for GalSGal on both sides of the membrane is unchanged. The inability of the cotransporter to bring the accumulation of galactoside into equilibrium with $\Delta\tilde{\mu}_{H^+}$ at high galactoside concentrations can be explained without postulating uncoupled fluxes of galactoside or H^+ across the membrane (leaks). The formation of the ternary carrier-H^+-galactoside complex on the cytoplasmic side of the membrane with increasing internal levels of sugar and the rapidity of galactoside exchange inhibit net influx of galactoside and favor exchange. Net transport is slow at high galactoside levels. Thus, the cotransporter can self-regulate transport without uncoupling H^+ and galactoside fluxes. Because the values of $\Delta\tilde{\mu}_{H^+}$ during binding and transport studies were measured, these results can be subjected to a quantitative analysis.

Introduction

Cells are able to concentrate many molecules considerably above the levels in the surrounding medium by virtue of membrane transport systems. Significant progress in identifying how cellular metabolism is coupled to active transport was stimulated by various proposals [1–3] positing that the energy required for the accumulation of some solutes resides in the transmembrane gradients of the electrochemical potential [4] of ions such as H^+ and Na^+. Thus, many transport proteins were envisioned as catalyzing the simultaneous movement of ions and solutes across the membrane.

The galactoside cotransporter of *E. coli* (lactose permease, product of the *lacY* gene) is located in the cytoplasmic membrane and catalyzes the transport of many α- and β-galactosides (cf. Ref. 5). The uptake of galactoside is coupled to the uptake of H^+ [6]. This coupling of the fluxes of H^+ and

Abbreviations: GalSGal, β-D-galactosyl-1-thio-β-D-galactoside; NpαGal, *p*-nitrophenyl α-D-galactoside; DnsEtOGal, 2′-*N*-dansylaminoethyl 1-*O*-β-D-galactoside; $ClHgBzSO_3$, sodium *p*-chlormercuribenzenesulfonate; $ClPhzC(CN)_2$, carbonyl cyanide *m*-chlorophenylhydrazone; Ph_4P^+, tetraphenylphosphonium ion; $\Delta\tilde{\mu}_{H^+}$, the transmembrane gradient of the electrochemical potential of H^+; $\Delta\mu_G$, the transmembrane gradient of the chemical potential of galactoside; k_{cat}, the maximal velocity expressed as a turnover number; K_T, the half-saturation constant for a transport process; Z, 59 mV/pH unit; K_H and K_G, dissociation constants for H^+ and galactoside; k_c and k_o, rate constants for the reorientation of loaded and unloaded cotransporter; * indicates $\Delta\tilde{\mu}_{H^+}$ is not zero.

galactoside across the membrane results in the coupling of the transmembrane gradient of the electrochemical potential of H^+ (in electrical units)

$$\Delta\tilde{\mu}_{H^+} = \Delta\psi - 2.3\frac{RT}{F}\Delta pH \quad (1)$$

to the transmembrane gradient of the chemical potential of galactoside

$$\Delta\mu_G = \frac{RT}{F}\ln\frac{G''}{G'} = -n_H \cdot \Delta\tilde{\mu}_{H^+} \quad (2)$$

where $\Delta\psi$ and ΔpH are the gradients of the electrical potential and pH, G''/G' is the ratio of internal to external galactoside concentrations, and n_H is the cotransport stoichiometry.

Because the cotransport stoichiometry is near 1 under many conditions [6–14], a general scheme (Fig. 1) for H^+/galactoside cotransport includes the binding and release of one galactoside and one H^+ and cotransporter isomerizations allowing the binding sites in the unloaded cotransporter or the fully loaded cotransporter to be presented to either of two compartments. Active transport occurs because the imposition of $\Delta\tilde{\mu}_{H^+}$ evokes changes in the parameters of the transport cycle directly related to the magnitude of the driving free energy. Thus the accumulation of galactoside, $\Delta\tilde{\mu}_{H^+}$, and the values of rate and dissociation constants at a

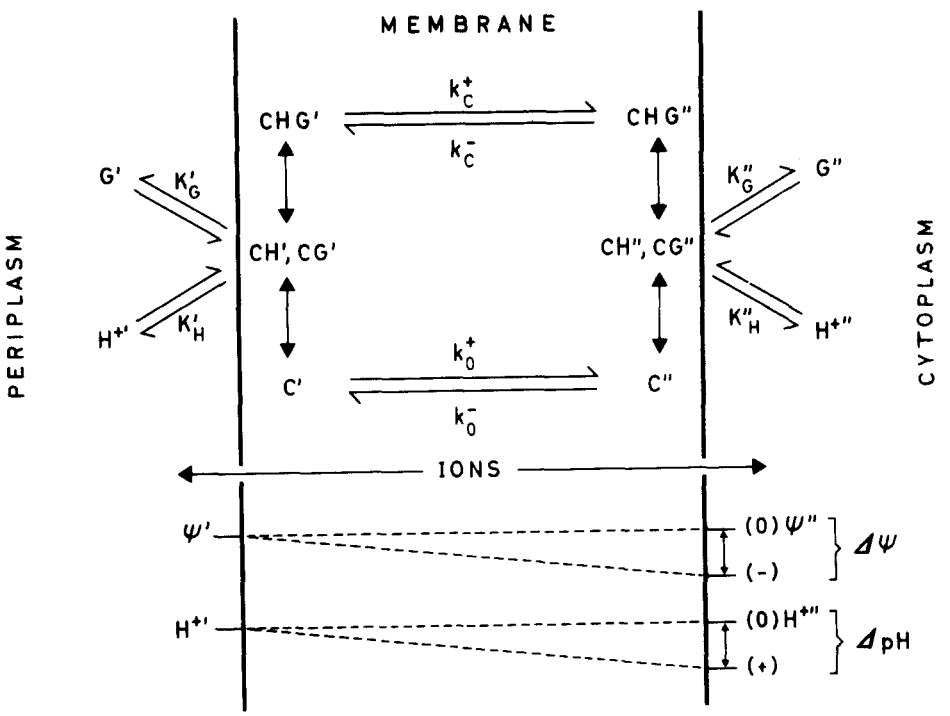


Fig. 1. A galactoside/H^+ cotransporter conveys H^+ and galactoside (G) between an outer compartment (H′ and G′ in periplasm) and an inner compartment (H″ and G″ in cytoplasm). The cotransport cycle consists of two types of step: binding or release of substrate and the reorientation of binding sites, symbolized by the dissociation constants (K'_G and K'_H on the outside and K''_G and K''_H on the inside) and by the rate constants (k_o^+ and the k_o^- for the unloaded cotransporters and k_c^+ and k_c^- for the ternary intermediates), respectively. The conversion of C to CHG may proceed via one or both of the binary complexes CH or CG. The order of substrate binding is not specified here. Net flow of galactoside involved the net flux of H^+. Parallel to this H^+ current, H^+ and many other ions transverse the membrane due to the action of pumps or ionophores, or uncouplers (arrow). In cells or in right-side-out vesicles the internal electrical potential (ψ'') is more negative than the external electrical potential so that the gradient of the electrical potential, $\psi'' - \psi' = \Delta\psi$, is negative. The internal concentration of H^+ (H″) is lower or equal to the external concentration (H′). Because, by convention, the pH gradient is defined as pH″ − pH′, ΔpH is positive or zero.

particular value of $\Delta\tilde{\mu}_{H^+}$ are related by

$$\frac{G''}{G'} = \exp(-F\Delta\tilde{\mu}_{H^+}/RT) = \frac{k_c^+ k_o^-}{k_c^- k_o^+} \cdot \frac{K_G'' K_H''}{K_G' K_H'} \cdot \frac{[H^+]'}{[H^+]''} \quad (3)$$

In fact, galactoside accumulation frequently deviates from the predictions of Eqn. 3. The internal concentration of galactoside in the steady state attains a maximal value (G''_{max}) with increasing external concentrations of galactoside. The accumulation ratio in the steady state depends upon the external concentration of galactoside (G')

$$\frac{G''}{G'} = \frac{G''_{max}}{G' + K_{ss}} \quad (4)$$

where K_{ss} is the half-saturation constant for this process [15]. The accumulation ratio also depends on the number of carriers in the membrane [16,17]. At first glance, this inefficiency is not compatible with a simple thermodynamic interpretation of secondary active transport. Theoretical explanations [18–20] for the disequilibrium between $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_G$ require either that the membrane is passively permeable for galactoside (outer leak) or that the binary complexes CH or CG catalyze uncoupled transport of one cosubstrate (inner leak or slip).

Therefore, a valid description of active transport must explain (a) how the lactose/H^+ cotransporter converts $\Delta\tilde{\mu}_{H^+}$ into $\Delta\mu_G$ under some conditions (Eqn. 3) and (b) why the cotransporter is unable to attain this equilibrium under other conditions (Eqn. 4). The strategy for elucidating the kinetic mechanism of cotransport in the present study is to examine cotransport function at levels of increasing complexity: binding, facilitated diffusion ($\Delta\tilde{\mu}_{H^+} = 0$, $G' = G''$ at equilibrium), and active transport ($\Delta\tilde{\mu}_{H^+} < 0$, $G' < G''$ at equilibrium). There are several reasons for re-examining binding and transport in this already well-studied system.

First, not all crucial experiments have been performed with the same substrate. To compare the results from these experiments with one another, a single galactoside is employed here. Also, in many previous studies the values of $\Delta\tilde{\mu}_{H^+}$ or $\Delta\mu_G$ under the ambient conditions were unknown. Such values are necessary for a quantitative evaluation of the data and are determined here. Additionally, new experiments are presented here, and in repeating earlier experiments different methods of measurement or analysis are used. Importantly, the results presented here point to an alternative for the disequilibrium of Eqn. 4 which does not rely on leak or slip. Furthermore, some previous results cannot be reconciled with one another or are actually contradictory, and results of transport measurements in vesicles and in cells sometimes differ. Here measurements in cells and vesicles are compared. Lastly, the experiments presented here are performed with β-D-galactosyl-1-β-D-thiogalactoside (GalSGal), which for the following reasons is preferred over lactose or most other galactosides.

GalSGal, like lactose, can be accumulated nearly 1000-fold in *E. coli* ML308-225 [13,21]. GalSGal, like lactose, has a negligible passive (non-carrier-mediated) permeability through the cytoplasmic membrane, because GalSGal is a hydrophilic disaccharide. GalSGal, unlike lactose, possesses an affinity for the cotransporter which is virtually independent of the magnitude of $\Delta\tilde{\mu}_{H^+}$. The dissociation constant for GalSGal, K_G, at $\Delta\tilde{\mu}_{H^+} = 0$ is similar to the half-saturation constant for active transport K_T ($\Delta\tilde{\mu}_{H^+} < 0$), whereas the K_G for lactose is about 150-fold larger than the K_T for active transport [13,21,22]. Also, GalSGal has a relatively high affinity for the cotransporter, so that binding can be measured directly and the galactoside binding sites can be saturated at substrate concentrations (less than 1 mM) where $\Delta\tilde{\mu}_{H^+}$ is not affected and cells and vesicles are not subject to osmotic shrinking or swelling.

Materials and Methods

Strains

For binding studies, the cotransporter overproducing *E. coli* T206 [17] was employed. For transport studies, the strain ML308-225 was grown on 4 mg/ml sodium succinate as the carbon source.

Preparation of membrane vesicles

Cytoplasmic vesicles from strain T206 were prepared as described [17]. Everted vesicles were prepared by lysis in a Ribi press operating at 60 MPa. ML308-225 vesicles were prepared according to the method of Short et al. [23] with the exception

that the vesicles were washed only once and then stored over liquid nitrogen. Repeated washings led to loss of transport activity. Vesicles were washed in the appropriate buffer for each experiment after rapid thawing.

Binding of galactosides

Substrate binding was generally measured by flow dialysis as previously described [21]. The pH dependence of GalSGal binding was measured by equilibrium dialysis for added precision. Here, the T206 vesicles in 50 mM potassium hydrogen phosphate, 10 mM sodium azide and 0.1 mM dithiothreitol of the appropriate pH were placed in lucite blocks and dialyzed with rotation overnight at 2°C. The blocks were warmed to 25°C, and the dialysis was continued for 3 h. After duplicate samples had been withdrawn for counting, vesicles were added to the samples from the buffer compartment to achieve equal quenching in all samples. Samples without vesicles exhibited a 17% higher counting yield for ^{3}H. Substrate binding to respiring, everted T206 vesicles (10–13 mg/ml) was also measured by flow dialysis. The binding of 2′-*N*-dansylaminoethyl 1-*O*-β-D-galactoside (DnsEtOGal) was measured fluorimetrically [24].

Facilitated diffusion (influx, efflux, exchange and countertransport at $\Delta\tilde{\mu}_{H^+} = 0$) in ML308-225 vesicles

Thawed vesicles were diluted 1:20 in buffer without $MgSO_4$, centrifuged for 15 min at 12000 × *g*, resuspended, and washed again. Vesicles were finally resuspended at a concentration of 2.5 mg protein/ml and transferred to an Erlenmeyer flask of at least 4-fold greater volume. The suspension was slowly stirred with a magnetic bar as long as the diameter of the flask. A 100 μM solution of gramicidin S from *Bacillus brevis* (Sigma, München, F.R.G.) in 50% ethanol was added dropwise over a period of 2–3 min to a final concentration of 1 μM (0.4 nmol gramicidin S/mg protein or 2 nmol gramicidin S/nmol lactose cotransporter). Vesicles were incubated for 90–120 min on ice to allow equilibration of the buffer across the membrane (0.35 M KCl/20 mM potassium hydrogen phosphate). The suspensions were brought to 25°C, and $MgSO_4$ was added to a final concentration of 1 mM. After centrifugation, the vesicles were suspended in buffer at 10–15 mg/ml. Influx was initiated by the addition of 10 μl 0.1–10 mM [^{3}H]GalSGal (8.6 MBq/ml) to 90 μl vesicles. For equilibrium exchange, vesicles were incubated 1–2 h with GalSGal and exchange was initiated by the addition of 10 μl [^{3}H]GalSGal of the same concentration to 90 μl vesicles. For efflux, vesicles (50 μl, 0.5–1.0 mg protein) preequilibrated with 10 μM–1 mM [^{3}H]GalSGal (1.8 kBq/sample) were rapidly mixed on a vortexer with 4 ml buffer. For countertransport, vesicles were incubated 2 h in 15 mM melibiose or 30 mM lactose and concentrated to 15–20 mg protein/ml by centrifugation. To 3 ml of buffer containing 10 μM–1 mM [^{3}H]GalSGal (0.9 kBq) and 15 or 30 mM glycerol to maintain isosmolarity were added 30 μl vesicles with rapid mixing. The $t = 0$ value was obtained by diluting vesicles into buffer containing 1 mM sodium *p*-chloromercuribenzene sulfonate ($ClHgBzSO_3$) or 2 mM $HgCl_2$. Corrections for passive fluxes of GalSGal were obtained from vesicles treated for 1 h with 5 mM *N*-ethylmaleimide or from vesicles derived from uninduced ML30 ($i^+z^+y^+$). After 3, 5 or 10 s, transport was halted by the addition of stopping buffer (see below).

Facilitated diffusion (influx, efflux, countertransport) in poisoned ML308-225 cells

ML308-225 cells were harvested, washed twice in 0.12 M Tris (pH 8.0) and subjected to the EDTA treatment of Leive [25] for 2 min at 37°C. The EDTA-treated cells were washed twice in 50 mM potassium hydrogen phosphate/2 mM $MgSO_4$ (pH 7.6). For influx, EDTA-treated cells were centrifuged for 10 min at 10000 × *g* and resuspended to about 10 mg protein/ml. To one half of this suspension, NpαGal was added as a solid to a final concentration of 2.2 mM or *N*-ethylmaleimide to a final concentration of 10 mM. To 180 μl of the suspension, 20 μl of 0.10–2.5 mM GalSGal (0.8 MBq/ml) was added, and transport was halted after 2 or 4 s by addition of stopping buffer. The carrier-specific influx of GalSGal was taken as the difference between the samples without and with NpαGal. For efflux, cells were suspended at 5 mg protein/ml, and GalSGal was added to final concentrations of 10 μM–1 mM. After a 20 min incubation, cells were poisoned by the introduc-

tion of 10 μM carbonyl cyanide *m*-chlorophenylhydrazone ($ClPhzC(CN)_2$), 1 mM tetraphenylphosphonium (Ph_4P^+) chloride and 10 mM NaN_3. The cells were collected by centrifugation at 10000 × *g* for 15 min at 20°C. Cells were resuspended at 45–50 mg/ml in the individual supernatants, and [^{3}H]GalSGal (0.15 MBq/ml) was added. To 3 ml 50 mM potassium hydrogen phosphate, 2 mM $MgSO_4$, 1 mM Ph_4PCl, 10 μM $ClPhzC(CN)_2$ (pH 7.6) were added 30 μl of cell suspension. Efflux was stopped after 3 to 20 s by the addition of stopping buffer. The $t = 0$ value was obtained by diluting cells into buffer containing 1 mM $ClHgBzSO_3$; passive efflux was assessed similarly by diluting cells into buffer with 1 mM $ClHgBzSO_3$ and waiting 20 s before filtering. For countertransport, EDTA-treated cells were washed in buffer containing 15 mM melibiose. After a 30 min incubation, the cells were poisoned as described above. Cells were concentrated to approx. 30 mg protein/ml. Countertransport was initiated by diluting 30 μl cells in 3.0 ml 10–250 μM GalSGal containing 22 kBq [^{3}H]GalSGal/ml in the above buffer lacking melibiose. Countertransport was stopped at the indicated times as for efflux measurements. All measurements were conducted at 25°C.

Active transport in ML308-225 vesicles and cells

Samples, 0.18 ml vesicles (2.5 mg protein/ml), were incubated with the indicated respiratory substrate in the presence of O_2 for 2 min before the addition of 20 μl 0.1–10 mM GalSGal containing 3.7 kBq [^{3}H]GalSGal/ml. Uptake was halted at 3, 5 or 10 s by addition of stopping buffer.

For EDTA-treated cells, a 0.9 ml suspension containing 0.8–1.0 mg protein/ml in the indicated buffer was aerated for 2 min in the presence of 10 mM sodium succinate. Transport was initiated by the addition of 100 μl 0.1–10 mM GalSGal containing 0.8 kBq [^{3}H]GalSGal/ml and halted at 3, 5, 10 or 15 s by the addition of stopping buffer. When active transport with pH was measured, 10 μl 3 mM $Ph_4P^+Cl^-$ was added to the cell suspension 3 min before aeration was commenced.

Stopping buffer

Stopping buffer had the same composition as the individual buffers, except that 2 mM $HgCl_2$ was present instead of $MgSO_4$, and metabolic poisons were absent. In the case of countertransport, KCl or glycerol were added to the stopping buffer to compensate for the high concentration of lactose or melibiose in the cells or vesicles. Transport was halted by rapidly mixing 5 ml stopping buffer with the sample. Vesicles or cells were collected on 25 mm diameter VG-6 glass-fiber filters (Schleicher & Schüll, Dassel, F.R.G.), the sample vessel was rinsed, and this solution added to the filter. Finally, the filter was washed twice with 5 ml stopping buffer.

Influx and influx during exchange in the presence of $\Delta\tilde{\mu}_{H^+}$

EDTA-treated ML308-225 cells were washed twice in 50 mM potassium hydrogen phosphate/2 mM $MgSO_4$/20 $\mu g \cdot ml^{-1}$ chloramphenicol (pH 6.0) and resuspended in this buffer at 12 mg protein/ml. The suspension was divided into three samples: 6.00 ml for uptake in unloaded cells; 6.00 ml + 0.66 ml 100 mM *N*-ethylmaleimide to inactivate the cotransporter and thus to correct for passive diffusion; and 18.00 ml + 0.18 ml 27.5 mM GalSGal to preload cells with galactoside. The last sample was divided further into two portions, one of which contained 0.74 MBq [^{3}H]GalSGal to monitor the intracellular level of GalSGal. Sodium succinate was added to all suspensions to a final concentration of 10 mM, and the cells were incubated 30 min. During this time, the extracellular concentration of GalSGal in the samples with galactoside sank to 58 μM and the intracellular level rose to about 3 mM. To measure influx in unloaded cells, 100 μl cells were mixed with 0.90 ml GalSGal to yield final extracellular concentrations of 15 or 96 μM containing additionally 3.7 kBq [^{3}H]GalSGal/ml. Samples were incubated for 0–120 s, and uptake was halted by stopping buffer (see above). Identical measurements were made with the *N*-ethylmaleimide-treated cells. Next, 100 μl cells preloaded with unlabelled GalSGal were diluted into 0.90 ml [^{3}H]GalSGal to yield a final, net extracellular concentration of 15 or 96 μM GalSGal, and the uptake was stopped between 0 and 120 s as above. Complementary to this measurement, the intracellular level of GalSGal was monitored under identical conditions. Cells preloaded with [^{3}H]GalSGal (100 μl)

were diluted into 0.90 ml unlabelled GalSGal to yield a final extracellular concentration of 15 or 96 μM. The sample was mixed with stopping buffer between 0 and 120 s.

Efflux and efflux during exchange in the presence of $\Delta\tilde{\mu}_{H^+}$

EDTA-treated ML308-225 cells were washed twice in 50 mM potassium hydrogen phosphate/2 mM $MgSO_4$/20 $\mu g \cdot ml^{-1}$ chloramphenicol (pH 5.5 or pH 7.5). Cells were resuspended at 11 mg protein/ml and kept at room temperature. Flow dialysis experiments (see below) under conditions described here disclosed that the steady state of GalSGal accumulation was attained within 12 min and was stable for an additional 11 min. To prepare for the efflux measurements, 10 μl 0.1–10 mM GalSGal, 10 μl 1 M sodium succinate (pH 7), and 0.15 MBq [^{3}H]GalSGal were added to 1.00 ml cell suspension. The suspension was placed in a 25°C water bath and was stirred by an obliquely standing magnetic bar under a gentle stream of H_2O-saturated air. After 12 min, steady-state intracellular levels between 10 μM and 1 mM GalSGal were achieved. Cells were withdrawn from the suspension only immediately before use and were never kept in the pipette for more than a few seconds to avoid anaerobiosis. Efflux was initiated by diluting 50 μl loaded cells in 5 ml 50 mM potassium hydrogen phosphate/2 mM $MgSO_4$ (pH 5.5 or 7.5). Efflux during exchange was measured by diluting 50 μl cells into buffer containing 0.90 mM GalSGal. Efflux was halted after 4, 7 or 10 s by addition of stopping buffer. The samples for $t = 0$ were obtained by directly diluting the cells into stopping buffer. The only feasible correction for passive efflux was obtained by diluting loaded cells into stopping buffer and waiting 20 s before filtering. Linearly interpolating between $t = 0$ and $t = 20$ s yielded values between 2 and 11% of the measured efflux velocities. Finally, the constancy of the intracellular GalSGal during measurements on a single sample was assessed by repeating the $t = 0$ measurement at the end of each series. The average initial concentration was taken as the average of the first and last $t = 0$ samples. The average variation of each of these samples was random and amounted to 1.7% from the mean.

Retarded efflux (trans effect of GalSGal)

EDTA-treated ML308-225 cells were washed in 50 mM potassium hydrogen phosphate/2 mM $MgSO_4$ (pH 7.6) and resuspended in this buffer at 5 mg protein/ml. Samples were made 1 or 20 mM in GalSGal and allowed to stand 60 min at 20°C. Cells were poisoned with 20 μM $ClPhzC(CN)_2$, 10 mM NaN_3 and 1 mM Ph_4PCl and were allowed to stand an additional 3 h. Samples were centrifuged for 15 min at $12000 \times g$ at 20°C, resuspended at 50 mg protein/ml in the supernatants and [^{3}H]GalSGal was added (0.15 MBq/ml). Efflux was initiated by diluting 50 μl cells in 3.00 ml buffer containing the indicated poisons.

Measurement of $\Delta\tilde{\mu}_{H^+}$ *and* $\Delta\mu_G$

The magnitudes of $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_G$ were determined by flow dialysis [9]. In cells and vesicles, $\Delta\psi$ was determined by the distribution of [^{3}H]Ph_4P^+ (0.6 μM, 55 kBq/ml) and ΔpH by the distribution of [1-^{14}C]butanoic acid (9.6 μM, 18 kBq/ml); $\Delta\mu_G$ was measured by the uptake of [6,6′-$^3H_2$]GalSGal (1 μM and 0.9 mM, 37 kBq/ml). In everted vesicles [26,27] $\Delta\psi$ was measured by the distribution of [^{35}S]thiocyanate (40–120 kBq/ml), ΔpH by the distribution of [^{14}C]methylamine (4.5 μM, 27 kBq/ml). In the presence of KCl, ΔpH in everted vesicles was also demonstrated qualitatively by the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine [28]. [^{3}H]Ph_4PBr was obtained from the Nuclear Research Center (Negev, Israel). GalSGal and NpαGal were labelled as described [29]. Other radiochemicals were obtained from New England Nuclear (Dreieich, F.R.G.). In each case, the ambient conditions correspond exactly to those of the transport or binding measurement with respect to aeration, concentration of substrates, and amounts of cells or vesicles.

Evaluation of results

Kinetic and bioenergetic data were analyzed as indicated in the references cited. The values for the internal volume used for calculations were: 2.2 μl per mg vesicle protein for right-side-out vesicles [30], 1.1 μl per mg vesicle protein for inside-out vesicles [27], and 5 μl per mg total cell protein for cells [8]. For the calculation of turnover numbers for transport in ML308-225 vesicles, a cotrans-

porter level of 0.2 nmol per mg vesicle protein was used [24]. Because 15% of the total cell protein is found in such preparations, a value of 0.03 nmol cotransporter per mg total cell protein for ML308-225 cells grown on succinate as the carbon source was employed. All bioenergetic data are reported in units of millivolts.

Results

Binding of GalSGal to the cotransporter ($\Delta\tilde{\mu}_{H^+} = 0$) in cytoplasmic membrane vesicles from strain T206: the dissociation constant is a reference value

The purpose of measuring substrate binding to an ion/solute cotransporter is to measure the affinity of the protein for a substrate, to determine whether the binding of one cosubstrate affects the binding of the other, and to obtain the number of cotransporters accessible to a substrate. Subsequently, the effect of $\Delta\tilde{\mu}_{H^+}$ upon these parameters can be determined.

Cytoplasmic membranes from the cotransporter overproducer *E. coli* T206 contain 3.4 nmol of binding sites/mg protein, and the dissociation constant for GalSGal is 69 μM at pH 7.6 (Table I). The Hill coefficient is 0.98, implying that only a single class of sites with respect to affinity is occupied. A similar affinity for GalSGal had been determined in previous studies [22,29]. Raising the concentration of H^+ 125-fold alters neither the affinity for GalSGal nor the number of accessible sites (Table I).

In conclusion, H^+ cannot affect the affinity of the cotransporter for GalSGal at physiological pH. This is consonant with the finding that GalSGal

TABLE I

BINDING OF GALACTOSIDES TO THE LACTOSE CARRIER IN VESICLES FROM STRAIN T206

Vesicle preparation	pH	Galactoside	K_G (μM)	Binding sites (nmol/mg protein)	Addition	$\Delta\psi$ (mV)	$-Z\Delta$pH (mV)
Cytoplasmic vesicles	5.5	GalSGal	87	3.7		0	0
	6.6	GalSGal	73	3.5		0	0
	7.6	GalSGal	69	3.4		0	0
Everted vesicles	5.5	NpαGal	26	1.4	–	0	0
					ATP [a]	48 [c]	38 [c]
					PMS/ascorbate [b]	76	29
	6.6	NpαGal	13	1.5	–	0	0
					ATP [a]	53	69
					PMS/ascorbate [b]	77	51
	7.6	NpαGal	19	1.8	–	0	0
					ATP [a]	57	74
					PMS/ascorbate [b]	73	70
	6.6	GalSGal	70	1.7	–	0	0
					ATP [a]	49 [d]	67 [d]
					PMS/ascorbate [b]	87	43
	6.6	DnsEtOGal	38	(100%) [e]	–	0	0
			38	(100%) [e]	10 mM D-lactate	+ [f]	+ [f]

[a] 5 mM.
[b] 0.2 and 10 mM, respectively.
[c] Measured in the presence of 40 μM NpαGal.
[d] Measured in the presence of 50 μM GalSGal.
[e] Relative specific fluorescence (see Ref. 24).
[f] Dye quenching in the presence and absence of 0.3 M KCl (see Ref. 28).

binding to the cotransporter does not evoke H^+ binding or release near neutral pH [21]. The magnitude of the observed dissociation constant (76 μM) provides a reference for the interpretation of transport constants in subsequent experiments.

Binding of galactosides to everted vesicles from strain T206 in the presence of $\Delta\tilde{\mu}_{H^+}$: estimation of $^*K''_G$

The purpose of these experiments is to determine whether the imposition of $\Delta\tilde{\mu}_{H^+}$ increases the dissociation constant for galactoside on the cytoplasmic side of the membrane ($^*K''_G$, Fig. 1 and Eqn. 3) or changes the number of carriers accessible to galactoside. Such changes could be detected as follows. Everted vesicles are prepared from strain T206 by high-pressure lysis. Such preparations present the cytoplasmic face of the cotransporter on the external surface of the vesicle (VI in Ref. 31), develop a $\Delta\tilde{\mu}_{H^+}$ of polarity opposite to that in the cell in the presence of MgATP or phenazine methosulfate plus ascorbate and O_2 (cf. Refs. 26,27), and contain 1.4 ± 0.4 nmol cotransporter per mg vesicle protein [21]. The lower specific activity reflects the presence of contaminating outer membrane absent in the preparation described in the previous section. If upon imposition of $\Delta\tilde{\mu}_{H^+}$ the affinity of the cotransporter for GalSGal or NpαGal decreases ($^*K''_G$), bound galactoside will be released from the cytoplasmic (here external) face of the protein.

A typical set of experiments appears in Fig. 2. The everted vesicles are incubated with thiocyanate to measure $\Delta\psi$ or methylamine to measure ΔpH. After several minutes MgATP is added (Fig. 2, arrow number 1), and the redistribution of labelled thiocyanate and methylamine across the cytoplasmic membrane as measured by the level of radioactivity in the effluent from the flow dialysis indicates the production of a reversed $\Delta\tilde{\mu}_{H^+}$. Subsequently, phenazine methosulfate plus ascorbate is added and a somewhat larger $\Delta\tilde{\mu}_{H^+}$ is produced (Fig. 2, arrow 2, Table I). Significantly, the levels of bound galactoside remain unchanged (Fig. 2, arrows number 1 and 2) as indicated by the unchanged levels of radioactive galactoside in the effluent. Addition of $ClPhzC(CN)_2$ collapses $\Delta\tilde{\mu}_{H^+}$, and $ClHgBzSO_3$ releases bound galactoside (Fig. 2, arrow number 3). By repeating these measurements at different galactoside concentrations,

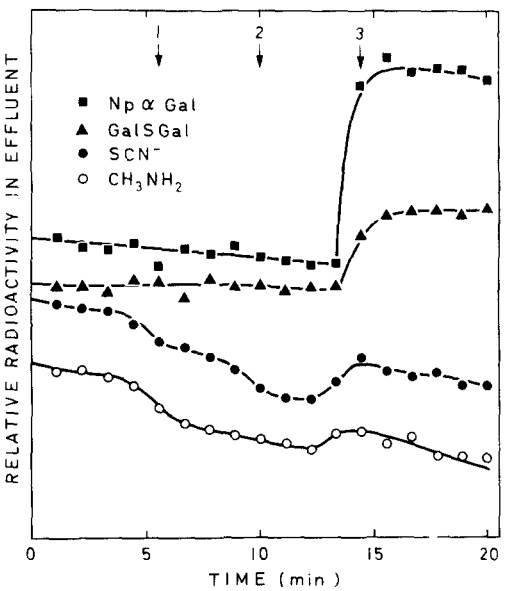


Fig. 2. The effect of $\Delta\tilde{\mu}_{H^+}$ on the affinity of the cotransporter for galactoside on the cytoplasmic side of the membrane can be measured by flow dialysis. Everted vesicles from the carrier-overproducing strain T206 are incubated with radioactive NpαGal (■) or GalSGal (▲) in the absence of $\Delta\tilde{\mu}_{H^+}$. When 5 mM Mg ATP (arrow 1) is added, $\Delta\psi$ and ΔpH of reversed polarity are formed, as seen from the uptake of thiocyanate (●) and methylamine (○). Addition of 10 mM sodium ascorbate in the presence of 0.2 mM phenazine methosulfate and O_2 (arrow 2) causes a slight increase in the reverse $\Delta\tilde{\mu}_{H^+}$. Addition of 0.4 mM $ClHgBzSO_3$ causes galactosides to be released from the cotransporter, and addition of 10 μM uncoupler $ClPhzC(CN)_2$ collapses $\Delta\tilde{\mu}_{H^+}$ (arrow 3). The vesicle concentration is 13 mg protein/ml.

the dissociation constant and number of binding sites are determined (Table I). Because NpαGal has a higher affinity for the cotransporter, these measurements are more accurate than those for GalSGal. Neither the imposition of a reversed $\Delta\tilde{\mu}_{H^+}$ nor variation of the external bulk pH between 5.5 and 7.6 alters the observed, overall dissociation constant (presumably $^*K''_G$) or the number of binding sites (Table I).

Because the time resolution of the flow dialysis experiments is not high (Fig. 2), this experiment is repeated under conditions where the binding of the substrate DnsEtOGal can be monitored continuously by its fluorescence (cf. Ref. 24). When a reversed $\Delta\tilde{\mu}_{H^+}$ is imposed by the addition of D-lactate in the presence of O_2, no change in substrate binding can be observed at the time resolu-

tion (2–5 s) of this experiment. Both the dissociation constant and the maximal specific fluorescence, a qualitative measure of the number of binding sites, are unchanged in the presence of a reverse $\Delta\tilde{\mu}_{H^+}$ (Table I). The presence of $\Delta\tilde{\mu}_{H^+}$ in this experiment is assessed by a fluorescence probe (Ref. 28; Table I).

In conclusion, the data on the binding of galactoside to everted vesicles from strain T206 in the presence of $\Delta\tilde{\mu}_{H^+}$ suggest that the imposition neither of $\Delta\psi$ nor of ΔpH results in a decrease in galactoside affinity for the cotransporter on the cytoplasmic side of the membrane ($^*K_G''$, K_G'' in Fig. 1 in the presence of $\Delta\tilde{\mu}_{H^+}$). Also, the number of cotransporters accessible to galactoside does not change. Lastly, there is only one class of identical binding sites in the presence and absence of $\Delta\tilde{\mu}_{H^+}$. This point is at variance with the views of Robertson et al. [32] and Ghazi and Schechter [33], but in accord with those of Winkler and Wilson [34].

The effects of $\Delta\tilde{\mu}_{H^+}$ on the transport cycle must be found elsewhere than at the level of galactoside binding. Therefore, galactoside transport in the absence and presence of $\Delta\tilde{\mu}_{H^+}$ should be compared to discover what steps are altered by the imposition of $\Delta\tilde{\mu}_{H^+}$.

Facilitated diffusion of GalSGal in right-side-out vesicles from strain ML308-225: rate of translocation in the absence of $\Delta\tilde{\mu}_{H^+}$ and the order of cosubstrate binding

The purpose of these experiments is to characterize the kinetics of cotransport when $\Delta\tilde{\mu}_{H^+}$ is zero and the effects of varying the concentration of the cosubstrate H^+. In the absence of $\Delta\tilde{\mu}_{H^+}$, the cotransporter mediates only the equilibration of galactoside across the membrane (cf. Ref. 35), but measurements indicate that the fluxes of galactoside and H^+ remain coupled [36–38]. Thus, the kinetics of facilitated diffusion can be interpreted by the same rules valid for soluble enzymes with two substrates such as kinases and dehydrogenases.

Here, inward and outward fluxes of galactosides are measured as a function of $[G]'$, $[G]''$, and $[H^+]'$ and $[H^+]''$ (ΔpH = 0). From such studies useful information can be obtained: the order of cosubstrate binding, the slowest (i.e., rate-determining) step(s), the functional symmetry of the cotransporter, and the apparent affinity of the cotransporter for H^+.

The rigorous interpretation of these data requires that true initial rates be measured and that the counterpotential arising from a net flow of H^+ be rapidly and completely dissipated. To avoid the growth of a counterpotential during cotransport, the membrane must be made permeable to ions without increasing the passive permeability for galactosides.

Use of gramicidin S as an ionophore

When right-side-out ML308-225 vesicles (Ref. 23; III in Ref. 31) loaded with 1 mM GalSGal are diluted 100-fold, efflux of galactoside is rapid with a half-time of 10–15 s (Fig. 3, left panel, filled circles). Inactivation of the cotransporter with $HgCl_2$ demonstrates that after a small, rapid loss of substrate initially (cf. Ref. 35) the passive efflux of GalSGal exhibits a half-time much longer than 125 s (Fig. 3, left panel, open squares). If the vesicle suspension and diluent contain 10 μM $ClPhzC(CN)_2$, a weak-acid protonophore, the time-course of efflux is the same (Fig. 3, left panel, open and closed circles; cf. Ref. 35).

If vesicles are pretreated with the ionophore gramicidin S (*cyclo*-(Val-Orn-Leu-DPhe-Pro)$_2$ [39]), the efflux of GalSGal is stimulated, the half-time decreasing to 2–3 s (Fig. 3, left panel, crosses). Gramicidin S is thus more effective than $ClPhzC(CN)_2$. If, in addition to phosphate, chloride is present, a stimulation of GalSGal efflux from untreated vesicles is discernible (Fig. 3, right panel, closed circles vs. left panel, closed circles). Passive or mediated fluxes of chloride are capable of reducing the counterpotential due to cotransport more effectively than $ClPhzC(CN)_2$. In the presence of KCl, gramicidin S still notably stimulates efflux (Fig. 3, right panel, crosses). Importantly, carrier-lacking vesicles from the uninduced strain ML30 ($i^+y^+z^+$), passively loaded with GalSGal, or ML308-225 vesicles loaded with GalSGal and treated with $HgCl_2$ to inhibit cotransporter-mediated GalSGal efflux do not exhibit an increased passive permeability to GalSGal as a result of pretreatment with gramicidin S (Fig. 3, right panel, open triangles and squares).

Therefore, to measure the facilitated diffusion

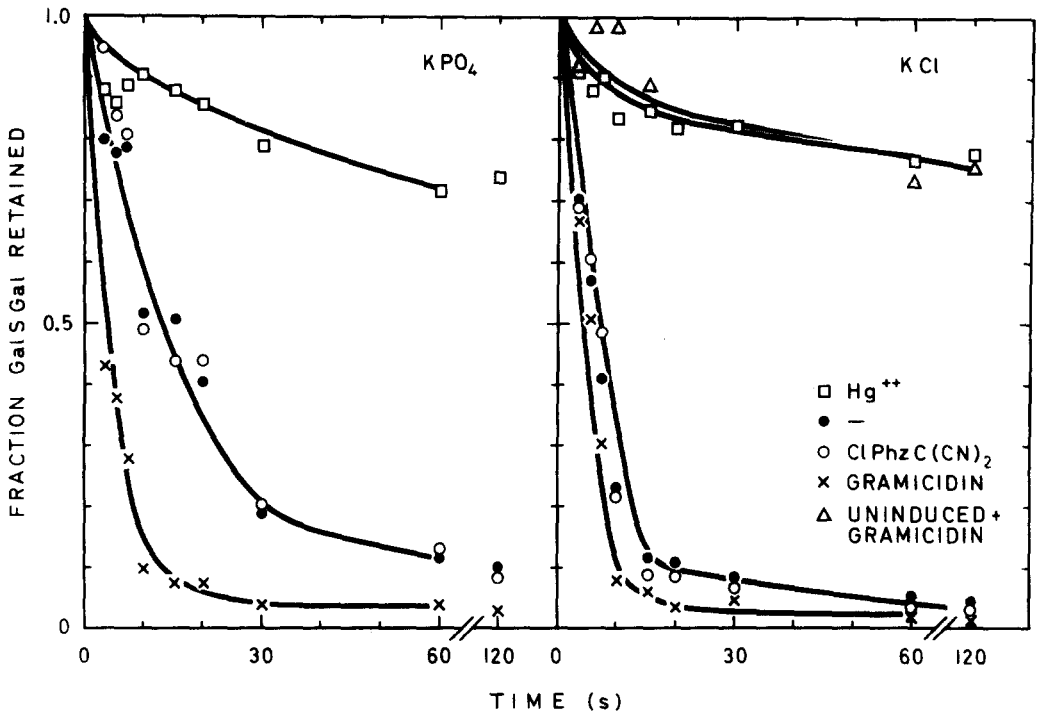


Fig. 3. To measure facilitated diffusion, the movement of galactosides must be studied in the presence of ionophores. Right-side-out ML308-225 vesicles are washed in 50 mM potassium hydrogen phosphate (pH 6.6) (left panel) or in 0.35 M KCl/20 mM potassium hydrogen phosphate (pH 6.6) (right panel) and loaded with 1 mM [^{3}H]GalSGal. Vesicles are diluted 100-fold, and efflux is stopped at the indicated times. Vesicles are untreated (●), treated with 10 μM ClPhzC(CN)$_2$ which is also present in the diluent (○), pretreated with 0.4 nmol gramicidin S/mg protein (×), or treated with 2 mM $HgCl_2$ (□). As a control, vesicles from ML30, not induced for the synthesis of the lactose/H$^+$ cotransporter, are pretreated with gramicidin S and passively loaded with [^{3}H]GalSGal (△).

TABLE II

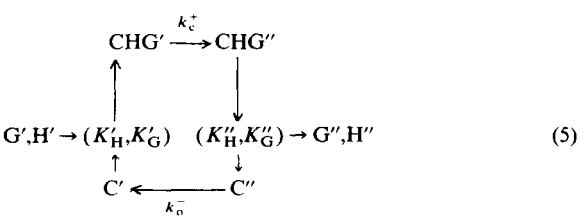
GalSGal FACILITATED DIFFUSION IN VESICLES AND POISONED CELLS (ML308-225)

Mode of facilitated diffusion	pH	K_T (μM)	k_{cat} (s^{-1})	
Vesicles				
Influx	5.5	115	0.6	Vesicles treated with gramicidin S, measure-
	6.6	108	0.5	ments performed in the presence of 0.35 M KCl
	7.6	118	0.8	
Efflux	6.6	140	0.4	
Exchange	5.5	149	2.2	
	6.6	103	2.9	
	7.6	172	2.5	
Countertransport	5.5	46	1.3	Vesicles loaded with 30 mM lactose
	5.5	50	1.4	Vesicles loaded with 15 mM melibiose
	7.6	54	1.1	Vesicles loaded with 30 mM lactose
	7.6	67	1.2	Vesicles loaded with 15 mM melibiose
Cells				
Influx	7.6	118	5.9	EDTA-treated cells poisoned with ClPhzC(CN)$_2$,
Efflux	7.6	217	14.7	NaN_3, and Ph_4PCl
Countertransport	5.5	44	12.1	Cells loaded with 15 mM melibiose
	7.6	63	14.0	Cells loaded with 15 mM melibiose

of GalSGal, vesicles are pretreated with 0.4 nmol gramicidin S per mg vesicle protein, and the buffer contains 0.35 M KCl (Fig. 3, right panel, crosses). Under these conditions no detectable $\Delta\tilde{\mu}_{H^+}$ is generated by such vesicles in the presence of phenazine methosulfate, sodium ascorbate, and O_2 by flow dialysis (data not shown). Furthermore, the above observations indicate (i) that $ClPhzC(CN)_2$ does not dissipate small values of $\Delta\tilde{\mu}_{H^+}$ rapidly enough and (ii) that vesicles must evince a small, non-negligible permeability for some ions, otherwise hardly any GalSGal efflux should occur due to the formation of a counterpotential in the absence of ionophores.

Influx

The catalytic cycle for net influx is



(5)

where the binding and release of substrates are indicated by the dissociation constants and translocation steps by the appropriate rate constants. GalSGal influx into gramicidin-S-treated vesicles from strain ML308-225 at pH 6.6 is characterized by a maximal velocity, expressed here as the turnover number, k_{cat}, of 0.5 cycles/s and a K_T of 108 μM (Table II). The value of K_T is similar to the dissociation constant for GalSGal, suggesting that this kinetic constant is primarily determined by the value of the thermodynamic quantity, K'_G.

Efflux

The efflux scheme resembles that of Eqn. 5 in reverse. The parameters for GalSGal efflux from gramicidin-treated vesicles at pH 6.6 are similar to those for influx: $k_{cat} = 0.4\ s^{-1}$, $K_T = 140\ \mu M$ (Table II). The determination of these values is less certain than those for influx because the intravesicular concentration of GalSGal changes rapidly (cf. Fig. 3) during the course of the measurement.

In conclusion, the similarity of the values of K_T for influx and efflux to K_G (Table I) indicates that the binding of GalSGal to the cotransporter is symmetrical in terms of affinity ($K'_G = K''_G$). Moreover, the similarity of k_{cat} for influx and efflux is consistent with the similarity of the magnitude of the rate-determining step(s) for the two cycles as well as suggesting that the occupancy of the proton binding site is similar in both cases.

H^+ is substrate and product

The purpose of measuring the pH dependence of influx is to assess how the cosubstrate H^+ interacts with the cotransporter. Inspection of the catalytic cycle for influx indicates that H^+ can bind to the cotransporter in two ways: on the external face to form CH′ or CHG′ and on the internal face to form CH″ or CHG″. External H^+ is an activator of influx because the rate of transport increases as CHG′ increases. Internal H^+, a product of influx, is an inhibitor, because the formation of CH″ prevents the completion of the cycle for net influx. Therefore, the pH dependence of influx when ΔpH is zero ($[H^+]' = [H^+]''$) could be complex, because the concentrations of a substrate (H′) and of a product (H″) are varied simultaneously.

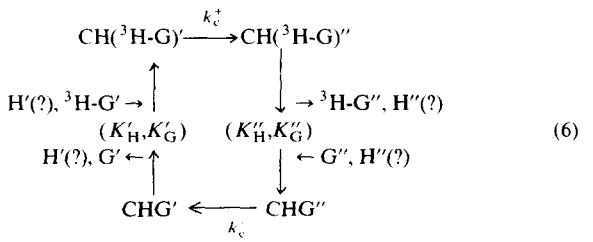
The k_{cat} for facilitated diffusion in the influx mode is only moderately dependent on the pH between pH 5.5 and pH 7.7, representing a 160-fold change in $[H^+]$ (Table II). At pH 7.7, k_{cat} is 0.8 s^{-1} and K_T is 118 μM, whilst at pH 5.5 k_{cat} is 0.6 s^{-1} and K_T is 115 μM. If the concentration of H^+ were subsaturating, then a 10-fold increase in its concentration should evoke a 10-fold change in the reaction velocity.

In conclusion, the relative invariance of k_{cat} for influx with pH suggests that $[H^+]'$ is saturating, i.e., $K'_H < 10^{-7.6}$. The relative invariance of k_{cat} with varying product concentration $[H^+]''$ suggests that H″ does not interact with the cotransporter under these conditions ($[H^+]' = [H^+]''$; $[G]'' = 0$). In kinetic terms, the conclusion is that the binary complex [CH]″ is not a significant intermediate in the influx cycle. One possible explanation would be that product release is ordered (H^+ off first, G second). Because galactoside is not present in the interior of the vesicles initially, internal H^+ would be unable to bind to the cotransporter. The invariance of efflux in the physiological range of pH [35]

suggests the same conclusion for that transport mode. However, as noted below, the absence of product inhibition by H^+ in influx and efflux is still not satisfactorily understood.

Exchange

In contradistinction to influx and efflux, no net fluxes of either galactoside or H^+ occur during equilibrium exchange. In these experiments, the unidirectional, inward flux of [^{3}H]GalSGal is measured as a function of galactoside and H^+ concentration. At saturating galactoside concentrations, carriers reorient exclusively as ternary complexes CHG′ and CHG″ according to the scheme:



(6)

whereby the cotransported proton must not of necessity also be exchanged. The principle kinetic difference in comparison to influx or efflux is that the translocation of the unloaded cotransporter (k_o steps in Fig. 1) does not determine k_{cat} and that the binding of H^+ to the cotransporter on both sides of the membrane should increase the rate of exchange by increasing CHG′ and CHG″.

As with influx, the K_T values for equilibrium exchange do not exhibit a pronounced pH dependence (range K_T = 103–172 μM; Table II). Marked is, however, the higher value k_{cat} for exchange (range 2.2–2.9 s^{-1}) as compared to influx. On the average, the maximal velocity of equilibrium exchange in vesicles is 4-fold faster than that of influx at the same pH. One of several possible explanations is that the ternary complexes reorient more rapidly than unloaded cotransporter ($k_c > k_o$) in vesicles.

The second important observation is that k_{cat} is not strongly pH-dependent. As exchange represents inward and outward galactoside fluxes via the ternary complexes CHG, both H′ and H″ can be regarded as substrates for this cycle, in contrast to the case for influx or for efflux. The value of k_{cat} represents the turnover number at saturating galactoside concentration: with increasing H^+ concentration the value of k_{cat} will increase until the proton binding site is also saturated. The constancy of k_{cat} for exchange implies that the effective pK for the symported proton is greater than 7.7.

Countertransport

The purpose of measuring countertransport is to examine exchange under conditions where the determination of initial velocities is more accurate than in equilibrium exchange. The transient accumulation of labelled galactoside occasioned by loading vesicles with saturating levels of an unlabelled substrate, prolongs the period in which true initial rates can be measured (see Methods).

Countertransport is measured in gramicidin-S-treated vesicles in the presence of buffered KCl, after loading with 30 mM lactose (about $2 \times K_G$) or 15 mM melibiose ($30 \times K_G$, [21]). Melibiose, like GalSGal, is a simple substrate with K_G nearly equal to K_T for active transport. The k_{cat} for GalSGal countertransport with melibiose or lactose does not vary between pH 5.5 and 7.6 and is somewhat smaller than the value measured for equilibrium exchange (Table II). The values for K_T are smaller than the values for equilibrium exchange, most likely due to a more accurate assessment of the initial rate of counterflow. Significantly, these values are similar to the observed dissociation constant K_G for GalSGal (Table I) and are independent of pH.

As indicated in the case of equilibrium exchange, the pH independence of k_{cat} suggests that the pK of the cotransported proton must be greater than 7.6, otherwise k_{cat} should increase with decreasing pH due to the further occupation of the H^+ substrate binding site and increase in the concentration of the CHG′ and CHG″.

Facilitated diffusion in poisoned ML308-225 cells: rapid rates pose problems

The purpose of examining facilitated diffusion in cells is three-fold. Previous studies indicated that the turnover number for active transport in cells is 10-fold higher than in vesicles [14,21]. Here a comparison is made in the absence of $\Delta\tilde{\mu}_{H^+}$. Also, the measurement of facilitated diffusion in

cells forms a necessary adjunct to the study of active transport in cells. Lastly, many transport measurements have been performed in poisoned cells, mostly with lactose-like substrates. The present study yields information on the facilitated diffusion of GalSGal which may be more readily interpreted for the reasons mentioned in the Introduction.

Collapsing $\Delta\tilde{\mu}_{H^+}$

In contrast to the case with vesicles, the complete collapse of $\Delta\tilde{\mu}_{H^+}$ in cells is very difficult under conditions in which the passive permeability of the membrane does not drastically increase (Ahmed, S. and Wright, J.K., unpublished data). In the presence of 10 μM $ClPhzC(CN)_2$, $\Delta\tilde{\mu}_{H^+}$ is collapsed at least to -20 to -30 mV. Cells so treated are further poisoned with 10 mM NaN_3, and the mobile, lipophilic cation tetraphenylphosphonium Ph_4P^+ is added at 1 mM to reduce $\Delta\psi$ further (cf. Ref. 33). While $\Delta\tilde{\mu}_{H^+}$ in cells cannot be measured by flow dialysis under these conditions, $\Delta\tilde{\mu}_{H^+}$ might not be exactly zero.

Influx

When [^{3}H]GalSGal is added to a final concentration of 50 μM, comparable to the observed dissociation constant at $\Delta\tilde{\mu}_{H^+}=0$ (Table I) to a suspension of poisoned cells (29 mg cell protein/ml) and transport is halted after 10 s, the calculated accumulation ratio is 1.6. This corresponds to a value of $\Delta\mu_G$ of 12 mV. This ratio remains constant for measurements up to 15 min, indicating that this is the equilibrium value under these conditions. Influx at different concentrations of GalSGal is halted after 2 or 4 s. The reciprocals of the measured rates at 2 or 4 s are plotted against the reciprocal of the GalSGal concentration (Fig. 4). Obviously, the two curves do not superimpose, conclusively demonstrating that true initial rates are not measured. Following the example of Page and West [36], better estimates of the true initial rates are obtained by extrapolating plots of log (observed rate) vs. time to $t = 0$. Analysis of these rates in the double-reciprocal plot yields a k_{cat} of 5.9 s^{-1}, and K_T is 118 μM (Fig. 4, open circles; Table II), a value similar to the dissociation constant (Table I). The value of k_{cat} is much higher than that observed for influx into vesicles (Table II), a discrepancy similar to that for active transport [14,21]. The reason for this difference is at present unknown. Also, the extrapolation of rates to zero time is probably responsible for the difference in the conclusions here and those in Ref. 33.

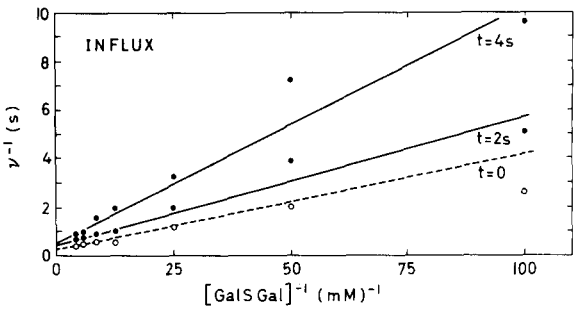


Fig. 4. Poisoned, EDTA-treated ML308-225 cells exhibit rapid influx of GalSGal. To quantitate this uptake, GalSGal entry is halted after 2 or 4 s at different concentrations (●). The apparent initial velocities are extrapolated [36] to zero time (○). The parameters for influx at pH 7.6 are a turnover number of 5.9 s^{-1} and a half-saturation constant, K_T, of 118 μM.

Efflux

The measurement of facilitated diffusion in poisoned cells is beset by a similar problem caused by the sheer rapidity of transport. During the course of efflux, the internal concentration of GalSGal decreases by up to 90% after 5 to 10 s. The time-courses of efflux at different initial GalSGal concentrations are evaluated by an integrated rate equation (Fig. 5A). The value of k_{cat} for efflux is recalculated as 14 s^{-1} with K_T equal to 217 μM (Table II). The efflux data can also be analyzed in a more straightforward, albeit mathematically less rigorous fashion. The average intracellular concentration of GalSGal is calculated for the interval between 0 and 3 s. The rate is defined as the change in the internal GalSGal concentration during this time. The plot of the reciprocal of the rate against the average GalSGal concentration as defined above is similar to that obtained using the integrated rate equation (cf. Fig. 5A and 5B). In conclusion, in spite of the difficulty in measuring the rapid rates of facilitated diffusion in poisoned cells, the cotransporter appears to function nearly symmetrically in cells as in vesicles, although the rates are 10-fold higher in cells.

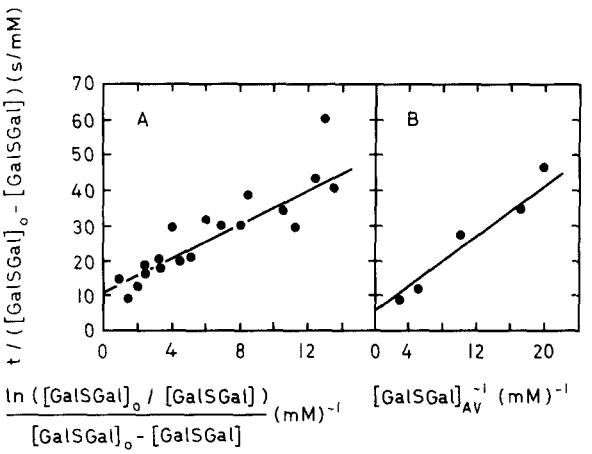


Fig. 5. When poisoned, EDTA treated ML308-225 cells are loaded with 10 μM to 1 mM labelled GalSGal and diluted 100-fold, the exit of substrate is rapid. In panel A, these data are plotted according to a linearized, integrated rate equation $t/(G_0'' - G'') = K_T/V_{max} \cdot \ln(G_0''/G'')/(G_0'' - G'') + 1/V_{max}$ where G_0'' is the internal GalSGal concentration at time zero and G'' at time t. The turnover number is 14.7 s^{-1} and K_T is 217 μM at pH 7.6. In panel B, the data for the first time points (3 s) are analyzed by plotting the reciprocal of the measured rate against the reciprocal of the average, internal concentration of GalSGal during the first 3 s.

Countertransport

As discussed above, in the absence of $\Delta\tilde{\mu}_{H^+}$, countertransport may be viewed as facilitated diffusion in the exchange mode. The presence of the overshoot makes the measurement of true initial velocities more facile. This is immediately obvious from a comparison of K_T values for GalSGal. Both in vesicles and in cells, the K_T values for countertransport are smaller than the values for other types of facilitated diffusion (Table II). The value of 44 μM and 63 μM measured at pH 5.5 and 7.6, respectively, are similar to the dissociation constant for GalSGal. The value of the turnover number is independent of pH between 5.5 and 7.6, 12.1 s^{-1} vs. 14.0 s^{-1}, and is also 10-fold greater than the corresponding values found in vesicles (Table II).

In conclusion, the affinity of the cotransporters in poisoned cells for GalSGal on the periplasmic face does not vary between 5.5 and 7.5 and is similar to the estimate from direct binding studies (Table I). Also the constancy of k_{cat} in the physiological pH region suggests that, in cells as in vesicles, the H^+ binding sites are fully occupied at these pH values.

pH dependence of exchange: estimate of the pK of the cotransported proton

The purpose of measuring the pH dependence of equilibrium exchange is to estimate the affinity of the cotransporter for the cosubstrate H^+. Several measurements above indicate that the pK of the cotransported proton must be greater than 7.6. The rate of equilibrium exchange should decrease by one-half when $[H^+]'$ and $[H^+]''$ fall from saturating values to K_H (cf. Ref. 20).

Gramicidin S-treated vesicles from ML308-225 in the presence of KCl buffered at pH values from 7.6 to 10.3 are loaded with 1 mM GalSGal and are diluted into the same buffered KCl solution containing 1 mM [^{3}H]GalSGal. The initial velocity sinks from 2.2 s^{-1} at pH 7.6 to 1.0 s^{-1} at pH 10.3 (Fig. 6, filled circles). The decrease in rate is not due to an irreversible denaturation of the cotransporters, as control vesicles washed at pH 10.3 and subsequently washed and loaded with GalSGal at pH 8.0 (Fig. 6, closed triangle) exhibit the same exchange rate as vesicles washed only at pH 8.0. A reversible inactivation of the protein cannot be ruled out, however. In conclusion, this experiment implies that the pK of the cotransported proton is at least 10.3.

Transport in the presence of $\Delta\tilde{\mu}_{H^+}$

The foregoing measurements of GalSGal transport in vesicles and in cells provide reference values of k_{cat} and K_T for influx, efflux, and exchange in the absence of $\Delta\tilde{\mu}_{H^+}$. The purpose of the following experiments is to determine by kinetic analysis which of the ten factors in the middle expression in Eqn. 3 are altered by the imposition of $\Delta\tilde{\mu}_{H^+}$. Comparing k_{cat}, K_T, and their variation with pH in the presence and absence of $\Delta\tilde{\mu}_{H^+}$ can identify these changes.

Influx in the presence of $\Delta\tilde{\mu}_{H^+}$ in vesicles (active transport)

In vesicles derived from ML308-225, the composition and magnitude of $\Delta\tilde{\mu}_{H^+}$ can be manipulated by the choice of substrate for the respiratory chain and of the external pH [40,41]. At an exter-

TABLE III

ACTIVE TRANSPORT OF GalSGal IN VESICLES AND CELLS (ML308-225)

Respiratory substrate (10 mM)	pH_{out}	pH_{in}	K_T (μM)	k_{cat} (s^{-1})	$\Delta\psi$ (mV)	$-Z\Delta pH$ (mV)	$\Delta\tilde{\mu}_{H^+}$ (mV)	$\Delta\mu_G$ (mV)	n_H [a]	[GalSGal] [b] (μM)
Vesicles										
D-Lactate	7.6	7.7			−76	−8	−84	+112	1.33	1
	7.6	7.6	90	1.15	−47–0	0	−47–0 [c]	+67–0 [c]	–	900
D-Lactate	5.5	6.2			−69	−40	−109	+82	0.75	1
	5.5	5.6	63	1.20	−39–0	−9	−48–0 [c]	+63–0 [c]	–	900
Succinate	5.5	5.6			−65	−9	−74	+84	1.14	1
	5.5	5.5	71	0.90	−35	0	−35–0 [c]	+45–0 [c]	–	900
Cells										
Succinate	5.5	7.3			−63	−106	−169	+184	1.09	1
	5.5	7.4	57	18.1	−73	−101	−174	+89	0.51	900
Succinate	7.6	7.8			−128	−12	−140	+176	1.25	1
	7.6	7.8	41	26.3	−102	−12	−124	+57	0.46	900
Succinate	5.5	7.5			−27	−117	−144	118	0.82	1
	5.5	7.2	59	17.5	−9	−100	−109	56	0.51	900

[a] Apparent H^+ symport stoichiometry.

[b] Concentration of GalSGal in flow-dialysis to determine $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_G$.

[c] These gradients collapse to zero within 4–6 min after addition of 0.9 mM GalSGal.

nal pH of 7.6 and during the oxidation of D-lactate by the respiratory chain, $\Delta\psi$ is −76 mV, ΔpH is −8 mV, and $\Delta\mu_G$ is +112 mV, which yields a

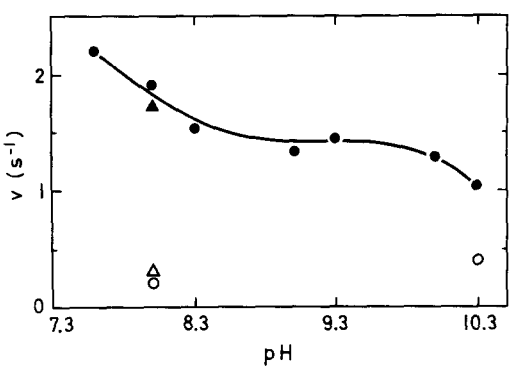


Fig. 6. The pH dependence of the equilibrium exchange of GalSGal can yield the pK of the symported H^+. When 1 mM radioactive GalSGal is added to a suspension of gramicidin S-treated ML308-225 vesicles loaded with 1 mM GalSGal, labelled GalSGal is exchanged for unlabelled GalSGal. The initial rate (v) of exchange decreases with increasing pH (●). Some vesicles are washed at pH 10.3 and measured at pH 8.0 as a control (▲). The magnitude of the correction applied for the passive flux of GalSGal in vesicles from uninduced ML30 under the same conditions is shown (○). Some of these vesicles are washed at pH 10.3 and subsequently measured at pH 8.0 (△).

cotransported stoichiometry of 1.33 H^+/GalSGal at low galactoside concentration (Table III). The parameters for active transport sustained by $\Delta\tilde{\mu}_{H^+}$ resemble those for facilitated diffusion in the influx mode: k_{cat} is 1.15 s^{-1} compared with 0.9 s^{-1} for influx; K_T values are 90 μM and 118 μM, respectively (Tables II and III). At pH 5.5 with D-lactate as the respiratory substrate, the driving force is composite (Table III), consisting of $\Delta\psi = -69$ mV and $\Delta pH = -40$ mV. The cotransport stoichiometry is 0.75 (cf. Ref. 9). The kinetic parameters do not differ from those at pH 7.6: $k_{cat} = 1.20$ s^{-1}, $K_T = 63$ μM (Table III). With succinate as the respiratory substrate at pH 5.5, ΔpH is depressed compared to that produced by the oxidation of D-lactate (−9 vs. −40 mV), whereas $\Delta\psi$ is virtually unchanged at −65 mV (Table III). The kinetic parameters, $k_{cat} = 0.90$ s^{-1} and $K_T = 71$ μM, are similar to those for facilitated diffusion (Table II) and for active transport supported by D-lactate oxidation (Table III). The cotransport stoichiometry is 1.14.

In conclusion, the similarity of K_T values for influx in the presence and absence of $\Delta\tilde{\mu}_{H^+}$ indicates that the imposition of $\Delta\tilde{\mu}_{H^+}$ does not decrease K'_G (cf. Fig. 1 and Eqn. 3). Likewise, $\Delta\tilde{\mu}_{H^+}$

slightly increases the rate of influx. This implies that either k_c^+ or k_o^- must increase. However, these effects alone are not commensurate with the imposed $\Delta\tilde{\mu}_{H^+}$ (Eqn. 3), therefore the major effects of $\Delta\tilde{\mu}_{H^+}$ have yet to be discovered. Lastly, the constancy of k_{cat} over a 125-fold variation in the cosubstrate H^+ demonstrates that at physiological pH, H^+ is saturating. The increase in k_{cat} with increasing pH observed by Robertson et al. [32] could not be documented for GalSGal.

To study efflux and exchange in the presence of $\Delta\tilde{\mu}_{H^+}$, vesicles or cells must be loaded with galactoside without depressing $\Delta\tilde{\mu}_{H^+}$. However, addition of 900 μM GalSGal to ML308-225 vesicles respiring on 10 mM D-lactate or succinate leads to a steady collapse of $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_G$ to unmeasurable values over a period of 4 to 6 min (Table III). Indeed, $\Delta\tilde{\mu}_{H^+}$ is known to be depressed by the cotransport of other galactosides with H^+ under a number of conditions [37,40–43]. Therefore, vesicles might be preferred for measuring facilitated diffusion, because $\Delta\tilde{\mu}_{H^+}$ may be easily collapsed. However, the instability of $\Delta\tilde{\mu}_{H^+}$ in vesicles at higher concentrations of galactoside make them inappropriate for certain experiments in the presence of $\Delta\tilde{\mu}_{H^+}$. Cells are now demonstrated to be better for such measurements.

Influx in cells in the presence of $\Delta\tilde{\mu}_{H^+}$ (active transport)

At pH 7.6, active transport in cells is supported almost solely by $\Delta\psi$ in EDTA-treated ML 308-225 cells (Table III). At an initial, external GalSGal concentration of 1 μM, the cotransport stoichiometry is measured as 1.25 H^+/GalSGal. Addition of 900 μM GalSGal to EDTA-treated ML308-225 cells respiring on 10 mM succinate does not measurably depress $\Delta\tilde{\mu}_{H^+}$ (Table III), although the *apparent* stoichiometry sinks to 0.46 H^+/GalSGal. GalSGal active transport by EDTA-treated cells in the presence of succinate is characterized by a K_T of 41 μM, similar to those observed for active transport by vesicles (Table III); the maximal velocity, however, lies considerably higher at 26.3 s^{-1}. This value is 10–20-fold greater than the corresponding values for vesicles (Table III). This difference is also observable for lactose [14,21].

At an external pH of 5.5, active transport is driven by a composite $\Delta\tilde{\mu}_{H^+}$ with ΔpH as the major component. The cotransport stoichiometry is 1.09 H^+/GalSGal at low galactoside concentrations, decreasing apparently to 0.51 at 900 μM GalSGal. The value of $\Delta\tilde{\mu}_{H^+}$ is hardly affected by the addition of galactoside, however (Table III).

An attempt can be made to sustain active transport nearly exclusively with ΔpH. By adding the lipophilic ion Ph_4P^+, $\Delta\psi$ can at least be partially collapsed [33]. At 0.3 mM Ph_4P^+, $\Delta\psi$ is depressed to between -43 and -11 mV in different experiments, while ΔpH rises to -114 to -119 mV. The stoichiometry is 0.82 H^+/GalSGal (Table III) at 1 μM GalSGal and apparently decreases to 0.51 at 900 μM. The values of k_{cat} and K_T, 17.5 s^{-1} and 59 μM, are similar to those observed at pH 7.6 (Table III). The pH-independence of k_{cat} in cells and vesicles agrees with many previous findings [21,44–46], but differs from a report that the V_{max} value of active transport for lactose increases 10-fold upon increasing the pH from 5.5 to 7.5 [32].

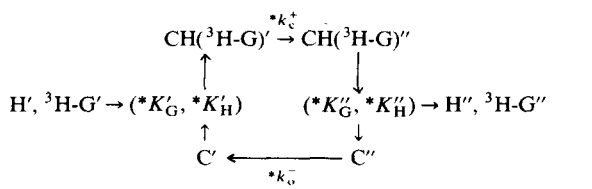
These data permit four conclusions. For active transport, $\Delta\psi$ and ΔpH appear thermodynamically and kinetically equivalent, because cotransport stoichiometry and kinetic parameters do not vary appreciably by interchanging $\Delta\psi$ and ΔpH. An explanation would be that the rate-determining step is nearly independent of $\Delta\psi$ and ΔpH. Subsequent analysis indicates that this is k_c^+. Also, cells are appropriate for kinetic studies requiring saturating levels of galactoside, because $\Delta\tilde{\mu}_{H^+}$ remains constant under these conditions. Next, at least one of the cotransporter reorientation steps (probably k_o^-) increases in the presence of $\Delta\tilde{\mu}_{H^+}$, because the turnover number for active transport is greater than for influx at $\Delta\tilde{\mu}_{H^+}$ near zero (cf. Tables II and III). Lastly, the accumulation of GalSGal does not come into equilibrium with $\Delta\tilde{\mu}_{H^+}$ at 0.9 mM GalSGal.

To identify the major effects of $\Delta\tilde{\mu}_{H^+}$ on the cotransport cycle, additional kinetic measurements must be made. This is achieved in the following two sets of experiments by comparing net fluxes, influx or efflux, where galactoside is present on only one side of the membrane, with the corresponding unidirectional fluxes during exchange, where labelled galactoside is present on one side and unlabelled galactoside on the other side of the membrane. A comparison of the relevant kinetic cycles (see below) discloses that such experiments

can reveal the apparent affinity of the cotransporter for galactoside on both sides of the membrane, the relative magnitudes of the cotransporter reorientation steps (k_o^-, k_o^+, k_c^-, k_c^+), the rate-determining step (cotransporter reorientation or cosubstrate binding or release), and the steps affected by $\Delta\tilde{\mu}_{H^+}$. Clearly, a straightforward comparison of data for net flux and exchange required that the value of $\Delta\tilde{\mu}_{H^+}$ be known and identical in both experiments.

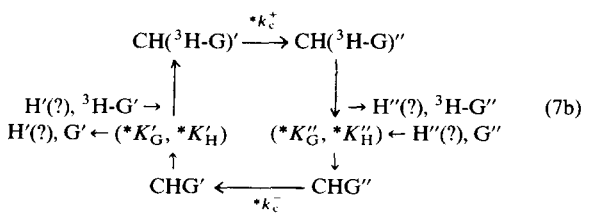
GalSGal influx into unloaded and preloaded cells in the presence of $\Delta\tilde{\mu}_{H^+}$

In these experiments, [^{3}H]GalSGal influx into EDTA-treated ML308-225 cells is followed in the absence and presence of saturating, intracellular, unlabelled GalSGal. The catalytic cycles are



(7a)

and



(7b)

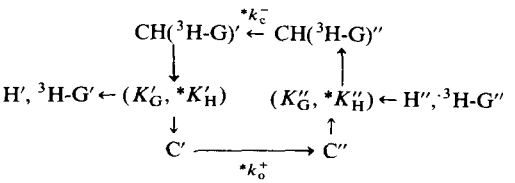
for influx during exchange.

The time-course of [^{3}H]GalSGal uptake at external concentrations of 15 and 96 μM into unloaded and preloaded cells is followed. Simultaneously, the intracellular concentration of GalSGal in cells preloaded with radioactive galactoside is monitored after dilution into unlabelled 15 or 96 μM GalSGal (Fig. 7). The time-course of GalSGal uptake over a period of 120 s is unaffected by preloading the cells with GalSGal. The initial rates are 4.2 s^{-1} at 15 μM external GalSGal and 16.4 s^{-1} at 96 μM external GalSGal. These values correspond to $k_{cat} = 28\ s^{-1}$ and $K_T = 90\ \mu$M (Table IV). The intracellular GalSGal in the preloaded cells remains at saturating levels of at least 3–4 mM during GalSGal influx (Fig. 7A and B, upper curves). Thus, the turnover number and half-saturation constant for GalSGal active transport are unchanged by the presence of saturating intracellular levels of GalSGal. In both experiments, $\Delta\tilde{\mu}_{H^+}$ is constant at -145 mV, although the apparent stoichiometry sinks from 1.00 to 0.49 H^+/GalSGal (Table IV).

The following conclusions can be derived. Both for net influx and influx during exchange (cf. countertransport) in the presence of the $\Delta\tilde{\mu}_{H^+}$, the values of K_T resemble those found with poisoned cells (Table II) and the values of the observed dissociation constant (Table I). Therefore, $\Delta\tilde{\mu}_{H^+}$ does not increase the affinity of the cotransporter for GalSGal on the periplasmic side of the membrane. Next, neither the binding nor release of H^+ or GalSGal can be rate-determining, because these steps occur a different number of times in cycles 7a and 7b. The identity of the turnover numbers for net influx and exchange is most readily explained by the fact that the common step *k_c^+, the inward reorientation of CHG′, is the slowest step. Lastly, the slightly faster rates in the presence of $\Delta\tilde{\mu}_{H^+}$ (cf. Tables II and IV) imply that $\Delta\tilde{\mu}_{H^+}$ increases at least k_c^+. Qualitatively similar results were obtained by Kepes [47] and by Winkler and Wilson [34]. However, in these instances GalSGal had not been tested and the values of $\Delta\tilde{\mu}_{H^+}$ were unknown. The similarity of the results also indicates that the kinetically complex galactosides employed in the work in Refs. 34 and 47 and the simple substrate GalSGal are accumulated by similar kinetic mechanisms.

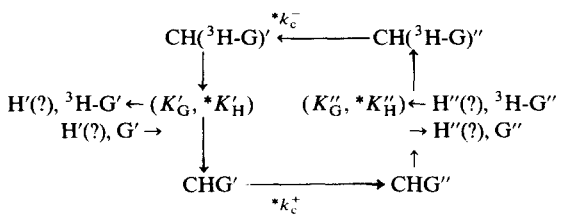
External GalSGal alters the kinetics of efflux in the presence of $\Delta\tilde{\mu}_{H^+}$

When [^{3}H]GalSGal efflux out of EDTA-treated ML308-225 is measured in the absence (Eqn. 8a) and presence (Eqn. 8b) of saturating extracellular, unlabelled GalSGal, the relevant kinetic schemes are:



(8a)

for efflux and



(8b)

for efflux during exchange.

EDTA-treated cells were loaded by active transport with various concentrations of [^{3}H]GalSGal up to 1 mM. Preliminary experiments disclosed that the steady state is attained after 11 min and is stable for 13 min thereafter. Bioenergetic data obtained under the prevailing conditions are determined (Table IV), showing that 0.9 mM external GalSGal does not collapse $\Delta\tilde{\mu}_{H^+}$, although the apparent stoichiometry sinks from 0.99 to 0.53 at pH 7.5 (Table IV). To determine the effect of both $\Delta\psi$ and ΔpH on the kinetics of efflux, measurements are conducted at pH 7.5, at which $\Delta\psi$ is the sole component of $\Delta\tilde{\mu}_{H^+}$, and at pH 5.5, at which both components contribute (Table IV).

Efflux at pH 7.5 is characterized by k_{cat} 1.4 s^{-1} and $K_T = 154$ μM (Fig. 8A, closed squares) as estimated from the lower left-hand portion of the curve. Imposing $\Delta\psi$ reduces the k_{cat} for efflux by a factor of 10 as compared to the value in poisoned cells (Table II, Fig. 5). The data for efflux at an external pH of 5.5 are similar (Table IV; Fig. 8A, closed triangles). However, efflux is moderately faster at pH 5.5.

The efflux experiments are repeated; however, 0.9 mM GalSGal ($11 \times K_G$) was included in the dilution buffer. This amount of galactoside does not depress $\Delta\tilde{\mu}_{H^+}$ (Table III, see also Ref. 37), but serves to shift the return step in the catalytic cycle from $^*k_o^+$ to $^*k_c^+$ (Eqns. 8a and 8b). The data for efflux during exchange conditions yield $k_{cat} = 26$ s^{-1} and $K_T = 125$ μM (Fig. 8B). These parameters are identical at pH 5.5 and 7.5 (external), although

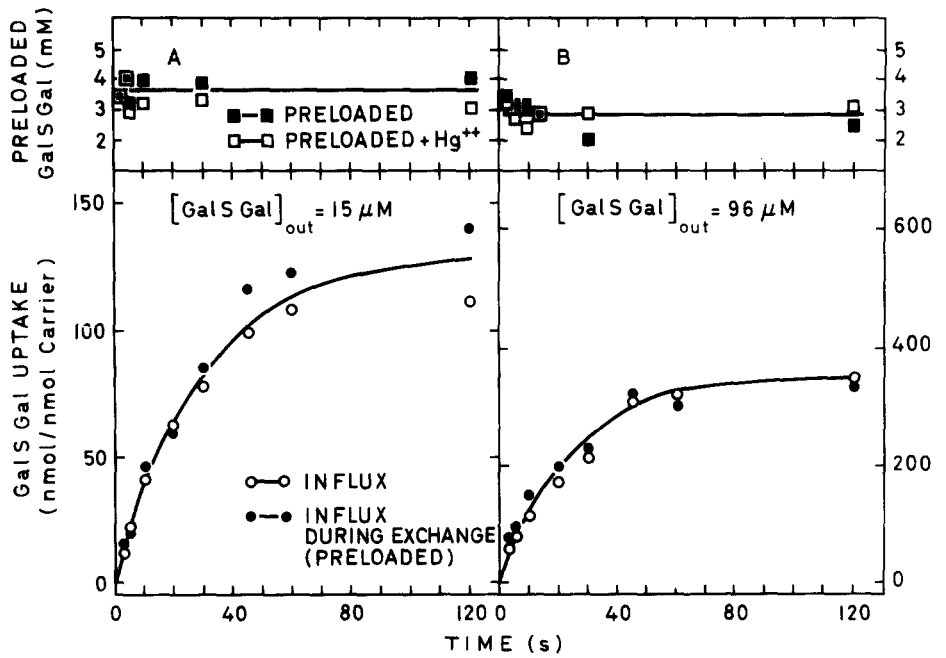


Fig. 7. The time-course of GalSGal active transport in EDTA-treated ML308-225 cells is not influenced by preloading cells with GalSGal. Unloaded (○) and preloaded (●) cells are diluted into buffer to final, external concentrations of GalSGal of 15 (panel A) or 96 μM (panel B), and uptake is followed for 120 s (A and B, lower panels). Simultaneously the internal concentration of GalSGal in the preloaded cells is monitored (upper panels, ■). To assess the passive flux of GalSGal out of the preloaded cells, some samples are treated with $HgCl_2$ and the efflux followed for 120 s (upper panels, □).

TABLE IV

EFFECT OF $\Delta\tilde{\mu}_{H^+}$ ON GalSGal TRANSPORT IN EDTA-TREATED ML308-225 CELLS

Experiment	pH_{out}	K_T (μM)	k_{cat} (s^{-1})	$\Delta\psi$ (mV)	$-Z\Delta pH$ (mV)	$\Delta\tilde{\mu}_{H^+}$ (mV)	$\Delta\mu_G$ (mV)	n_H	[GalSGal]
Influx	6.0			−64	−82	−146	+145	1.00	1 μM [a]
	6.0	90	28	−69	−75	−144	+71	0.49	0.9 mM [a], 15 and 96 μM (out) [b]
Influx during exchange	6.0	90	28	−69	−75	−144	+104	0.72	3–5 mM (in) [b]
Efflux	5.5	330	3.3	−69	−63	−132	+101	0.77	1 μM [a], 10 μM–1 mM (in) [b]
Efflux during exchange	5.5	125	26	−69	−60	−129	+56	0.43	0.9 mM [a], 0.9 mM (out) and 10 μM–1 mM (in) [b]
Efflux	7.5	154	1.4	−105	+4	−101	+100	0.99	1 μM [a], 10 μM–1 mM (in) [b]
Efflux during exchange	7.5	125	26	−97	0	−97	+51	0.53	0.9 mM [a], 0.9 mM (out) and 10 μM–1 mM (in) [b]

[a] Concentration of GalSGal in flow dialysis experiment to determine $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_G$.

[b] Concentration of GalSGal inside and outside of cells in the samples for the kinetic measurements.

the composition of $\Delta\tilde{\mu}_{H^+}$ is different. The value for k_{cat} is similar to that for active transport (Table III).

The immediate conclusions of the findings for GalSGal efflux in the presence of $\Delta\tilde{\mu}_{H^+}$ can be summarized in four points. The affinity of GalSGal for the cotransporter on the inner surface of the membrane is unchanged by the imposition of $\Delta\tilde{\mu}_{H^+}$. The average value of K_T for efflux in both modes is 184 μM (Table IV). This value is comparable to those measured for efflux in the absence of $\Delta\tilde{\mu}_{H^+}$ (Table II) as well as to the overall dissociation constant and $^*K_G''$ (Table I). Next, the imposition of $\Delta\tilde{\mu}_{H^+}$ slows down efflux but not exchange. Also, GalSGal efflux in the presence and absence of $\Delta\tilde{\mu}_{H^+}$ is a saturable process. These results are qualitatively in agreement with studies on lactose efflux in the presence of $\Delta\tilde{\mu}_{H^+}$ from vesicles by Kaczorowski et al. [48] and from reconstituted proteoliposomes by Viitanen et al. [49] and differ from those of Winkler and Wilson [34]. Lastly, because efflux (Eqn. 8a) is slower than efflux during exchange (Eqn. 8b) the common step $^*k_c^-$ cannot be rate-determining. Therefore, the inward reorientation of the unloaded cotransporter ($^*k_o^+$ in Eqn. 8a) must be slower than the inward reorientation of the ternary complex CHG′ ($^*k_c^+$ in Eqn. 8b). This implies that $\Delta\tilde{\mu}_{H^+}$ decreases k_o^+.

Kinetic inefficiency: the disequilibrium between $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_G$ in the steady state

The following experiments have two purposes. The steady-state accumulation of GalSGal is demonstrated to follow Eqn. 4 when $\Delta\tilde{\mu}_{H^+}$ is constant. Next, a kinetic explanation is sought for this behavior which does not rely upon the existence of uncoupled flows of galactoside and H^+ across the membrane (leaks).

The accumulation ratio as a function of [GalSGal]

In EDTA-treated ML308-225 cells the stoichiometry of H^+ : GalSGal cotransport is 0.97 $\pm$ 0.15 H^+ per galactoside under a variety of conditions (Tables III and IV) and 1.07 $\pm$ 0.22 in the case of cotransport measured with right-side-out vesicles (Table III). These measurements are performed at an initial, external concentration of 1 μM GalSGal. In the case of cells, when the external concentration of GalSGal is raised to 900 μM, $\Delta\tilde{\mu}_{H^+}$ is not significantly altered, but $\Delta\mu_G$ decreases so that the apparent stoichiometry sinks to 0.48 $\pm$ 0.03 H^+ per GalSGal (Tables III and IV). In the case of vesicles, $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_G$ decrease to unmeasurable values within 4 to 6 min (Table III). If $\Delta\mu_G/\Delta\tilde{\mu}_{H^+}$ is determined for cells as a function of the external GalSGal concentration in the steady

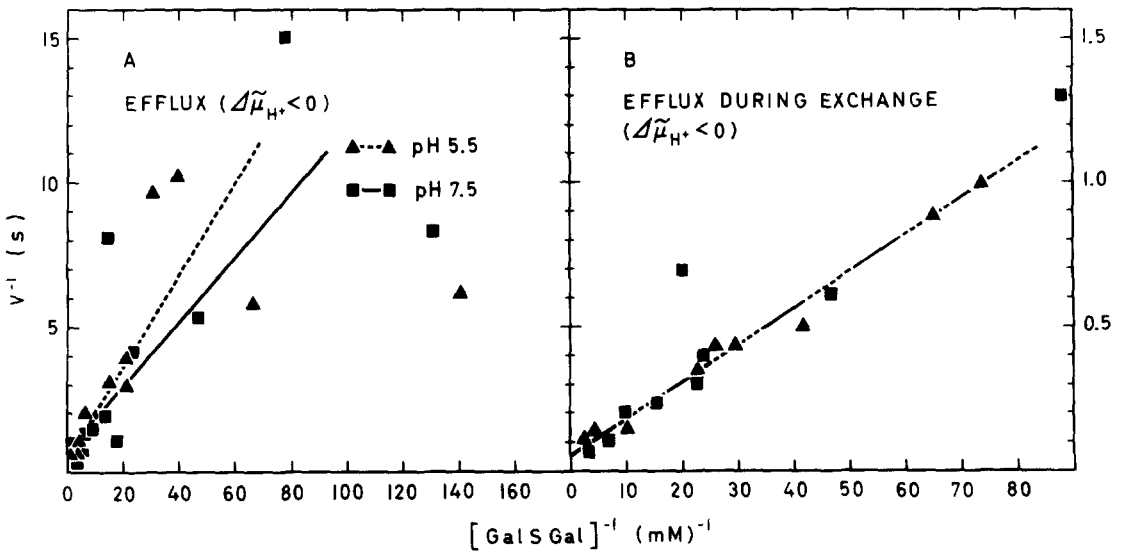


Fig. 8. Efflux of GalSGal in the presence of $\Delta\tilde{\mu}_{H^+}$ is more rapid in the presence of external galactoside. EDTA-treated ML308-225 cells incubated with various concentrations of labelled GalSGal are diluted into buffer without GalSGal (panel A) or with 0.9 mM GalSGal (panel B). The rate (v) is measured as a function of the internal concentration of GalSGal. Experiments are conducted at pH 5.5 (▲) and 7.5 (■).

state, this value decreases from 0.97 at 0.2 μM to 0.6 at 355 μM GalSGal (Fig. 9, upper panel). Under these conditions, $\Delta\tilde{\mu}_{H^+}$ remains virtually constant (Fig. 9, lower panel, open circles). If these data are analyzed according to Eqn. 4 by plotting the reciprocal of the accumulation ratio against the actual external concentration of GalSGal in the steady state, certain limiting values can be obtained. In the limit of zero external GalSGal, the maximal accumulation ratio for these ambient conditions (carrier level, O_2 tension, 10 mM succinate, pH 7.6) is 587, corresponding to a stoichiometry of 1.03 H^+ per galactoside. The maximal internal concentration of GalSGal is 24.5 mM. The half-saturation constant for this process is 43 μM for GalSGal (Fig. 9, lower panel; Eqn. 4). This value is similar to K_T for active transport (Tables III and IV) and to the observed dissociation constant at $\Delta\tilde{\mu}_{H^+}=0$ (Table I).

In conclusion, at low concentrations of GalSGal, galactoside accumulation tends to come into equilibrium with $\Delta\tilde{\mu}_{H^+}$ in a fashion suggesting a 1 : 1 stoichiometry during cotransport. However, at higher concentrations of GalSGal the equilibrium predicted by Eqn. 3 is not attained, and the deviation follows Eqn. 4.

Net galactoside fluxes can be slow

Three features of galactoside/H^+ cotransport suggest that the cotransporter may be kinetically inefficient, that is, unable to catalyze rapid net uptake of galactoside at high external and internal concentrations of substrate: The affinity of the cotransporter for galactoside on both sides of the membrane is high (Tables I, II and IV), at physiological pH H^+ is saturating, and efflux in the presence of high external levels of GalSGal is as rapid as net influx (Table IV). Therefore, as the internal levels of GalSGal rise above the values of K_T for efflux during exchange, efflux (Eqn. 8b) competes effectively with influx (Eqn. 7a). The cotransporter is simply unable to attain the equilibrium of Eqn. 3 rapidly. The following experiment is designed to show that under conditions where unidirectional transport is rapid, net transport is slow. The experiment is easier to perform if efflux rather than influx is measured.

When gramicidin-S-treated vesicles or poisoned cells, loaded with up to 1 mM [^{3}H]GalSGal are diluted into buffer with 10 μM galactoside, internal galactoside exits with a half-time of about 5 s (Figs. 3 and 5). if poisoned, EDTA-treated ML308-225 cells loaded in the presence of 1 mM

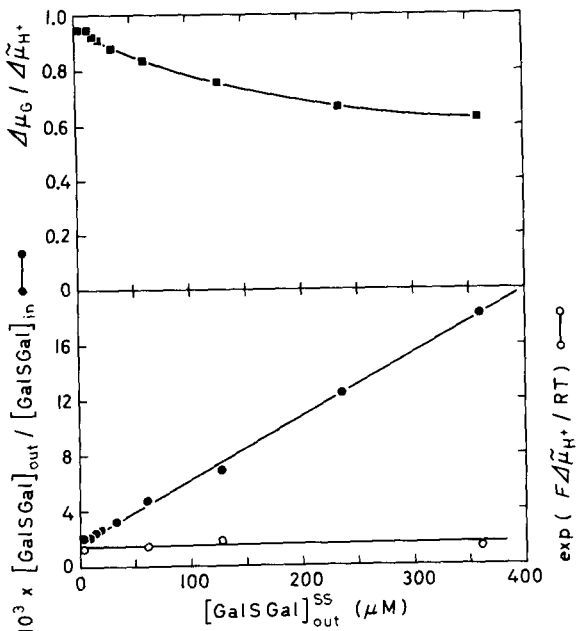


Fig. 9. GalSGal accumulation does not always come into equilibrium with $\Delta\tilde{\mu}_{H^+}$. The internal and external concentration of GalSGal in EDTA-treated ML308-225 cells in the steady state is measured by flow dialysis. Upper panel: The apparent stoichiometry $\Delta\mu_G/\Delta\tilde{\mu}_{H^+}$ (■) is determined as a function of the actual, external concentration of GalSGal. Lower panel: The reciprocal of the accumulation ratio is plotted as a function of the actual, external concentration of GalSGal (●, cf. Eqn. 4). Under the same conditions, $\Delta\tilde{\mu}_{H^+}$ is determined. The reciprocal of the accumulation ratio expected for 1:1 H^+/galactoside cotransport is plotted as a function of the external GalSGal concentration (○).

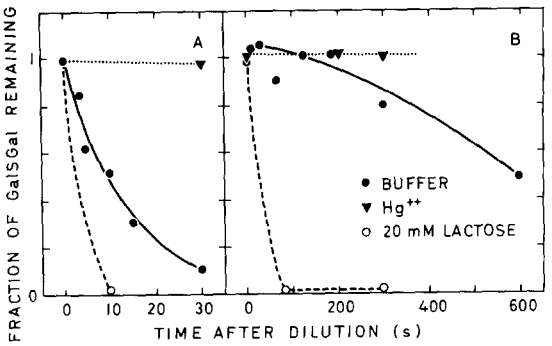


Fig. 10. Net transport of galactoside can be slow. Poisoned, EDTA-treated cells are incubated with 1 mM (A) or 20 mM (B) radioactive GalSGal at pH 7.6. Cells are then diluted 60-fold into buffer (●), into buffer containing 2 mM $HgCl_2$ (▼), or buffer containing 20 mM lactose (○) and the amount of radioactive GalSGal remaining after different times is determined.

[^{3}H]GalSGal are diluted only 60-fold, so that the external concentration is 17 μM ($0.21 \times K_G$) instead of 10 μM ($0.13 \times K_G$); the half-time for efflux increases to about 11 s (Fig. 10A, ●——●). This slight inhibition is not due to the formation of an external, binary cotransporter-galactoside complex (CG′ in Fig. 1), which would decrease the concentration of the unloaded cotransporter C′, thus inhibiting efflux. Including 20 mM lactose in the dilution buffer evokes a stimulation of the efflux of [^{3}H]GalSGal (Fig. 10A, open circles). These effects are more pronounced when the cells are incubated with 20 mM [^{3}H]GalSGal before dilution (Fig. 10B). In this case, the external concentration of [^{3}H]GalSGal after dilution is 330 μM or $4.3 \times K_G$. The half-time for efflux is about 600 s (Fig. 10B, closed circles). If the diluent contains 20 mM lactose which inhibits the binding of [^{3}H]GalSGal to the cotransporter externally, the half-time for efflux is reduced by at least a factor of 12 (Fig. 10B, open circles).

In conclusion, the retardation of net efflux by external [^{3}H]GalSGal is due to the inhibition of the net flux (i.e., outward flux minus inward flux) and not to a simple inhibition of the outward, unidirectional flux as evidenced by the stimulation of efflux by external lactose. Therefore, the saturation observed for the internal concentration of GalSGal in the steady-state (Eqn. 4, Fig. 9) as a function of the external concentration may be ascribed to the cycling of ternary complexes CHG′ and CHG″ at high galactoside concentrations, with resulting low net fluxes of GalSGal. Thus, the kinetic inefficiency (i.e., slowness of *net* influx) of the cotransporter, and not thermodynamic inefficiency (leaks), could be responsible for the observed disequilibrium. That this kinetic inefficiency can be described by Eqn. 4 can be demonstrated by a theoretical analysis [59].

Discussion

A qualitative description of the activity of an ion/solute cotransporter addresses the following points: the relative rates of cotransporter reorientations, the affinities of the cotransporter for ion and solute, the order of binding of the cosubstrates,

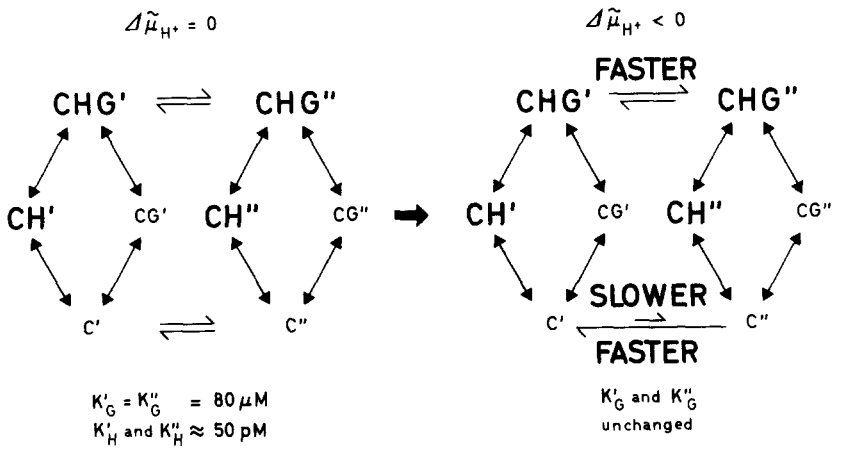


Fig. 11. A qualitative model for cotransport. See text for details.

and the rate-determining step(s).

In poisoned cells, the rates for the reorientation of the unloaded cotransporter (k_o) and the ternary complex (k_c) are similar. Because the maximal velocities of net influx, net efflux, and exchange in the countertransport mode differ maximally by a factor of 2.5, the individual translocation rate constants (k_o^+, k_o^-, k_c^+ and k_c^-) should not differ appreciably from one another (Fig. 11). For unknown reasons, turnover numbers in vesicles are only about one-tenth of those in cells (cf. Refs. 14, 21, 50).

In the absence of $\Delta\tilde{\mu}_{H^+}$, the affinities of the cotransporter for GalSGal on both sides of the membrane are comparable. The values of K_T for influx, efflux, and exchange are very similar in vesicles and cells (Table II). Furthermore, these kinetic constants appear to be determined by the value of the dissociation constant (76 μM). In this context, the report that the facilitated diffusion of GalSGal is characterized by values of K_T near 20–25 mM and by maximal velocities 3- to 5-fold higher than those for active transport [32] is difficult to reconcile with data reported here and elsewhere [21,22,29,51].

Physiological values of pH represent saturating concentrations of the cotransported H^+. Turnover numbers for facilitated diffusion exhibit no pronounced dependence on pH. Kaczorowski and Kaback [35] and Page and West [46] have made similar observations with other galactosides. The dissociation constant for H^+ must, therefore, be smaller than 10 nM ($pK > 8$). The value of K_H the dissociation constant for the cotransported H^+ can best be estimated from the pH dependence of equilibrium exchange. The data in Fig. 6 suggest that the apparent dissociation constant is about 50 pM. The significance of this finding is that the cotransporter is completely protonated at physiological pH, existing nearly exclusively as either CH or CHG (Fig. 11).

Galactoside and H^+ bind randomly to the cotransporter. The order of cosubstrate binding or release cannot be

$$C \rightleftharpoons CG \rightleftharpoons CHG \tag{9}$$

or

$$C \rightleftharpoons CH \rightleftharpoons CHG \tag{10}$$

In the case of Eqn. 9, both the equilibrium dissociation constant and half-saturation constants must exhibit a pronounced dependence on pH. Moreover, when the concentration of H^+ is saturating, galactosides would appear to have infinite affinity for the cotransporter (K_G or $K_T = 0$). However, neither K_G nor K_T varies significantly over a 160-fold change in the concentration of H^+. In the case of Eqn. 10, a pronounced, so-called *trans*-inhibition of net influx or efflux must occur at H^+ concentrations comparable to K_H. For instance, the presence of the binary complex CH″ would inhibit the completion of the influx cycle by blocking formation of C″. *Trans*-inhibition is well-docu-

mented for Na^+/glucose cotransport, where the presence of the internal binary complex CNa″ is demonstrated to inhibit net influx [52–54]. Therefore, binding and release of H^+ and galactoside probably proceed randomly on both faces of the cotransporter (Fig. 11). Such an assignment is favored by Page and West [55].

The rate-determining step for cotransport in the absence of $\Delta\tilde{\mu}_{H^+}$ is one of the protein isomerizations (k_c or k_o). If the release or binding of one of the cosubstrates were the slowest step during cotransport, then exchange would have to be faster or slower than a net flux of galactoside, due to the different number of release and binding steps in the two types of cycle (compare Eqns. 9a and 9b). Because exchange (countertransport) and net influx or efflux in poisoned cells proceed with similar turnover numbers (Table II), the step specified by k_c or k_o must be rate-limiting. As noted above, the rates of these steps appear to be similar in cells, so that no particular one is solely rate-determining. The data in Figs. 7 and 8 also suggest that in the presence of $\Delta\tilde{\mu}_{H^+}$ cotransporter reorientations and not binding or release of cosubstrates are the slowest steps in cotransport.

These findings are summarized in Fig. 11 for the cotransporter in cells. The inward and outward reorientations of the unloaded and fully loaded cotransporter (k_o and k_c) occur at similar rates and are the slowest steps in the cycle. The cosubstrates bind randomly to the cotransport on both sides of the membrane. The dissociation constant of the symported H^+ may be as small as 50 pM. Therefore, at physiological pH, the predominant cotransport intermediates are CH and CHG. The dissociation constant for GalSGal is the same on both sides of the membrane at about 80 μM.

The chief difference between this model for the facilitated diffusion of GalSGal and many previous descriptions is that in the absence of $\Delta\tilde{\mu}_{H^+}$ the cotransporter is not viewed as assuming a conformation having a low affinity for GalSGal. The value of K_T for facilitated diffusion is suggested to be determined by the dissociation constant for galactoside. The proposed model conforms to the views of Page and West [46], who suggested the cotransporter is fully protonated at physiological pH. However, there are differences with two other studies. In one case [48], the values of K_T for influx and efflux were suggested to differ by a factor of 10, the release of cosubstrates on the exterior was proposed to be ordered according to Eqn. 10, and the order of cosubstrate binding on interior was not specified. In another case [33], facilitated diffusion is viewed as catalyzed by a class of cotransporter sites which are not rapidly interconvertible with those which catalyze active transport. Also, facilitated diffusion is postulated to have a much lower maximal velocity and to involve the cotransport of fewer H^+ than during active transport. Some data presented by Winkler and Wilson [34] support the model in Fig. 11 for facilitated diffusion.

Active transport

The imposition of $\Delta\tilde{\mu}_{H^+}$ across the membrane causes galactoside to be accumulated by cotransport with H^+. The kinetic and binding experiments reported here allow the changes in the cycle responsible for the concentrative uptake of GalSGal to be identified.

The imposition of $\Delta\tilde{\mu}_{H^+}$ changes at least three rate constants for the reorientation of the cotransporter. The rate of exchange in cells increases by a factor of 1.8 in the presence of $\Delta\tilde{\mu}_{H^+}$ (Fig. 1). This implies that k_c^+ or k_c^- must increase. The rate of influx increases by more than a factor of 4, implying that both k_o^- and k_c^+ are increased by $\Delta\tilde{\mu}_{H^+}$. Lastly, the decrease in the rate of net efflux by up to 10-fold implies that $\Delta\tilde{\mu}_{H^+}$ decreases k_o^+. In the case of lactose active transport, an increase in k_o^- is one possibility suggested in the analysis of Kaczorowski et al. [35,48].

The imposition of $\Delta\tilde{\mu}_{H^+}$ does not affect the affinity of the cotransporter for GalSGal. The half-saturation constants for influx, efflux, and exchange are similar in the presence and absence of $\Delta\tilde{\mu}_{H^+}$ (Fig. 11). The dissociation constant for GalSGal on the cytoplasmic membrane is not altered in the presence of $\Delta\tilde{\mu}_{H^+}$ (Table I). Similarly, K_T for active transport is similar to the dissociation constant ($\Delta\tilde{\mu}_{H^+} = 0$). Therefore, in the presence of $\Delta\tilde{\mu}_{H^+}$, K'_G and K''_G are both about 80 μM. A possible effect of $\Delta\tilde{\mu}_{H^+}$ upon the affinity of the cotransporter for H^+ cannot be ruled out by this qualitative analysis. Active transport exhibits a broad maximum with respect to pH, as do binding and facilitated diffusion, implying that physiologi-

cal pH still represents saturating levels of the symported H^+. However, because the affinity of the cotransporter for H^+ is so high, even changes in K'_H or K''_H of 100-fold would hardly alter the degree of saturation. Therefore, changes in the affinity of the cotransporter for the symported H^+ would be difficult to detect.

The changes in the cotransport cycle in the presence of $\Delta\tilde{\mu}_{H^+}$ are summarized in Fig. 11. A distinctive feature of this model is that a change in the affinity of the cotransporter for galactoside is not incorporated as an essential feature of energy transduction. A challenging problem is to determine how the cotransporter becomes deprotonated to complete the cycle for net influx and how, in view of the low dissociation constant, the cotransporter can deprotonate rapidly. Interestingly, Elferink et al. [56] have suggested that rapid galactoside transport may be associated with electron flow in the membrane. The release of H^+ from the cotransporter may be influenced by ion fluxes on the membrane surface. This possibility is currently being examined.

The data here for active transport differ significantly from some previously reported. Robertson et al. [32] observed that lactose active transport in vesicles evinces a pronounced dependence on pH and $\Delta\tilde{\mu}_{H^+}$. The maximal velocity was observed to increase by a factor of 10 as the concentration of H^+ was decreased 100-fold (pH 5.5 to 7.5) and to be a function of $(\Delta\tilde{\mu}_{H^+})^2$. Other studies, including some with lactose [21,22,29,51], provide data similar to those reported here. Therefore, the discrepancy between the findings of GalSGal here and lactose in Ref. 32 does not appear to be due to the use of different galactosides.

Disequilibrium between $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_G$

At low concentrations of GalSGal (1 μM), the accumulation of this galactoside comes into equilibrium with $\Delta\tilde{\mu}_{H^+}$, as expected for 1 : 1 cotransport. At higher concentrations this equilibrium is not attained (Fig. 9). Two frequently proposed explanations for this disequilibrium rely on thermodynamic inefficiency, imperfect coupling of H^+ and galactoside fluxes [15,18–20]. Either the galactoside leaks out of the cell (outer leak) or occasionally the cotransporter transports either H^+ or galactoside alone (inner leak). In the case of the lactose/H^+ cotransporter, the data of Booth and Hamilton [44] suggest that for a hydrophilic disaccharide like GalSGal the outer leak is too small to explain the magnitude of the disequilibrium. On the other hand, theoretical considerations indicate that the magnitude of the disequilibrium for an inner leak (reorientation of CH or CG parallel to that of C and CHG) is independent of the cotransporter level in the membrane because the ratio of cotransport to inner leak is independent of the number of carriers. However, Maloney and Wilson (Ref. 16, cf. Ref. 17) demonstrated that the magnitude of galactoside accumulation is a function of the level of cotransporter. Clearly, neither inner nor outer leaks are sufficient to explain the disequilibrium between $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_G$.

Here, a third possibility is offered. The two principal features of this explanation are that galactoside and H^+ fluxes remain strictly coupled under all conditions (thermodynamic efficiency 100%) and that at higher galactoside concentrations net influx is unable to compete with efflux of accumulated galactoside via the cotransporter (low kinetic efficiency). Thus, although the fluxes of H^+ and galactoside are tightly coupled (thermodynamic efficiency), cycling of the cotransporters as ternary complexes

$$H' + G' \rightleftharpoons (CHG' \rightleftarrows CHG'') \rightleftharpoons H'' + G'' \qquad (11)$$

prevents the attainment of equilibrium within a reasonable time (kinetic inefficiency). The physiological importance of this effect is that the cell may be able to protect itself from high internal levels of galactosides without wasting energy by uncoupling H^+/galactoside cotransport to reduce the accumulation levels at higher galactoside concentrations.

Two experimental observations also are in agreement with this model for the disequilibrium of $\Delta\tilde{\mu}_{H^+}$ and $\Delta\mu_G$. GalSGal is able to protect cells against lactose-induced cell death [57,58]. By virtue of the higher affinity of GalSGal for the cotransporter, an electroneutral exchange of GalSGal (cycling of CHG) is favored, protecting the cell from the collapse of $\Delta\tilde{\mu}_{H^+}$ by net transport of lactose. Also, at high levels, GalSGal causes much smaller pH excursions when added to cells as compared to lactose, although the GalSGal : H^+ stoichiometry

is close to unity. At the saturating, external levels of GalSGal in Ref. 6, influx rapidly raises the intracellular concentration to levels greater than K''_G. The subsequent cycling of the ternary complex is accompanied by little net flux of H^+.

Because the values of $\Delta\tilde{\mu}_{H^+}$ under the ambient conditions of these experiments are known, a quantitative analysis can be undertaken. In a following publication this analysis will be conducted. This quantitative analysis confirms the qualitative interpretations offered here and permits the values of the individual parameters of the cotransport cycle and their dependence on $\Delta\psi$ to be assigned.

Acknowledgements

I am grateful to many colleagues for helpful discussions, especially K. Dornmaier, R. Seckler, and P. Overath. S. Meessen and J. Fehling provided invaluable technical assistance, and U. Hieke typed the manuscript with great patience.

References

1 Mitchell, P. (1973) J. Bioenerg. 4, 63–91
2 Williams, R.J.P. (1978) Biochim. Biophys. Acta 505, 1–44
3 Crane, R.K. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 99–159
4 Guggenheim, E.A. (1929) J. Phys. Chem. 33, 842–849
5 Sandermann, H. (1977) Eur. J. Biochem. 801, 507–515
6 West, I.C. (1970) Biochem. Biophys. Res. Commun. 41, 655–661
7 West, I.C. and Mitchell, P. (1973) Biochem. J. 132, 587–592
8 Zilberstein, D., Schuldiner, S. and Padan, E. (1979) Biochemistry 18, 669–673
9 Ramos, S. and Kaback, H.R. (1977) 16, 4271–4275
10 Booth, I.R., Mitchell, W.J. and Hamilton, W.A. (1979) Biochem. J. 182, 687–696
11 Ahmed, S. and Booth, I.R. (1981) Biochem. J. 200, 573–581
12 Ten Brink, B., Lolkema, J.S., Hellingwerf, K.J. and Konings, W.N. (1981) FEMS Microbiol. Lett. 12, 237–240
13 Overath, P. and Wright, J.K. (1983) Trends Biochem. Sci. 8, 404–408
14 Wright, J.K. and Overath, P. (1984) Eur. J. Biochem. 138, 497–508
15 Rickenberg, H.V., Cohen, G.N., Buttin, G. and Monod, J. (1956) Ann. Inst. Pasteur (Paris) 91, 829–857
16 Maloney, P.C. and Wilson, T.H. (1973) Biochim. Biophys. Acta 330, 196–205
17 Teather, R.M., Bramhall, J., Riede, I., Wright, J.K., Fürst, M., Aichele, G., Wilhelm, U. and Overath, P. (1980) Eur. J. Biochem. 108, 223–231
18 Eddy, A.A. (1977) Curr. Top. Membrane Transp. 10, 279–360
19 Kotyk, A. (1983) J. Bioenerg. Biomembranes 15, 307–319
20 Heinz, E. (1978) in Mechanics and Energetics of Biological Transport, pp. 98–119, Springer Verlag, Berlin
21 Wright, J.K., Riede, I. and Overath, P. (1981) Biochemistry 20, 6404–6415
22 Carter, J.R., Fox, C.F. and Kennedy, E.P. (1968) Proc. Natl. Acad. Sci. USA 60, 725–732
23 Short, S.A., Kaback, H.R. and Kohn, L.D. (1975) J. Biol. Chem. 250, 4291–4296
24 Overath, P., Teather, R.M., Simoni, R.D., Aichele, G. and Wilhelm, U. (1979) Biochemistry 18, 1–11
25 Leive, L. (1965) Biochem. Biophys. Res. Commun. 21, 290–296
26 Singh, A.P. and Bragg, P.D. (1979) Arch. Biochem. Biophys. 195, 74–80
27 Reenstra, W.W., Patel, L., Rottenberg, H. and Kaback, H.R. (1980) Biochemistry 19, 1–9
28 Schairer, H.U., Friedl, P., Schmid, B.I. and Vogel, G. (1976) Eur. J. Biochem. 66, 257–268
29 Kennedy, E.P., Rumley, M.K. and Armstrong, J.B. (1974) J. Biol. Chem. 249, 33–37
30 Kaback, H.R. and Barnes, E.M. (1971) J. Biol. Chem. 246, 5523–5531
31 Seckler, R. and Wright, J.K. (1984) Eur. J. Biochem. 142, 269–279
32 Robertson, D.E., Kaczorowski, G.J., Garcia, M.-L. and Kaback, H.R. (1980) Biochemistry 19, 5692–5702
33 Ghazi, A. and Schechter, E. (1981) Biochim. Biophys. Acta 645, 305–315
34 Winkler, H.H. and Wilson, T.H. (1966) J. Biol. Chem. 241, 2200–2211
35 Kaczorowski, G.J. and Kaback, H.R. (1979) Biochemistry 18, 3691–3697
36 Page, M.G.P. and West, I.C. (1980) FEBS Lett. 120, 187–191
37 Ahmed, S. and Booth, I.R. (1981) Biochem. J. 200, 583–589
38 Patel, L., Garcia, M.L. and Kaback, H.R. (1982) Biochemistry 21, 5805–5810
39 Hull, S.E., Karlsson, R., Main, P., Woolfson, M.M. and Dodson, E.J. (1978) Nature 275, 206–207
40 Schuldiner, S. and Kaback, H.R. (1975) Biochemistry 14, 5451–5461
41 Ramos, S. and Kaback, H.R. (1977) Biochemistry 16, 854–859
42 Ghazi, A., Therisod, H. and Schechter, E. (1983) J. Bacteriol. 154, 92–103
43 Ahmed, S. and Booth, I.R. (1983) J. Gen. Microbiol. 129, 2521–2529
44 Booth, I.R. and Hamilton, W.A. (1980) Biochem. J. 188, 467–473
45 Bentaboulet, M. and Kepes, A. (1981) Eur. J. Biochem. 117, 233–238
46 Page, M.G.P. and West, I.C. (1981) Biochem. J. 196, 721–731
47 Kepes, A. (1960) Biochim. Biophys. Acta 40, 70–84
48 Kaczorowski, G.J., Robertson, D.E. and Kaback, H.R. (1979) Biochemistry 18, 3697–3704
49 Viitanen, P., Garcia, M.L., Foster, D.L., Kaczorowski, G.J. and Kaback, H.R. (1983) Biochemistry 22, 2531–2536

50 Wright, J.K., Seckler, R. and Overath, P. (1986) Annu. Rev. Biochem. 55, 225–248
51 Wright, J.K., Dornmaier, K., Mitaku, S., Möröy, T., Neuhaus, J.-M., Seckler, R., Vogel, H., Weigel, U., Jähnig, F. and Overath, P. (1985) Ann. NY Acad. Sci. 456, 326–341
52 Kessler, M. and Semenza, G. (1983) J. Membrane Biol. 76, 27–56
53 Dorando, F.C. and Crane, R.K. (1984) Biochim. Biophys. Acta 772, 273–287
54 Kaunitz, J.D. and Wright, E.M. (1984) J. Membrane Biol. 79, 41–51
55 Page, M.G.P. and West, I. (1982) Biochem. J. 204, 681–688
56 Elferink, M.G.L., Hellingwerf, K.J., Nano, F.E., Kaplan, S. and Konings, W.N. (1983) FEBS Lett. 164, 185–190
57 Wilson, D.M., Putzrath, R.M. and Wilson, T.H. (1981) Biochim. Biophys. Acta 649, 377–384
58 Ahmed, S. and Booth, I.R. (1983) J. Gen. Microbiol. 129, 2521–2529
59 Wright, J.K. (1986) Biochim. Biophys. Acta 854, 219–230