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The lactose/H^+ carrier of *Escherichia coli*: *lacY*^{UN} mutation decreases the rate of active transport and mimics an energy-uncoupled phenotype

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The *Escherichia coli* K12 strain X71-54 carries the *lacY*^{UN} allele, coding for a lactose/H^+ carrier defective in the accumulation of a number of galactosides [Wilson, Kusch & Kashket (1970) *Biochem. Biophys. Res. Commun.* **40**, 1409–1414]. Previous studies proposed that the lower accumulation in the mutant be due to a faulty coupling of H^+ and galactoside fluxes via the carrier. Immunochemical characterization of the carriers in membranes from mutant and parent strains with an antibody directed against the *C*-terminal decapeptide of the wild-type carrier leads to the conclusion that the mutant carrier is similar to the wild-type in terms of apparent M_r, *C*-terminal sequence, and level of incorporation into the membrane. The pH-dependence of galactoside transport was compared in the mutant and the parent. At pH 8.0–9.0, mutant and parent behave similarly with respect to the accumulation of β-D-galactosyl 1-thio-β-D-galactoside and to the ability to grow on the carrier substrate melibiose. At pH 6.0, both the maximal velocity for active transport and the level of accumulation of β-D-galactosyl-1-thio-β-D-galactoside are lower in the mutant. The mutant also is unable to grow on melibiose at pH 5.5. However, at pH 6.0 and low galactoside concentrations, the symport stoichiometry is 0.90 H^+ per galactoside in the mutant as compared with 1.07 in the parent. These observations suggest that symport is normal in the mutant and that the lower rate of transport in the mutant is responsible for the phenotype. At higher galactoside concentrations, accumulation is determined not only thermodynamically but also kinetically, contrary to a simple interpretation of the chemiosmotic theory. Therefore lower rates of active transport can mimic the effect of uncoupling H^+ and galactoside symport. Examination of countertransport in poisoned cells at pH 6.0 reveals that the rate constants for the reorientation of the loaded and unloaded carrier are altered in the mutant. The reorientation of the unloaded carrier is slower in the mutant. However, the reorientation of the galactoside–H^+-carrier complex is slower for substrates like melibiose, but faster for substrates like lactose. These findings suggest that lactose-like and melibiose-like substrates interact with the carrier in slightly different ways.

Mitchell (1973) and Crane (1977) indicated that the energy for the active transport of many solutes may reside in the transmembrane gradients of the electrochemical potential of ions such as H^+ and Na^+. Implicit in these theories is the concept that transport proteins contain binding sites for solutes and the co-transported ion and that solute and ion may be simultaneously conveyed across the membrane through the carrier.

The lactose/H^+ carrier of *Escherichia coli* is a well-studied example of solute/ion symport (recently reviewed by Overath & Wright, 1983; Wright *et al.*, 1984). However, the location of the binding site(s) for H^+ and the mode of H^+ conduction through the protein matrix of the carrier are presently unknown. The interaction of H^+ with the carrier might be perturbed in two ways: chemical modification of the protein or the introduction of mutations into the *lacY* gene coding for the carrier.

Abbreviations used: GalSGal, β-D-galactosyl 1-thio-β-D-galactoside; NpαGal, *p*-nitrophenyl α-D-galactoside; NpβGal, *o*-nitrophenyl β-D-galactoside; MeSGal, methyl β-D-thiogalactoside.

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In either case, an uncoupling of galactoside transport from the simultaneous movement of H^+ will result in the inability of the carrier to concentrate galactosides within the cell.

Wong *et al.* (1970) and Wilson *et al.* (1970) have isolated two mutants which exhibit impaired accumulation of galactosides. Subsequently, the mutation in *E. coli* X71-54 was mapped by Hobson *et al.* (1977) to a region of the *lacY* gene corresponding to the *C*-terminus of the carrier or as a deletion between the *lacY* and *lacA* genes. These mutations are designated Y^{UN}, for energy-uncoupled phenotype.

Both of the independently isolated mutants behave similarly: the accumulation of galactoside in the mutant is 10–20% of that in the parent. The addition of galactosides to cell suspensions results in a smaller uptake of H^+ by the mutant than in the parent (West & Wilson, 1973). The defect in the mutant is more pronounced at acid pH (Wilson & Kusch, 1972). Contradistinctively, the rate of transport and hydrolysis of NpβGal, a common index of carrier function, is somewhat higher in the mutants than in the parents (Wong *et al.*, 1970; Wilson & Kusch, 1972).

The mechanistic interpretation offered to explain these observations is that the transport of galactosides in the mutants is uncoupled from the obligate co-transport of H^+ and, hence, from a source of energy. Further studies suggest that this uncoupling, and the consequent depressed accumulation of galactosides, are kinetically manifested by an increased rate of efflux in the mutants (Wong *et al.*, 1970; Wilson *et al.*, 1970).

Two developments prompt us to re-examine the mutant carrier. Antibodies against the synthetic *C*-terminal decapeptide of the carrier are available (Seckler *et al.*, 1983; Seckler & Wright, 1984). These can be employed to probe the *C*-terminus of the mutant, where the mutation may reside. Additionally, the kinetic mechanism of active transport is now known (J. K. Wright, unpublished work; Wright *et al.*, 1984). Importantly, the carrier is kinetically designed to self-limit galactoside accumulation at high substrate levels (see below), perhaps as a form of protection for the cell. Studies of galactoside transport can, therefore, map the effect of the Y^{UN} mutation to a specific point in the symport cycle.

Theory

The catalytic cycle for galactoside (G)/H^+ symport appears in the upper portion of Fig. 1.

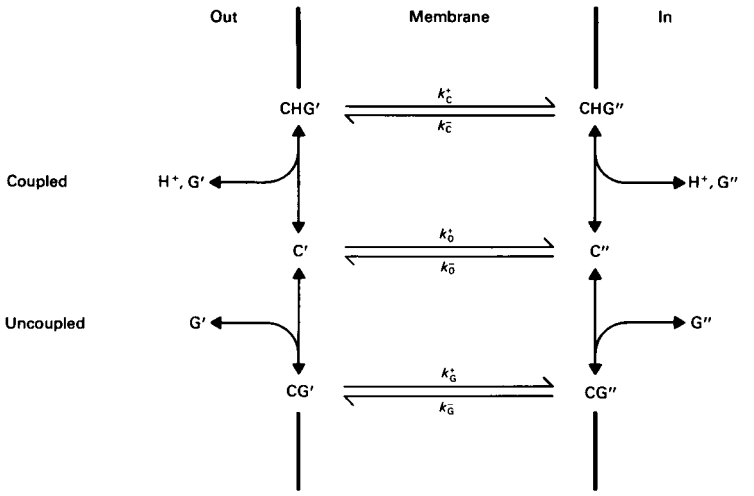


Fig. 1. *Comparison of transport cycles for H^+/galactoside symport and carrier-mediated facilitated diffusion*
During coupled transport of H^+ and galactoside (upper cycle), outward-oriented carrier, C′, binds H^+ and galactoside (G′) on the outer face of the membrane. The ternary complex CHG′ reorients with rate constant k_C^+ to become the inward-oriented ternary complex CHG″. Galactoside G″ and H^+ are released in the inner compartment. To complete the catalytic cycle, the inward-oriented unloaded carrier C″ reorients with rate constant $k_{\bar{O}}$ to become the outward-oriented form C′. If mutation or chemical modification could uncouple the translocation of galactoside from the obligate co-transport of H^+, the lower cycle describes galactoside transport. In this case, the binary complex CG is permitted to reorient with rate constants designated by k_G^+ and $k_{\bar{G}}$. The upper cycle leads to accumulation of galactoside (G″ > G′), the lower cycle to mere equilibration (G″ = G′). If the uncoupling is only partial, so that galactoside transport is a combination of both cycles, an impaired accumulation of galactoside would be observed. As noted in the text, kinetic factors modulate the ability of the lactose/H^+ carrier to obtain the maximal accumulation expected when transport occurs exclusively via the upper cycle.

According to the chemiosmotic theory, the accumulation ratio for galactoside at equilibrium is:

$$\frac{[G]''}{[G]'} = \exp(-nF\Delta\bar{\mu}_{H^+}/RT) \quad (1)$$

where n is the stoichiometry of symport, $\Delta\bar{\mu}_{H^+}$ is the transmembrane gradient of the electrochemical potential of H^+, F is 96490 A·s·mol^{-1}, R is 8.314 J·mol^{-1}·K^{-1} and T is the absolute temperature. The catalytic cycle for a facilitator of galactoside transport appears in the lower portion of Fig. 1. Because, in this case, galactoside uptake is not coupled to a source of energy at equilibrium, the accumulation ratio is merely:

$$\frac{[G]''}{[G]'} = 1 \quad (2)$$

For the case that a carrier operates partially in a coupled and partially in an uncoupled mode, the accumulation ratio can vary between the extremes of eqns. (1) and (2).

Importantly, however, the equilibrium of eqn. (1) is only attained by the wild-type carrier at very low galactoside concentrations. In fact, the accumulation ratio in the steady state is observed to be a function of the external concentration of galactoside (Rickenberg *et al.*, 1956) according to:

$$\frac{[G]''}{[G]'} = \frac{[G_{in}^{max.}]}{[G]' + K_S} \quad (3)$$

where $G_{in}^{max.}$ is the maximal, internal concentration of galactoside in the steady state and K_S is the half-saturation constant for this process. Additionally, the accumulation depends on the carrier level in the membrane (Maloney & Wilson, 1973; Teather *et al.*, 1980).

Finally, for later reference we note that the lactose/H$^+$ carrier actually recognizes a large number of α- and β-galactosides (Sandermann, 1977). A large body of evidence bespeaks the presence of a single binding site for all galactosides per carrier monomer (Hobson *et al.*, 1977; Teather *et al.*, 1978; Overath *et al.*, 1979; Wright *et al.*, 1981; Wright & Overath, 1984). These galactosides may be divided into two classes, both of which are actively transported: kinetically simple substrates (e.g. melibiose) have an affinity for the carrier which is nearly independent of $\Delta\bar{\mu}_{H^+}$. Kinetically complex substrates (e.g. lactose) have an apparent affinity for the carrier which increases in the presence of $\Delta\bar{\mu}_{H^+}$ (Wright *et al.*, 1981). Because these two classes of substrates interact with the carrier in slightly different ways, kinetic studies should include a representative of each.

Therefore, at galactoside levels of interest to microbiologists in determining phenotypes, the accumulation is both thermodynamically and kinetically determined, namely by the degree of coupling, by the level of carrier in the membrane, and by the turnover number for active transport. The purpose of the present paper is to determine which of these three factors is responsible for reduced accumulation in the *lac Y^{UN}* mutant X71-54.

Material and methods

Strains

The parental strain *E. coli* X71(i$^-$z$^+$y$^+$a$^-$, *ProC*, *trp*$^-$, B$_1^-$, F$^-$) and the mutant X71-54 carrying the Y^{UN} allele were obtained from Dr. T. H. Wilson, Harvard University, Boston, MA, U.S.A. Both strains were grown in Cohen–Rickenberg minimal salts medium containing proline (100 μg/ml), tryptophan (10 μg/ml), thiamin (0.5 μg/ml), 1 mM-$MgSO_4$ and 10 mM-disodium succinate. When the stock solutions of proline and tryptophan were sterilized by filtration and not autoclaved, the generation time of both strains at 37°C was 90 min. The phenotypes of parent and mutant were routinely tested on minimal melibiose plates, supplemented as indicated above (Hobson *et al.*, 1977), on which the mutant does not grow at 42°C.

Plates for comparing growth at different pH values contained 1.5 mg of melibiose/ml and twice the normal buffer concentration at pH 7.0 and supplemented with 30 mM-Mes at pH 5.5 and with 30 mM-Taps at pH 8.5.

Immunochemical methods

The polyclonal antibody raised against the synthetic *C*-terminal decapeptide of the wild-type carrier in rabbits was described by Seckler *et al.* (1983). Electrotransfer of proteins from polyacrylamide gels and immunoblotting were conducted as described by Wright & Overath (1984), with the following modifications. The transfer was performed in 25 mM-Na_2HPO_4/NaH_2PO_4, pH 6.5, and the second antiserum was goat anti-rabbit immunoglobulin, conjugated to alkaline phosphatase (Medac, Hamburg, Germany). The nitrocellulose filters were developed as described by Blake *et al.* (1984). The dilutions of first and second antibody were 1:500 and 1:1000. To compare the amount of carrier in membrane fractions (VI in Seckler & Wright, 1984) from parent and mutant, the carrier bands were cut from the nitrocellulose filter after they had been revealed and further cut into smaller (about 1 cm × 1 cm) pieces. These were shaken for 30 min at 37°C in 2 ml of *p*-nitrophenyl phosphate (0.5 mg/ml, analysis grade); Sigma, München, Germany)/50 mM-$NaHCO_3/Na_2CO_3$/2 mM-$MgSO_4$, pH 9.5. The reaction was halted

by the addition of 50μl of 5M-NaOH and 50μl of 0.5M-sodium EDTA. The absorbance was measured immediately at 420nm, and the results evaluated as described by Blake *et al.* (1984).

Transport measurements

The basic methodology has been described by Wright *et al.* (1981) and Wright & Overath (1984). It is crucial that the stopping buffer contains 2mM-$HgCl_2$ to inhibit the carrier and prevent loss of galactoside. Iso-osmolarity in countertransport experiments was maintained by the addition of glycerol to the stopping buffer. Uptake was halted after 3, 5, or 10s or as otherwise indicated. For the measurement of active transport, cells were aerated for 2min in the presence of 10mM-sodium succinate before the addition of galactoside. Cells were subjected to the EDTA treatment of Lieve (1965) and were washed in 50mM-K_2HPO_4/KH_2PO_4 containing 20mM-Mes at pH6.0, 20 mM-Tris at pH7.0 and 8.0, and 20mM-Taps at pH9.0. The accumulation in the steady state and $\Delta\bar{\mu}_{H^+}$ were measured by flow dialysis (Ramos *et al.*, 1976; Wright *et al.*, 1984).

To measure countertransport, EDTA-treated cells were washed in buffer at pH6.0 containing 10mM-melibiose or 10mM-MeSGal, resuspended in the same solution containing galactoside, and left for 1h at ambient temperature at a concentration of about 1.5mg of protein/ml. Cells were then poisoned with 20μM-carbonyl cyanide *m*-chlorophenylhydrazone/10mM-NaN_3/1mM-tetraphenylphosphonium chloride (added last), and concentrated to 15–20mg of protein/ml by centrifugation. Countertransport was initiated by diluting 20μl of preloaded cells into 2ml of buffer containing 1μM-[6,6′-$^3H_2$]GalSGal (3kBq/ml) or 0.1mM-[*methyl*-^{14}C]MeSGal (0.8kBq/ml), and the uptake halted at the indicated time with stopping buffer.

The transport and hydrolysis of NpβGal was measured discontinuously at pH6.0 as described by Wright *et al.* (1981). In poisoned cells, the concentration of carbonyl cyanide *m*-chlorophenylhydrazone was decreased to 5μM to avoid interference with the absorbance measurements.

Results

Immunochemical analysis of the C-terminus of mutant and wild-type carriers

The *lac* Y^{UN} mutation resides in a region of the gene encoding a section of the polypeptide at or near the *C*-terminus of the carrier. The *C*-terminus of the wild-type carrier is an immunodominant epitope (Seckler & Wright, 1984) and cross-reacts strongly with antibodies prepared against the synthetic *C*-terminal decapeptide (Seckler *et al.*, 1983). Hence these antibodies can be used to examine the *C*-terminus of the mutant. When the proteins in membrane envelopes from mutant and parent strains are subjected to electrophoresis in the presence of sodium dodecyl sulphate and transferred to nitrocellulose filters, broad bands, migrating with an apparent M_r of 31000 and typical for the lactose/H^+ carrier, are found (arrow in Fig. 2; cf. Seckler & Wright, 1984) after exposure to antibody directed against the synthetic *C*-terminal decapeptide of the wild-type carrier in each case.

The relative amount of carrier in cell envelopes from mutant and parent strains can be determined in the following way (Fig. 3). After electrophoresis the membrane proteins are transferred to a nitrocellulose filter. The filter is bathed in antibody directed against the synthetic *C*-terminal decapeptide, washed, and bathed in the second antibody conjugated to alkaline phosphatase. After washing, the carrier bands are revealed as described in the Materials and methods section. The bands are cut out of the filter, placed in solutions of a chromo-

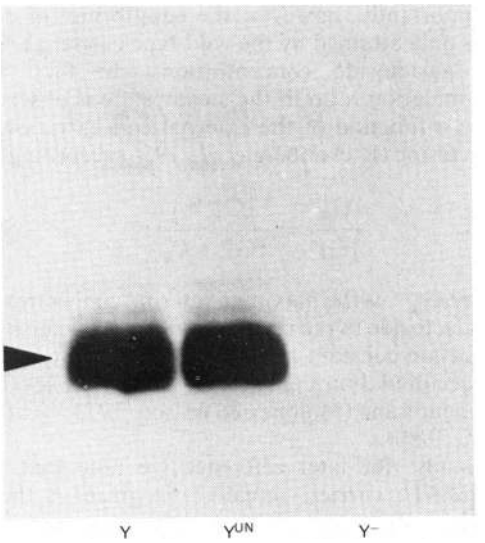


Fig. 2. *Quantification of normal (Y) and mutant (Y^{UN}) carriers after electrophoresis of crude membranes in the presence of sodium dodecyl sulphate*

Proteins were electrotransferred to a nitrocellulose filter. The filter was bathed in rabbit antibody directed against the synthetic *C*-terminal decapeptide and subsequently in goat anti-(rabbit immunoglobulin) antibody conjugated to alkaline phosphatase. The cross-reacting proteins are revealed by incubating the filter in the phosphatase substrate 4-chloroindol-3-yl phosphate in the presence of Nitro Blue Tetrazolium. The broad band migrating with an apparent M_r of 31000 (arrow) corresponds to the lactose/H^+ carrier. As a control, an equal amount of membrane lacking the carrier (Y^-) was also loaded on the gel. No cross-reaction was observed.

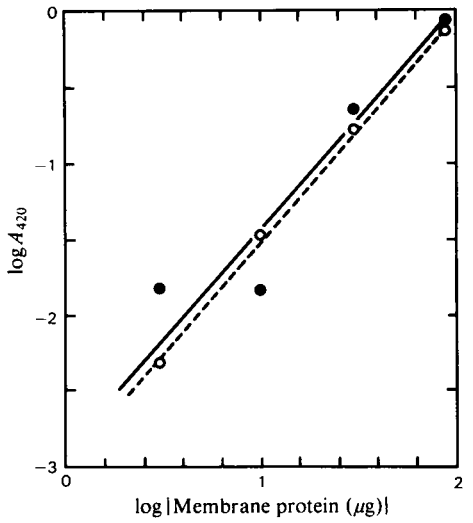


Fig. 3. *Comparison of the amount of carrier in membranes from the parent* (●) and the mutant (○)
Different amounts of membrane were subjected to electrophoresis in the presence of sodium dodecyl sulphate, and proteins were transferred to nitrocellulose filters. After being revealed (Fig. 2), bands were cut from the filter, incubated with *p*-nitrophenyl phosphate, a substrate of the phosphatase conjugated to the second antibody, and finally the amount of product formed was measured by the absorbance increase at 420nm (A_{420}).

genic substrate of the phosphatase, and shaken. The colour development is related to the amount of carrier present (Fig. 3; cf. Blake *et al.*, 1984). No binding of antibody to the filter can be detected if the membrane samples are pretreated with carboxypeptidase A to remove the epitope (Seckler & Wright, 1984). Significantly, the curves for the membranes from the mutant and from the parent are nearly superimposable, indicating that, to within 15%, the same amount of carrier is present in both membranes.

Therefore the mutant and wild-type carriers have similar M_r and *C*-termini and are present in nearly equal amounts in the membrane. This last finding eliminates the possibility that the lower accumulation of galactoside in the mutant be due to a lower level of carrier in the membrane (cf. Maloney & Wilson, 1973).

pH-dependence of defect in transport

Wilson & Kusch (1972) demonstrated that parent and mutant accumulate MeSGal equally well at pH8.2, while at pH5.8 the mutant accumulates only 10–20% as much as the parent. A similar behaviour is observed with GalSGal (Figure 4*a*; ●, parent; ○, mutant). At pH6.0, where the difference in GalSGal accumulation in the mutant and parent is the greatest, $\Delta\bar{\mu}_{H^+}$ is measured and the stoichiometry of symport *n* is determined from:

$$n = -\frac{\Delta\mu_G}{\Delta\bar{\mu}_{H^+}} \quad (4)$$

where μ_G, the transmembrane gradient of the chemical potential of galactoside, is given by:

$$\Delta\mu_G = \frac{RT}{F}\ln\left(\frac{[G]''}{[G]'}\right) \quad (5)$$

At pH6.0, $\Delta\bar{\mu}_{H^+}$ amounts to −147mV in the mutant and −154mV in the parent, essentially identical values (Fig. 4*a*, □ and ■ respectively). The 186-fold accumulation (113mV) in the mutant corresponds to a symport stoichiometry of 0.90 H^+ per GalSGal molecule; the 652-fold (165mV) accumulation in the parent corresponds to a symport stoichiometry of 1.07 H^+ per GalSGal molecule. Thus the symport stoichiometry in the mutant is close to 1. A mere 16% decrease in the stoichiometry could not explain the observations of West & Wilson (1973). However, a decreased rate of active transport in the mutant could. The difference between parent and mutant must, therefore, reside in their kinetic properties.

The maximal velocity of transport, $V_{max.}$, of GalSGal in the mutant and parent are similar at pH9 (Fig. 4*b*; ▽ and ▼ respectively). In the parent, $V_{max.}$ initially decreases with increasing pH and then remains nearly constant between pH6 and 8 at about 40nmol/min per mg of protein. In the mutant, $V_{max.}$ goes through a small maximum at pH8.0 and decreases to 6.5nmol/min per mg of protein at pH6.0. Whereas the half-saturation constants for active transport in the parent lie near 80μM (Fig. 4*b*, ▲), those for active transport in the mutant are significantly lower at 10–20μM (Fig. 4*b*, △).

Lastly, the ability of parent and mutant to grow on a substrate of the carrier can be correlated with the above transport measurements. Hobson *et al.* (1977) found that parent and mutant could be most readily distinguished by testing the strains for growth on solid minimal-medium containing melibiose. The medium of such plates is pH7. Indeed, the wild-type grows well on solid medium at pH7.0 (Fig. 4*c*), whereas the mutant does not grow at all. If the melibiose plates are prepared with buffer adjusted to pH5.5, the parent grows more rapidly than at pH7.0, whereas the mutant fails to grow at all. At pH8.5, where the maximal velocities of GalSGal active transport are similar in parent and mutant, both strains grow well.

These observations also suggest that the pH optimum for the active transport of galactoside by the mutant is actually at pH8.5. Most importantly, the impaired accumulation of galactosides in the

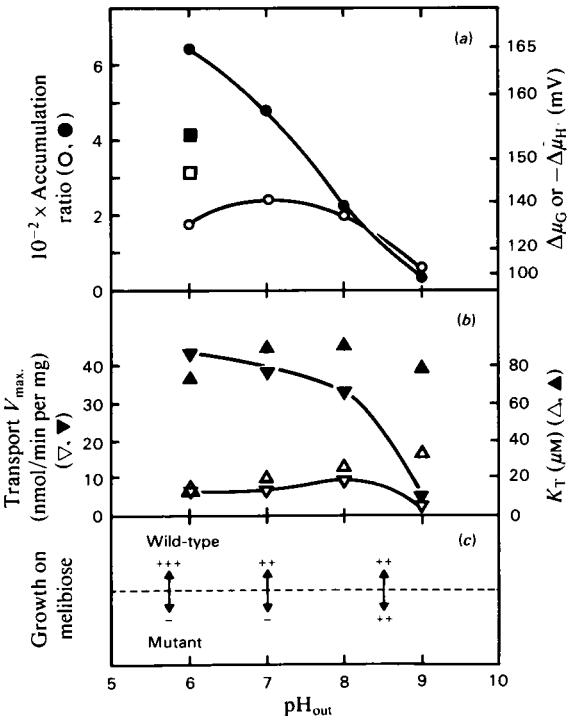


Fig. 4. *pH-dependence of various parameters in parent and mutant*

(*a*) The accumulation ratio of GalSGal in the steady state was measured by flow dialysis (●, parent; ○, mutant) at an initial, external concentration of GalSGal of 1 μM. The values of $\Delta\bar{\mu}_{H^+}$ were also measured at pH6.0 in the parent (■) and in the mutant (□). The scale on the right expresses values in mV. (*b*) The maximal velocity [$V_{max.}$ (▼, parent; ▽, mutant), in units of nmol of GalSGal/min per mg of cell protein] and half-saturation constant [K_T (▲, parent; △, mutant), unit μM] for the active transport of GalSGal in cells was measured. Continuous lines connect values of $V_{max.}$. (*c*) The parent (upper panel) grows well on solid medium with melibiose, a substrate of the carrier, as a carbon source (++) and very well at pH5.5 (+++). The mutant (lower panel) does not grow either at pH5.5 or 7.0 (−), but grows at pH8.5 (++).

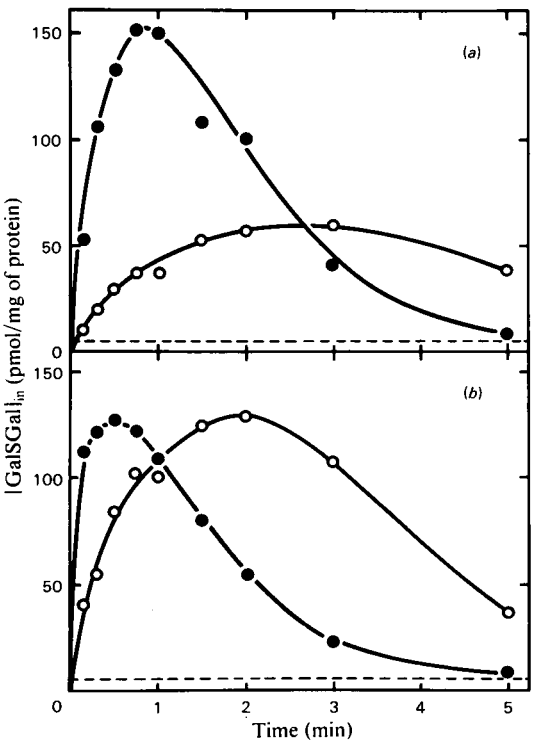


Fig. 5. *Countertransport in poisoned cells at pH6.0*

(*a*) Parent (●) and mutant (○) were preloaded with 10mM unlabelled melibiose, a kinetically simple galactoside, and diluted 100-fold into buffer containing 1 μM labelled GalSGal, also a simple substrate. (*b*) Parent (●) and mutant (○) were preloaded with 10mM unlabelled MeSGal, a kinetically complex galactoside, and diluted 100-fold into buffer containing 1 μM labelled GalSGal.

mutant is apparently related to a decrease in the maximal velocity of active transport.

Transport of various galactosides at pH6.0 in the presence of $\Delta\bar{\mu}_{H^+}$

Although parent and mutant cells contain β-galactosidase, lactose active transport can be measured. The sampling time must be 10s or less, and transport must be halted by the addition of $HgCl_2$, which inhibits the carrier, thus trapping lactose and its hydrolysis products in the cell. The maximal velocity of lactose transport so measured in the mutant is 62nmol/min per mg of cell protein, a factor of 5 slower than in the parent (Table 1). The half-saturation constant, K_T, is smaller in the mutant than in the parent. Similarly, the maximal velocity for the active transport of MeSGal is smaller in the mutant than in the parent by a factor of 2.8 and K_T is somewhat smaller in the mutant (Table 1).

When the transport and hydrolysis of NpβGal is measured, the maximal velocity in the mutant is 1.4-fold greater than in the parent (Table 1), in agreement with the observations of Wilson & Kusch (1972). Thus we face a dilemma: why does the mutant transport some substrates slower and, nevertheless, others faster than the parent? To clarify this point, the facilitated diffusion of galactosides was measured. Here, the intrinsic ability of the carrier to catalyse substrate translocation is studied without the complication of the effect of $\Delta\bar{\mu}_{H^+}$ on the carrier.

Table 1. *Comparison of kinetic parameters for galactoside transport by the mutant (strain X71-54) and the parent (X71) at pH6.0*

Substrate	Mutant		Parent	
	$V_{max.}$ (nmol/min per mg of protein)	K_T (mM)	$V_{max.}$ (nmol/min per mg of protein)	K_T (mM)
Lactose	62	0.25	316	0.59
MeSGal	121	1.2	343	2.6
GalSGal	6.5	0.011	43	0.062
NpβGal	410	1.2	283	0.9
NpβGal*	320	0.8	190	1.1
GalSGal (Melibiose)*†	8	0.019	56	0.046

* Cells were poisoned with 10μM-carbonyl cyanide *m*-chlorophenylhydrazone/10mM-NaN_3/1mM-tetraphenylphosphonium chloride.

† Countertransport in cells loaded with 10mM unlabelled melibiose.

Table 2. *Comparison of parameters for countertransport by poisoned cells at pH6.0*

The initial-velocity ('v_0') values and the internal concentrations of labelled substrate at the peak of the overshoot in the mutant ('peak') are reported as percentages of the values observed for the parent.

External, labelled substrate	Internal, unlabelled substrate: Melibiose (simple)		MeSGal (complex)	
	v_0	peak	v_0	peak
GalSGal (simple)	18	37	35	103
MeSGal (complex)	34	93	208	170

Facilitated diffusion of galactosides at pH6.0

In cells, $\Delta\bar{\mu}_{H^+}$ can be reduced by incubation with carbonyl cyanide *m*-chlorophenylhydrazone, tetraphenylphosphonium chloride, and NaN_3 together. The value of $\Delta\bar{\mu}_{H^+}$ decreases from values of nearly -200mV to -20 to -30mV (J. K. Wright, unpublished work). Under these conditions, galactoside transport may be viewed, to a good approximation, as facilitated diffusion.

NpβGal transport and hydrolysis in both the parent and mutant strains remain essentially unchanged after collapsing $\Delta\bar{\mu}_{H^+}$ (Table 1). The maximal velocity in the mutant is 1.7-fold faster than in the parent. The faster rate of NpβGal transport in the mutant is, therefore, not greatly dependent on $\Delta\bar{\mu}_{H^+}$.

Another form of facilitated diffusion catalysed by the carrier is countertransport. Here a labelled, external galactoside is exchanged for an internal, unlabelled galactoside.

Wright & Overath (1984) have demonstrated that GalSGal–melibiose countertransport is a reliable assay for carrier function in the facilitated diffusion mode. The kinetic parameters for countertransport in poisoned cells resemble those for GalSGal active transport (Table 1): the maximal velocity for countertransport in the mutant is 7-fold smaller than that in the parent, and K_T is also lower in the mutant (Table 1).

The observation that GalSGal–melibiose countertransport is much slower in the mutant than in the parent appears at first glance to contradict the observation that MeSGal–MeSGal countertransport (Wilson & Kusch, 1972) and NpβGal transport in poisoned cells are more rapid in the mutant than in the parent.

However, there is an interesting correlation based on the observations of Wright *et al.* (1981) regarding various substrates. Both NpβGal and MeSGal are kinetically complex substrates exhibiting $\Delta\bar{\mu}_{H^+}$-dependent apparent affinities for the carrier, whereas GalSGal and melibiose are kinetically simple substrates with $\Delta\bar{\mu}_{H^+}$-independent affinities for the carrier. There is, therefore, *a priori*, reason to suspect that these two types of galactosides interact in different ways with the carrier. To resolve this problem, countertransport is measured in poisoned cells by using different combinations of kinetically simple and complex substrates.

Countertransport of simple and complex galactosides at pH6.0

In the first series of experiments, the influx of a kinetically simple substrate, GalSGal, during countertransport was measured in poisoned cells that had been preloaded with an unlabelled, simple substrate melibiose (Fig. 5*a*) or with an unlabelled

complex substrate, MeSGal (Fig. 5*b*). The curves for countertransport in the parent and in the mutant are quite distinct.

In the case of [^{3}H]GalSGal–melibiose (termed simple–simple) countertransport, the initial velocity, v_0, in the mutant (Fig. 5*a*, ○) is only 18% of that in the parent (Fig. 5*a*, ●; Table 2); the peak of the overshoot in the mutant is only 37% of that found in the parent (Fig. 5*a*, Table 2). In the case of [^{3}H]GalSGal–MeSGal (termed simple–complex) countertransport, the initial velocity in the mutant (Fig. 5*b*, ○) increased to 35% of that of the parent (Fig. 5*b*, ●; Table 2). The peak of the overshoot in the mutant significantly increased to 103% of that in the parent (Fig. 5*b*, Table 2).

In the second series of experiments, the influx of a kinetically complex substrate, MeSGal, during countertransport was measured in poisoned cells that had been preloaded with an unlabelled simple substrate (Fig. 6*a*) or with an unlabelled complex substrate (Fig. 6*b*).

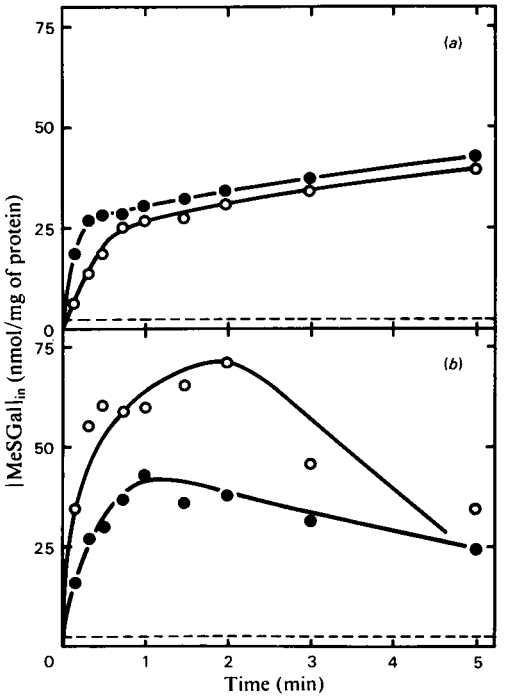


Fig. 6. *Countertransport in poisoned cells at pH 6.0*
(*a*) Parent (●) and mutant (○) were preloaded with 10 mM unlabelled melibiose, a kinetically simple galactoside, and diluted 100-fold into buffer containing 0.1 mM-labelled MeSGal, a complex substrate. (*b*) Parent (●) and mutant (○) were preloaded with 10 mM-unlabelled MeSGal and diluted 100-fold into buffer in which the final concentration of labelled MeSGal was 0.1 mM.

In the case of [^{14}C]MeSGal–melibiose (termed complex–simple) countertransport (Fig. 6*a*; ●, parent; ○, mutant), the initial velocity in the mutant is 34% of that in the parent; at the highest point in the overshoot measured, the peak in the mutant is 93% of that in parent (Fig. 6*a*, ○ versus ●, Table 2). In the case of [^{14}C]MeSGal–MeSGal (termed complex–complex) countertransport (Fig. 6*b*; ●, parent; ○, mutant), the initial velocity in the mutant is 208% of that in the parent; the peak of the overshoot in the mutant is 170% of that in the parent (Fig. 6*b*, Table 2).

Inspection of Table 2 reveals an interesting correlation: the countertransport (i.e, exchange) of two kinetically simple galactosides is poorer in the mutant than in the parent in terms of initial velocity and of the peak of the overshoot. The countertransport of two kinetically complex galactosides is better in the mutant than in the parent by the same criteria. Simple–complex or complex–simple countertransport are intermediate cases.

Discussion

Experimental observations reported here permit a localization of the effect of the Y^{UN} mutation in strain X71-54 on the symport cycle in Fig. 1.

(*a*) The mutant carrier in strain X71-54 evinces the same electrophoretic mobility in the presence of sodium dodecyl sulphate as the carrier in the parent strain. The mutant carrier cross-reacts with antiserum prepared against the synthetic *C*-terminal decapeptide of the native carrier, suggesting that these sequences are similar in wild-type and mutant carriers. Therefore the mutation is not a deletion, frameshift or a premature termination, which alters or eliminates this determinant in the *C*-terminus.

This conclusion is supported by two other observations. Seckler & Wright (1984) demonstrated that removing three to seven of the *C*-terminal residues of the wild-type carrier suppressed antibody binding without altering either galactoside binding or countertransport. This suggests that the Y^{UN} mutation in strain X71-54 must change an amino acid at a more internal position.

(*b*) Quantification of the binding of the anti-peptide antibody to parent and mutant carriers discloses that, within 15%, the expression of both *lac Y* and *lac* Y^{UN} is identical (Fig. 3). The mutation present in the *lac* Y^{UN} allele does not affect the biosynthesis of the mutant carrier or its insertion into the membrane. Most importantly, this finding eliminates the possibility that the depressed accumulation in the mutant is due to a decrease in the number of carriers (cf. Maloney & Wilson, 1973).

(*c*) The defect in the accumulation of the kinetically simple substrate GalSGal is pH-dependent, as Wilson & Kusch (1972) also demonstrated for the kinetically complex substrate MeSGal. The mutation thus impairs the active transport of both types of substrates.

A comparison of the accumulation of GalSGal in the steady state, of initial-velocity kinetic parameters for the active transport of GalSGal, and of the ability to grow on melibiose as a function of pH reveals the following trend: the defects in the mutant correlate most strongly with the decrease in the maximal velocity of active transport (Fig. 4).

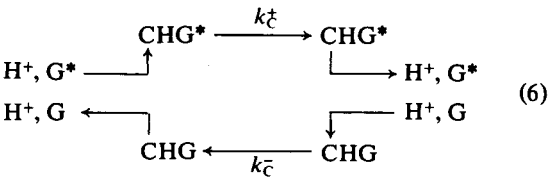
(*d*) At pH 6.0, where the difference between parent and mutant is most pronounced, the symport stoichiometry in the mutant is 0.90 H$^+$ per galactoside molecule. The differences in the apparent stoichiometries for parent and mutant can be interpreted in two ways. The effect of the mutation could be to lower the stoichiometry by 16% in the mutant. This small change adequately explains the depressed galactoside accumulation (cf. eqn. 4). However, the galactoside-induced flow of H$^+$ in the mutant due to symport would be 84% of that in the parent, contrary to the observation of West & Wilson (1973). Alternatively, the symport stoichiometry in the mutant could be normal, but a lower maximal velocity of symport prevents the attainment of the maximal concentration gradient of galactoside (cf. eqn. 3). This lower maximal velocity in the mutant would explain the results of West & Wilson (1973).

Therefore, of the three possible causes for the defective accumulation in the mutant, namely lower carrier levels, galactoside transport without H$^+$, or reduced turnover number for symport, the latter kinetic effect appears the likeliest choice.

The Y^{UN} mutation causes complex alterations in the symport cycle. For instance, whereas the active transport of GalSGal and MeSGal are slower in the mutant than in the parent (Table 1), GalSGal countertransport is slower (Table 1) and MeSGal countertransport is faster (Wilson & Kusch, 1972) than in the parent.

(*e*) The effects of the mutation appear to be opposite for different galactosides. Because of correlations discussed in the Results section and as a working hypothesis, we posit that galactosides designated by Wright *et al.* (1981) as kinetically complex ($\Delta\bar{\mu}_{H^+}$-dependent affinity for the carrier) will exhibit more rapid countertransport or exchange in the mutant than in the parent and that galactosides designated simple ($\Delta\bar{\mu}_{H^+}$-independent affinity for the carrier) will exhibit slower countertransport or exchange in the mutant than in the parent.

The catalytic cycle for countertransport is initially an exchange:



(6)

where G* is the external, labelled galactoside and G is the internal, unlabelled galactoside, k_C^+ and k_C^- are the forward and reverse rate constants for the translocation of galactoside in the ternary complex. Wright *et al.* (1981) and Wright *et al.* (1984) have presented evidence that the translocation rate constants are rate-determining. Therefore the faster or slower rates of countertransport are interpreted as increases or decreases in k_C^+ and k_C^-. Examination of the results presented here and in previous studies suggests the following generalizations.

(*f*) The rate constant for the reorientation of ternary complex containing a kinetically simple galactoside is smaller in the mutant than in the parent. This explains why GalSGal–melibiose countertransport (Fig. 5*a*) is slower in the mutant. The mutation decreases k_C^+ for GalSGal and k_C^- for melibiose.

(*g*) The rate constant for the reorientation of a ternary complex containing a kinetically complex galactoside is larger in the mutant. This explains why MeSGal–MeSGal countertransport is faster in the mutant than in the parent (Fig. 6*b*). The values of k_C^+ and k_C^- for MeSGal are larger in the mutant.

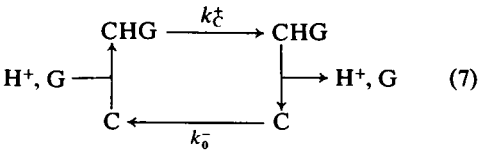
A comparison of the countertransport of kinetically simple against kinetically complex galactosides, or the reverse, permits the above interpretations to be tested. Because the translocation step for the simple substrate is slower and that for the complex substrate is faster in the mutant, intermediate effects are expected. The countertransport in the mutant under these conditions should not be as slow as seen for the exchange of simple galactosides or as rapid as the exchange of complex galactosides. As anticipated, the relative rates of simple–complex or complex–simple countertransport as well as the relative height of the overshoot in the mutant as compared with those in the parent lie between the relative values for simple–simple and complex–complex countertransport (Table 2).

Recent studies by Smith-Gill *et al.* (1984) on lysozyme and Quiocho & Vyas (1984) on the arabinose-binding protein disclose that sugars may bind to a single binding site so that some functional groups of similar substrates interact with different parts of the binding site. Slightly different interactions of simple and complex galactosides with the carrier may explain the different effects of the mutation on the translocation of these two classes of substrates.

(*h*) Inspection of the data in Table 1 indicates that the lower rate of GalSGal transport and the higher rate of NpβGal transport in the mutant are observable even if $\Delta\bar{\mu}_{H^+}$ is collapsed. Therefore these changes in the mutant do not require the presence of $\Delta\bar{\mu}_{H^+}$ for their expression (cf. Table 2).

(*i*) The maximal velocities for the active transport of simple and complex galactoside are slower in the mutant (Table 1), with the exception of NpβGal, which is discussed below, than in the parent. As indicated in the introduction, a lower turnover number for active transport is postulated to decrease the efficiency of the conversion of $\Delta\bar{\mu}_{H^+}$ into $\Delta\mu_G$ without altering the intrinsic ability of the carrier to respond to $\Delta\bar{\mu}_{H^+}$ (cf. Overath & Wright, 1983). Because $k_C^{\ddagger}$ and $k_{\bar{C}}$ for MeSGal are increased in the mutant, the slower rate of MeSGal active transport must be due to the effect of the mutation on yet another step.

The catalytic cycle for active transport is:



(7)

where $k_{\bar{0}}$ is the reorientation step for the unloaded carrier. This step completes the influx cycle, whereas $k_{\bar{C}}$ completes the cycle for the exchange portion of countertransport in eqn. (6). Therefore, to decrease the rate of active transport of all galactosides, the mutation must also decrease $k_{\bar{0}}$.

A comprehensive model for the effects of the Y^{UN} mutation on the catalytic cycle can be presented (Fig. 7). The magnitude of the rate constants for the reorientation of the unloaded carrier (C), of the ternary complex (CHL) with a lactose-like ($\Delta\bar{\mu}_{H^+}$-dependent affinity) substrate, and of the ternary complex (CHT) with a GalSGal-like ($\Delta\bar{\mu}_{H^+}$-independent affinity) substrate is roughly indicated by the length of the arrows. The Y^{UN} mutation in strain X71-54 causes one rate constant to increase and two to decrease. Because the active transport of simple and complex galactosides involves the reorientation of the unloaded carrier (C″→C′), this mode of transport will be slow for all substrates in the mutant. Countertransport or exchange proceeding by reorientation of ternary complexes will be faster or slower in the mutant depending on whether complex or simple galactosides are involved.

Interesting in this context is the fact that the binding of a monoclonal antibody to the carrier induces changes in lactose transport (Carrasco *et al.*, 1984) similar to those reported here. Perhaps similar conformational changes are induced in the carrier by the mutation and by the binding of this antibody.

The model in Fig. 7 is capable of explaining all previous observations on the mutant X71-54. In the initial report by Wilson *et al.* (1970), the higher rate of the transport and hydrolysis of NpβGal in

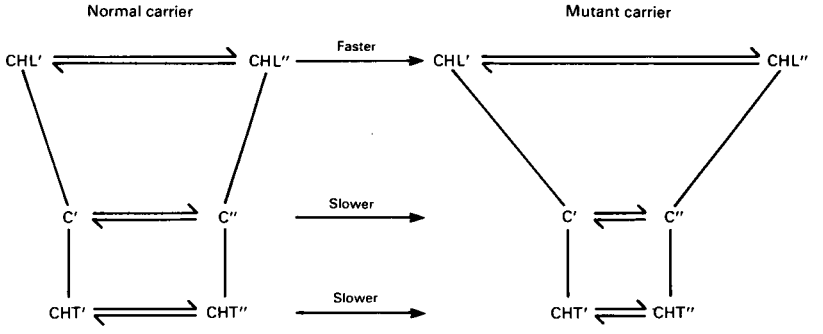


Fig. 7. *Model for the effect of the Y^{UN} mutation in strain X71-54*

In the normal lactose/H^+ carrier in the parent, the rate for the reorientation of the ternary complex CHL with lactose-like (kinetically complex) substrate is probably somewhat faster than that for reorientation of the unloaded carrier, C′, or for ternary complexes, CHT, with GalSGal-like (kinetically simple) substrates. By using the notation in Fig. 1, k_C for lactose is somewhat larger than k_0 or k_C for GalSGal. Galactoside transport in the mutant appears to be well coupled with H^+ transport. Therefore the rate constant for the translocation of galactoside uncoupled from H^+ symport (k_G in Fig. 1) must be small or zero. The Y^{UN} mutation changes three pairs of rate constants for the reorientation of various carrier species: the rate constant for the reorientation of ternary complexes CHL with lactose-like substrates increases in the mutant. The rates of the reorientation of the unloaded carrier C and of ternary complexes CHT with GalSGal-like substrates are smaller in the mutant. The transport of both lactose-like and GalSGal-like galactoside is slower in the mutant than in the parent, because the reorientation of the unloaded carrier k_0 is slower in the mutant. However, the exchange of lactose-like substrates is faster in the mutant.

the mutant was noted. NpβGal, like MeSGal and lactose, is a kinetically complex substrate (Wright *et al.*, 1981). Because only transport in the exchange mode is faster in the mutant, NpβGal$\rightleftharpoons$ transport must be an exchange, perhaps NpβGal–galactose exchange, as previously suggested by Wright *et al.* (1981). The report of more rapid efflux of MeSGal in the mutant can also be understood (Fig. 2 in Wilson *et al.*, 1970). Here unlabelled MeSGal was present in the external medium to prevent recapture of labelled substrate. Because MeSGal is a complex substrate and because actually efflux *during exchange* was measured, the rate was faster in the mutant. On the other hand, GalSGal efflux with or without GalSGal in the external medium should be slower in the mutant. This prediction could be tested. Similarly, the transport data of Wilson & Kusch (1972, Fig. 4 and 5 therein) can be explained by the model in Fig. 7. In the mutant, the slower decrease in the internal concentration of MeSGal after the peak in the overshoot is due to the fact that this is merely efflux and not efflux during exchange, as long as the external concentration of the unlabelled galactoside is subsaturating. This type of efflux contains a reorientation of the unloaded carrier (C′→C″) and, hence, must be slower in the mutant. Lastly, the observations of West & Wilson (1973) that the galactoside-induced flux of H$^+$ in the mutant is smaller than in the parent is due to the fact that the maximal velocity of influx in the mutant is smaller than in the parent (Table 1).

A theoretical analysis of countertransport by a sugar/H$^+$ symporter reveals that the peak height is proportional to the rate of the carrier-mediated exit of the internal, unlabelled galactoside, under certain conditions; that is:

$$\text{Peak} \propto k_{\bar{C}}\,[\text{CHG}]'' \qquad (8)$$

or:

$$\propto k_{\bar{C}}\,[\text{H}^+]_{\text{in}}[\text{G}]_{\text{in}} \qquad (9)$$

(J. K. Wright, unpublished work). Inspection of Figs. 5(*a*) and 6(*b*) discloses that the strain with the larger k_C in the model in Fig. 7 exhibits the higher overshoot. More importantly, Seto-Young *et al.* (1984) demonstrated that, in parent and mutant, the overshoot peak decreased with increasing pH. Because this effect is due to the binding of the symported H$^+$ to the carrier (eqn. 8), the similarity of this decrease in mutant and parent indicates that H$^+$ symport occurs during countertransport in both strains. This supports our contention that H$^+$/galactoside coupling is hardly altered in the mutant (Fig. 4*a*).

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