*Biochem. J.* (1973) **132**, 587–592
*Printed in Great Britain*



# Stoicheiometry of Lactose–Protein Symport Across the Plasma Membrane of *Escherichia coli*

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(*Received* 8 *December* 1972)

The rate of [$^{14}$C]lactose inflow into anaerobic, non-metabolizing, *Escherichia coli* has been correlated with the rate of the simultaneous effective inflow of $H^+$. It is shown that the initial rates of the two transport processes are essentially the same, and it is concluded that the two processes are strictly coupled with a 1:1 stoicheiometry.

Recent observations on the specific translocation of β-galactosides across the plasma membrane of non-metabolizing *Escherichia coli* (West, 1970; West & Mitchell, 1972; West, 1973) have indicated that the carrier system for β-galactoside uptake previously known as β-galactoside permease (Rickenberg *et al.*, 1956) is a symporter system that catalyses the tightly coupled translocation of $H^+$ ions with β-galactoside molecules (or a β-galactoside/$OH^-$ antiporter system effectively catalysing the same overall reaction).

The object of the work described in the present paper was to characterize the β-galactoside porter system further by measuring the stoicheiometric relationship between the number of lactose molecules and the effective number of $H^+$ ions participating in the porter-coupled reaction.

As before (West & Mitchell, 1972), the movement of $H^+$ in one direction and the movement of $OH^-$ in the opposite direction are both described as 'effective' movements of $H^+$, as they cannot be distinguished in the present study.

## Materials and Methods

### *Reagents*

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr. P. G. Heytler of E. I. du Pont de Nemours and Co., Inc. (Wilmington, Del., U.S.A.); carbonic anhydrase was obtained from Sigma London Chemical Co. Ltd. (Kingston-upon-Thames, Surrey, U.K.); [1-$^{14}$C]lactose (14.9 μCi/μmol) was obtained from The Radiochemical Centre, Amersham, Bucks; the other chemicals were of A.R. grade, where available, and obtained from Hopkin and Williams (Chadwell Heath, Essex, U.K.).

### *Organism*

The bacterial strain used in this work was *E. coli* ML 308-225 ($i^-$,$z^-$,$y^+$), constitutive for β-galactoside accumulation but lacking β-galactosidase, kindly provided by Dr. T. H. Wilson, Harvard Medical School, Boston, Mass., U.S.A.

### *Culture and preparation of bacteria*

Stock cultures were maintained on solid medium [medium C of Roberts *et al.* (1955), containing 0.4g of glucose and 1g of agar per 100ml]. The liquid culture medium used was that of Mager & Magasanik (1960), with succinate as carbon source, and contained (in 1 litre): 7.0g of $KH_2PO_4$, 3.2g of $Na_2HPO_4$, 2.0g of $(NH_4)_2SO_4$, 0.41g of $MgSO_4$,-$7H_2O$, 1mg of $CaCl_2$ and 2.5g of succinic acid. The medium was adjusted to pH7.0 with KOH. A small inoculum from the agar slope was grown for 24h at 25°C in 4ml of liquid medium, which was then added to 200ml of medium in a 1-litre flask. This was incubated at 37°C for 17h under aerated conditions. The stationary phase of growth, corresponding to an $E_{700}$ of about 1.0 (0.5–0.7mg cell dry weight/ml), was reached 1h before harvesting. Cells were harvested by centrifugation at 4°C for 5min at 15000*g*. Under these conditions the harvested cells were depleted of carbohydrate reserves and the rate of endogenous glycolysis was very low. The harvested cells were washed twice at 4°C with 150mM-KCl buffered with 3.0mM-glycylglycine (adjusted to pH7.0 with KOH), and suspended at a cell density of 40–50mg dry wt. ml in the same KCl–glycylglycine medium. This stock bacterial suspension was stored at 4°C, and was used for experiments for up to 5h.

### *Stock solutions, standards and inhibitors*

Stock aqueous lactose solution, both unlabelled and labelled with $^{14}$C (0.1 μCi/μmol), was made up at a concentration of 0.33M and adjusted to pH7.0 with 50mM-KOH. Carbonic anhydrase was made up in water at a concentration of 20mg/ml; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was made up at a concentration of 1 μmol/ml in ethanol. For pH titrations, standard HCl (50.0mM) and KOH (50.0mM) were made up in 100mM-KCl.

Potassium iodoacetate solution (0.1M) was prepared within 4h of use by dissolving iodoacetic acid in 0.1M-KOH. The effectiveness of this iodoacetate

solution as an inhibitor of glycolysis was found to diminish significantly within 12h of preparation, presumably because of decomposition of the iodoacetate.

Solutions of *p*-chloromercuribenzoate were found to be unstable and were used within 4h of preparation. Further, although that compound dissolved more quickly in alkaline than in neutral solutions, the brief exposure to alkali was found to lead to considerable inactivation. Therefore *p*-chloromercuribenzoic acid was dissolved at neutral pH to a final concentration of 0.1mM in the weakly buffered incubation medium (100mM-KCl, 50mM-KSCN, 2mM-glycylglycine adjusted to pH7.0 with 0.1M-KOH) by stirring at 65–75°C for 1h. On cooling, the pH was readjusted to 7.0 with 0.1M-KOH.

The HCl and KOH standards, the lactose solutions, and the incubation medium were freed of $O_2$ by evacuating, and flushing with $O_2$-free $N_2$.

*Equipment and method for measuring pH changes*

The experimental vessel, electrodes, electrometers and recording systems were as described previously (Mitchell & Moyle, 1969). The closed, temperature-controlled electrode-vessel (final volume 4.0ml, temperature 25°C), was filled with anaerobic incubation medium (100mM-KCl, 50mM-KSCN, 2mM-glycylglycine, adjusted to pH7.0 with 0.1M-KOH), which was dispensed from a glass syringe through a narrow inlet-tube. Stock cell suspension was added and the pH was adjusted to 7.0–7.1. Air was excluded by directing a stream of $O_2$-free $N_2$ at the mouth of the inlet tube, and residual $O_2$ in the well-stirred suspension was consumed by the bacteria within 1–2min. Although iodoacetate may react to some extent with the $\beta$-galactoside carrier (Kennedy, 1970), it does not greatly inhibit transport, and was required in the present experiments to decrease endogenous glycolytic metabolism and inhibit any possible residual metabolism of the added lactose by the $\beta$-galactosidase-negative bacteria. Carbonic anhydrase was added (25$\mu$g/ml, final concentration) as a routine, to catalyse the equilibration of the $CO_2$–$H_2CO_3$–$HCO_3^-$ system as described by Scholes & Mitchell (1970).

Anaerobic HCl and KOH standards and lactose solutions were added to the experimental vessel from Agla micrometer syringes (Burroughs Wellcome and Co., Dartford, Kent, U.K.), fitted with glass needles.

*Equipment and method for measurement of intracellular [$^{14}C$]lactose*

As it was the intention to follow the time-course of lactose inflow into completely anaerobic cells treated with iodoacetate, where there was expected to be little or no accumulation but nevertheless rapid equilibration of lactose between intracellular and extracellular media, the conventional Millipore-filtration technique (Kepes, 1960) was unsatisfactory. A technique was developed whereby any movement of lactose after sampling was prevented by inhibiting the $\beta$-galactoside carrier with *p*-chloromercuribenzoate, which acts extremely rapidly (see the Results section). It was then possible to wash the cells thoroughly and thus remove the large excess of extracellular [$^{14}C$]lactose, without further inflow or outflow of lactose.

Samples (0.25ml) of the cell suspension were taken from the anaerobic experimental vessel through a glass needle into a glass syringe, which had been thoroughly flushed with $O_2$-free $N_2$. The sample was injected rapidly into 3.0ml of 'stopping' solution, having the same composition as the incubation medium except for the addition of 0.1mM-*p*-chloromercuribenzoate, and stirred rapidly for 5s with a magnetic stirrer. These diluted suspensions were then taken into a syringe and forced through a Millipore filter (0.6$\mu$m pore size, 25mm diameter) in a Swinnex filter holder [Millipore (U.K.) Ltd., London N.W.10, U.K.]. A syringeful of air (10ml) was then forced through the filter followed by a second 3.0ml portion of the 'stopping' solution, and a second syringeful of air. The filters were dried under a lamp, but then left for 12h to equilibrate to the relative humidity of the room air before the $^{14}C$ content was estimated with a mica-window Geiger–Müller tube and scaler (Panax, Redhill, Surrey, U.K.). The dried filters were held in aluminium planchettes by metal rings that decreased the surface area of the sample to 70% but excluded the spurious counts in the incompletely washed, outer portion of the filter. The counting efficiency ($0.0372 \pm 0.0017$ counts/disintegration; S.D., $n = 12$) was determined by applying known amounts of [$^{14}C$]lactose solution of known specific radioactivity to filters that were subsequently dried and counted in the same manner as the samples.

Estimates of the amount of intracellular lactose are shown in the Figures, with bars which, with the exception of Fig. 3(*b*), give the standard error estimated from the number of counts recorded above the background count. In Fig. 3(*b*), where the average values from several experiments are shown, the bars represent the standard deviation of the individual experimental values from the mean.

## Results

*p-Chloromercuribenzoate inhibition of lactose transport*

In the present work, the technique used for following the time-course of the rapid lactose inflow down the lactose concentration gradient into metabolically

inactive cells depends on our ability to stop the lactose inflow via the β-galactoside porter after appropriate time-intervals. It is well known that *p*-chloromercuribenzoate inhibits β-galactoside transport (Kepes, 1960), but there was uncertainty about the completeness of this inhibition. Kepes (1969) observed that the half-time for outflow of thiomethylgalactoside from cells treated with uncoupler (20mM-$NaN_3$) was increased from 2.2 to 6.5min by 0.2mM-*p*-chloromercuribenzoate, suggesting that β-galactoside translocation was inhibited only by some 66%. In preliminary experiments designed to test the effectiveness of this mercurial as a stopping reagent, we, likewise, apparently obtained only partial inhibition by using 0.1mM-*p*-chloromercuribenzoate. However, after we suspected that our stock solutions were decomposing before use, we applied the precautions discussed in the Materials and Methods section and observed that a freshly prepared 0.1mM solution caused essentially complete inhibition of the lactose outflow from cells treated with uncoupler, as shown in the following experiment.

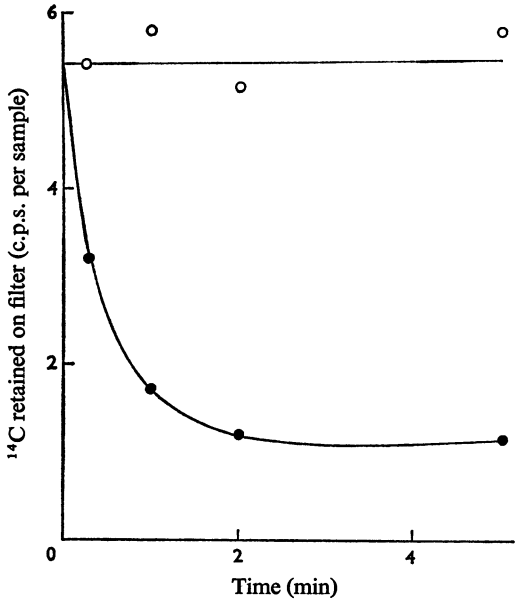


Fig. 1. *Inhibition of the uncoupler-induced outflow of [$^{14}$C]lactose from E. coli*

*E. coli* was suspended as described in the text, at a cell density of 3.74mg dry wt./ml. At zero time a 0.5ml portion of the suspension was added to 3.0ml of incubation medium containing 2μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (●), or 2μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 0.1mM-*p*-chloromercuribenzoate (○). Samples were filtered after the intervals shown.

The stock bacterial suspension (0.8ml) was added to 8ml of incubation medium (100mM-KCl, 50mM-KSCN, 2mM-glycylglycine, adjusted to pH7.0 with KOH) and [$^{14}$C]lactose was added to a final concentration of 0.45mM (0.125μCi/μmol). This suspension was maintained aerobic by stirring rapidly with a magnetic stirrer in a small beaker for 50min. Samples of 0.5ml were then transferred with a syringe into 3.0ml of incubation medium containing either uncoupler (2μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), or uncoupler and 0.1mM-*p*-chloromercuribenzoate, in a set of beakers, where they were incubated, with stirring, for a further period of 0.25–5.0min, as indicated. These diluted samples were then filtered, washed and prepared for the measurement of radioactivity. Dilution of samples into the medium containing uncoupler caused rapid outflow of lactose from the cells (Fig. 1, ●), while the inclusion of *p*-chloromercuribenzoate in this medium completely stopped that outflow for the 5min duration of the experiment (Fig. 1, ○).

Further confirmation of the effectiveness of 0.1mM-*p*-chloromercuribenzoate in stopping the translocation of lactose is provided by the experiment shown

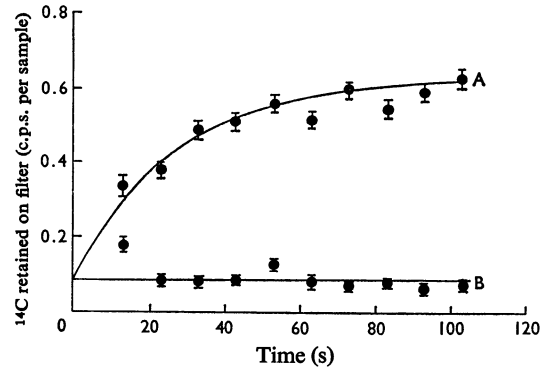


Fig. 2. *Inhibition of the inflow of [$^{14}$C]lactose into anaerobic E. coli*

The temperature-controlled experimental vessel, volume 4.0ml, contained: 16.0mg cell dry weight, 100mM-KCl, 50mM-KSCN, 2mM-glycylglycine, 1mM-iodoacetate and 0.1mg of carbonic anhydrase. After anaerobic incubation at 25°C, pH7.0–7.1, for 40min, 40μl of anaerobic 0.33M-lactose solution labelled with [$^{14}$C]lactose, 0.1μCi/μmol, was injected (zero time). The ordinate shows count rate above a background of 0.18c.p.s. Curve A, without mercurial; curve B, with 0.1mM-*p*-chloromercuribenzoate in the incubation medium. Vertical bars indicate the standard errors of the $^{14}$C determinations as explained in the Materials and Methods section.

in Fig. 2. The inflow of lactose into non-metabolizing cells under anaerobic conditions was followed by the procedure described in the section on Materials and Methods. It can be seen (Fig. 2, curve A) that, in the absence of the mercurial, [$^{14}$C]lactose flowed into the cells with an approximately exponential time-course (half-time, 20s). However, when the cells were suspended in incubation medium containing 0.1 mM-*p*-chloromercuribenzoate, there was no increase in the amount of [$^{14}$C]lactose associated with the cells during the 100s of the experiment (Fig. 2, curve B), and it is concluded that there was no penetration of lactose into the cells during that time. The difference between curves A and B in Fig. 2 would then represent the intracellular [$^{14}$C]lactose, corrected for the small amount of extracellular $^{14}$C contamination of the cells and the filter.

*Correlation between [$^{14}$C]lactose inflow and effective $H^+$ inflow*

The effective movement of $H^+$ into the cells, when an anaerobic solution of lactose (or other β-galactoside) is added to anaerobic suspensions of non-metabolizing *E. coli*, has been described previously (West, 1970; West & Mitchell, 1972). The objective of the present work was to examine the stoicheiometry between that effective inflow of $H^+$ and the inflow of lactose. It was not possible to measure both [$^{14}$C]lactose and effective $H^+$ movements simultaneously in the same suspension because withdrawal of portions of the suspension for [$^{14}$C]lactose determinations disturbed the baseline of the very sensitive pH measuring and recording system. Parallel sets of measurements were therefore done on cells from the same stock suspension, treated identically except that in one case the anaerobic lactose solution was labelled with [$^{14}$C]lactose and samples were withdrawn for $^{14}$C determination, as described above, while in the other case the lactose was unlabelled, and the pH of the suspensions was monitored as described previously (West & Mitchell, 1972).

The result of a typical experiment, showing the time-course of lactose inflow and effective $H^+$ inflow, is given in Fig. 3(*a*). The results of six experiments were averaged and the mean values plotted in Fig. 3(*b*), with an indication of the standard deviation of the individual results. Initially the rate of inflow of lactose and the rate of effective inflow of $H^+$ were practically equivalent, but as the experiment proceeded, the effective inflow of $H^+$ slowed sooner than did the inflow of lactose, and the stoicheiometric

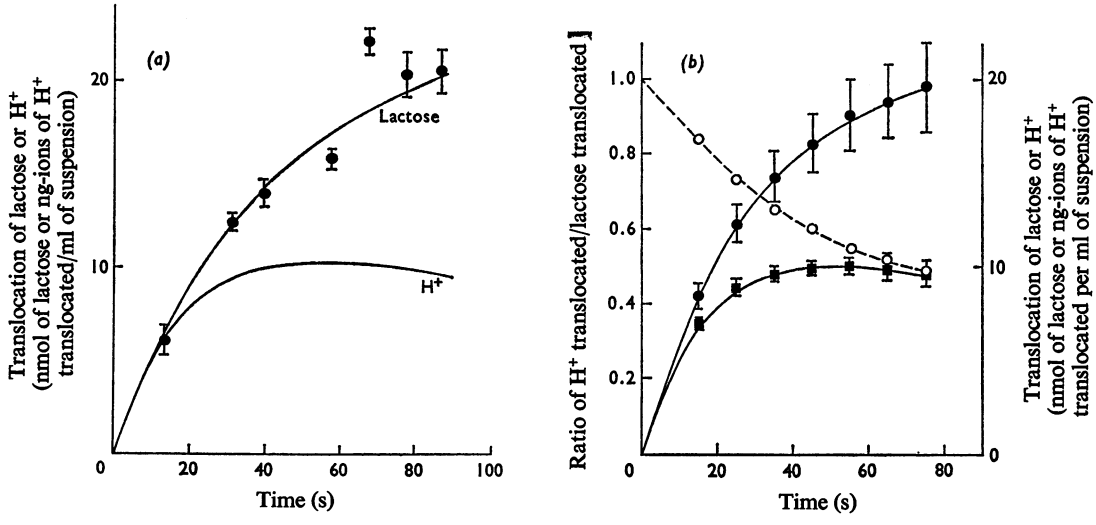


Fig. 3. *Correlation between [$^{14}$C]lactose inflow and effective inflow of $H^+$ into anaerobic E. coli*

(*a*) Details as in Fig. 2 without mercurial. The $^{14}$C count rate was divided by the specific radioactivity and the counting efficiency to obtain the quantity of lactose translocated. The continuous trace showing the quantity of $H^+$ effectively translocated was derived from the continuous recording of extracellular pH by titration with standard acid and alkali as described previously (West & Mitchell, 1972). Vertical bars indicate the standard errors of the $^{14}$C estimations, as explained in the Materials and Methods section. (*b*) The averaged results of six experiments like that of Fig. 3(*a*); ●, lactose translocation; ■, effective $H^+$ translocation; ○, stoicheiometric ratio of effective $H^+$ translocation/lactose translocation. The bars indicate the standard deviation of the individual experiments about the mean.

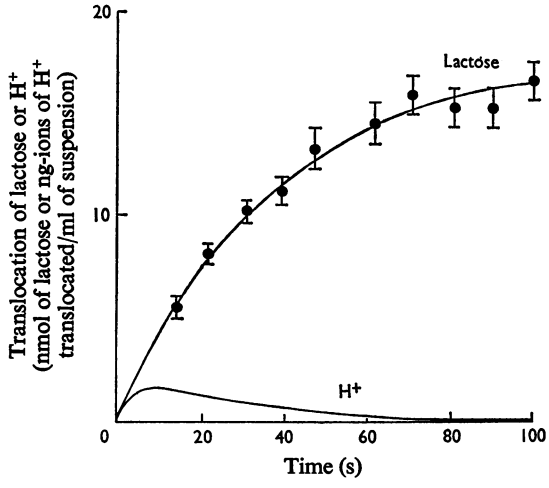


Fig. 4. *Effect of uncoupler on the correlation between inflow of lactose and effective inflow of $H^+$ into anaerobic E. coli*

Details are as in Fig. 3(*a*) but with the addition of 2 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to the incubation medium.

ratio of effective $H^+$ translocation/lactose translocation fell from 1.0. A point was reached when the effective $H^+$ inflow was reversed, and $H^+$ appeared to flow out of the cells. At eventual equilibrium, the lactose presumably remains in the cells, while the pH difference across the membrane returns to its original equilibrium value.

The stoicheiometric ratio of effective $H^+$ translocation/lactose translocation is also plotted in Fig. 3(*b*). At the earliest experimental point (at 15s), the stoicheiometric ratio is $0.84 \pm 0.07$ (S.D., $n = 6$) $H^+$ per lactose. But, extrapolation of the curve back to zero time gives a stoicheiometric ratio very close to 1.0 $H^+$ per lactose.

It is concluded that in this strain of *E. coli* the inflow of lactose molecules is stoicheiometrically coupled to the inflow of an equal number of $H^+$ ions, or the outflow of an equal number of $OH^-$ ions.

Fig. 4 shows the inflow of lactose and the effective inflow of $H^+$ when lactose is added to cells suspended in incubation medium containing 2 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Although the rate of lactose inflow appeared slightly inhibited, the inhibition was not statistically significant. The effective inflow of $H^+$, on the other hand, was much decreased by this proton-conducting uncoupler. As before, the ratio of effective $H^+$ translocation/lactose translocation was greater at shorter times after lactose addition, but by 15s it had already fallen to 0.13.

As shown in Fig. 2, lactose inflow was completely inhibited by 0.1 mM-*p*-chloromercuribenzoate. The corresponding inhibition of the effective movement of $H^+$ on adding lactose solution to cells treated with 0.1 mM-*p*-chloromercuribenzoate was investigated. It was confirmed that the rate of effective $H^+$ inflow was decreased to less than 0.1 ng-ion/min per ml of suspension, an inhibition of 99.6%.

## Discussion and Conclusions

In previous publications reporting the effective inflow of $H^+$ ions on adding lactose to anaerobic suspensions of non-metabolizing *E. coli* (West, 1970; West & Mitchell, 1972), the inflow of lactose was inferred from the effects of compounds known to inhibit the β-galactoside porter, and from the use of a mutant lacking the β-galactoside transport function. The experiments reported in the present paper confirm that there was, in fact, an inflow of lactose.

The initial rate of lactose translocation was 10.6nmol/mg dry weight per min; lower than that obtained in normal aerobic cells, but similar to that in cells treated with 1mM-iodoacetate and 30mM-$NaN_3$ (Winkler & Wilson, 1966). Lactose flowed into the cells with an exponential time-course that approached a plateau value of 21.7nmol/ml of suspension or 5.4nmol/mg dry weight of bacteria.

The results presented in this paper indicate that the initial rate of effective inflow of $H^+$ is equal to the initial rate of lactose inflow, and thus confirm the preliminary conclusion (West, 1970) that the two flows are stoicheiometrically coupled by the porter, with a ratio of 1:1. As expected, the close equality of the two inflow rates was only observed initially when the observed flow rates should correspond to those of the porter-catalysed reaction. When lactose molecules and $H^+$ ions effectively pass in together via the porter, the pH difference that is consequently built up across the membrane (acid inside) presumably causes an effective outward leakage of $H^+$ through the membrane and accounts for the progressive fall in the observed ratio of effective net $H^+$ translocation to lactose translocation.

The presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which is known to increase greatly the rate of proton leakage through artificial, mitochondrial, and bacterial membranes (Mitchell, 1970), greatly increased the discrepancy between net effective $H^+$ translocation and lactose translocation, presumably by greatly increasing the effective outward leakage of $H^+$ ions. Indeed, the effect of this uncoupler on the time-course of effective $H^+$ inflow can be described quantitatively in terms of its observed effect on the effective $H^+$ ion conductance of the membrane, by using a simple and orthodox differential equation. However, in the presence, as

in the absence of uncoupler, the initial rate of the observed net effective inflow of $H^+$ is equal to that of lactose; and it should be emphasized that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone does not affect the strict 1:1 coupling of the two fluxes on the β-galactoside porter system.

We are grateful to Dr. Jennifer Moyle for helpful discussion, and to Mr. Alan Jeal and Miss Stephanie Phillips for expert assistance. We thank the Science Research Council for a Special Research Grant in partial support of this work and Glynn Research Ltd. for facilities and general financial support.

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