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COUNTER-TRANSPORT MEDIATED BY THE LACTOSE PERMEASE OF *ESCHERICHIA COLI*

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Summary

When the two main energy yielding pathways, respiration and the membrane ATPase of *Escherichia coli* are poisoned, the lactose permease is unable to accomplish accumulative transport of thiogalactosides, but the efflux of preloaded substrate can be coupled to a transiently uphill transport of exogenous substrate. This transient uphill transport, called overshoot has been reexamined with the possibility of an obligate H^+ cotransport in mind. Overshoot can be diminished but not suppressed by a proton-conducting uncoupler, carbonyl cyanide *m* chlorophenylhydrazone, (CCCP) and by a liposoluble cation, triphenyl-methyl phosphonium ($TPMP^+$). The effect of other factors, such as temperature, amount of permease and pH were also explored. The overshoot was found to decrease with increasing pH, until at pH 8 it became negligible. This is in sharp contrast with the relatively flat pH dependence of uphill and downhill transport in unpoisoned cells. CCCP and $TPMP^+$ had no inhibitory effect on the overshoot at pH 6 and below.

Introduction

Lactose permease was usually pictured as a transport mechanism using a mobile carrier embedded in the membrane of *Escherichia coli*. One of the best criteria of the operation of a mobile carrier mechanism was the possibility, once metabolic energy was made unavailable, to drive a transient uphill transport by a downhill outflux of a preloaded homologous or identical substrate [1,2].

Since it was established that metabolic energy could be provided to the

Abbreviations: TMG, methyl β-D-thiogalactopyranoside; IPTG, isopropyl-β-D-thiogalactopyranoside; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; $TPMP^+$, triphenyl-methyl phosphonium ion; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; TDG, galactosyl-β-D-thiogalactopyranoside.

membrane to drive uphill transport of lactose and of a number of other solutes from two main sources, (1) respiration and (2) ATP via the membrane-bound energy transducing ATPase [3—6] the concept of an "energized state of the membrane" as the common precursor of energy for transport has evolved [7]. According to the most widespead interpretation this energized state is an electrochemical gradient of H^+ [8] also called a "proton-motive force" composed of a pH difference and of a transmembrane potential difference.

Supporting this view was the inhibition of active transport by proton-conducting uncouplers of oxidative phosphorylation [9] even in anaerobiosis, where oxidative phosphorylation plays obviously no role, and the possibility of driving a temporary uphill transport of lactose in membrane vesicles with no metabolic energy source, by the establishment of a transmembrane potential difference through the outflux of K^+ carried by valinomycine [10,11] or by forcing an inward flux of H^+ by an acid pulse [12].

Moreover, H^+ movements carried by a downhill lactose flux penetrating into deenergized *E. coli* were described [13,14].

The molecular mechanism of the coupling of an H^+ flux to a flux of sugar was usually, although not obligatorily, associated to the concept of mobile carrier [15] having two sites [16], one for the proton and one for the sugar, the two sites being translocated together. It was interesting therefore to examine the effect of the H^+ concentration in the medium, which is likely to influence H^+ symport, upon the process which most convincingly suggests the operation of a mobile carrier, i.e. countertransport in deenergized cells, more specifically the temporary uphill transport termed "entrance counterflow" by Wong and Wilson [17], called overshoot hereafter.

In the overshoot experiment the passive outflux of the preloaded substrate can be considered the primary flux and the temporary uphill influx of the outer substrate as the secondary flux. The potential energy drop of the preloaded substrate is thought to be channeled to the work necessary to accomplish the temporary uphill flux via a gradient of available substrate sites on the two sides of the membrane. But if the primary and secondary fluxes are also accompanied by symport of H^+ (or any other common partner) a temporary gradient of H^+ (or the partner) also contributes to the moving force. Therefore, it was concievable to dissipate this contributing energy intermediate by dissipating the gradient. Two inhibitors were used to achieve this goal, a classical uncoupler or "proton conductor" carbonylcyanide-*m*-chlorophenylhydrazone, (CCCP) and a lipid-soluble pentrating cation, triphenyl-methyl phosphonium ($TPMP^+$) capable to short-circuit the transmembrane potential difference.

Materials and Methods

The experiments reported below were carried out with three different strains of *E. coli*, ML308, K12 3300 and AR13, a gluconatekinase-deficient derivative of DF 1070 [18]. All three strains exhibited the same pattern; therefore, for each relevant experiment only the results obtained with one strain will be described. Bacteria were grown on mineral medium 63 (M63) [19] with 4 g/l glycerol as the carbone source and 2 mg/l thiamin at 37° C. For induction of

lactose permease 0.2 mM isopropyl-β-D-thiogalactoside was added. Growth was followed by absorbance measurements at 600 nm.

Bacteria were harvested during the exponential phase, centrifuged at 6000 × *g* for 5 min, washed once and resuspended in the medium used for incubation and equilibrated at 25° C, unless otherwise stated, with constant aeration.

Countertransport experiments were carried out in two steps, the preloading and the countertransport proper. Preloading with methyl β-D-thiogalactopyranoside (TMG) was made by one of two methods. The first consisted in the active accumulation of the substrate by normally energized bacteria (no inhibitor added). After 25 min, when a plateau of accumulation was reached, cells were centrifuged and the pellet resuspended in 1/200 of the original volume in the appropriate medium. The countertransport was initiated by dilution in 200 vol. of the appropriate medium containing the energy inhibitors and 0.5 mM final concentration of [^{14}C]TMG.

The second method of preloading, designed to avoid pH shift, osmotic shock and other drastic treatments for the bacteria at the start of the overshoot included first a centrifugation and resuspension of the bacteria in a minimal volume (A_{600} = 100—200) of a medium, the composition of which was as close as possible to the final dilution medium. This medium included enough inhibitors to preclude active accumulation, and it contained 100 mM non-radioactive TMG. This passive preloading was carried on for approximately 40 min. The countertransport experiment started with 200-fold dilution in the appropriate medium including [^{14}C]TMG. 0.5—2 ml samples generally containing 100—300 μg bacteria (dry weight) were filtered on millipore filters, 0.45 μm pore size, rinsed twice with 3 ml of the incubation medium with TMG omitted, dryed and counted in a liquid scintillation counter.

Control runs were made in exactly the same conditions as the countertransport experiments except that non radioactive TMG in the preloading period was replaced by H_2O and a proportional amount of TMG was added to the dilution medium, so that final concentration and specific activity of TMG were the same in experiment and control.

Downhill transport of *o*-nitrophenyl-β-D-galactopyranoside (ONPG), the hydrolyzable chromogenic substrate, was measured in aerated suspensions by the rate of liberation of *o*-nitrophenol measured as A_{420} after alkalinization by 0.4 M (final concentration) of Na_2CO_3. A blank value obtained in the presence of 2 mM galactosyl-β-D-thiogalactopyranoside (TDG) (80 × K_m) was substracted.

Results

Two variants of the basic experiment of countertransport are represented in Fig. 1. In both, cells in a concentrated suspension were preloaded with TMG while active accumulation was prevented by two different sets of energy poisons. One was inhibited with a mixture of sodium cyanide and sodium azide, the other, by 50 mM NaF. Both inhibitors gave substantially 100% inhibition as shown in the non-preloaded control. The 40 min of preincubation permitted the penetration of the lactose analog until near equilibrium with the environment was reached. This level is slightly below maximal levels of accumulation by energized cells exposed to saturating concentration of the same substrate [21].

When such preloaded cells are diluted, e.g. 200-fold, without removing the energy poison, a passive exit starts so that the intracellular concentration drops with pseudo-first order kinetics, i.e. exponentially to a 200-times lower level, with a $t_{1/2}$ which can range between 0.5 and 5 min. The final level is in equilibrium with the final concentration of substrate. The radioactive substrate present in the dilution medium is seen to penetrate into the cell far beyond its equilibrium value. After reaching a maximum, the concentration of the radioactive substrate falls and its rate of exit approaches the rate of exit of the preloaded substrate, and both reach an equilibrium concentration. When the radioactive substrate is chemically identical to the substrate used for the preload, the first ascending phase of the curve can be considered as an approach to isotopic equilibrium, where the exchange is much more rapid than the net flux of the substrate. But the same phenomenon can be observed when the preload and the countertransport are made with two different lactose analogs (e.g., TMG and TDG). Therefore, the flux of the second (radioactive) substrate, which is a "passive" flux for a second or so from time zero until equilibrium with the outer concentration is reached, becomes an active transport from the equilibrium concentration up to the maximum since it moves against its own concen-

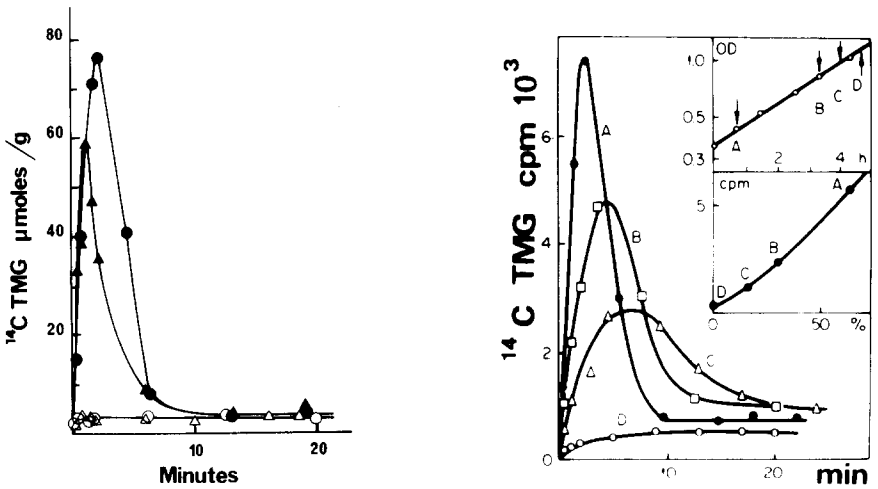

Fig. 1. [^{14}C]TMG uptake in poisoned cells with or without preloading with non-radioactive TMG. Cells of strain ML308 were washed with medium 63, resuspended in 1/200 vol. of the same medium and exposed for 40 min to the inhibitors and 10^{-1} M TMG (or no sugar), at 22°C. Samples were then diluted in 200 vol. of mineral medium containing the same inhibitors and [^{14}C]TMG at a final concentration of 0.5 mM. Cells preloaded with TMG: (●———●), sodium azide 2 mM plus KCN 2 mM (medium 63, pH 7.35); (▲———▲), NaF 50 mM (medium 63, pH 6.0). Cells not preloaded: (○———○), sodium azide 2 mM plus KCN 2 mM (pH 7.35); (△———△), NaF 50 mM (pH 6.0).

Fig. 2. Overshoot experiments with *E. coli* 3000 at various stages of induction of the *lac* operon. Samples of the culture were arrested with 50 µg/ml chloramphenicol, centrifuged and resuspended in 1/200 vol. of mineral medium containing $4 \cdot 10^{-2}$ M sodium azide and 10^{-1} M non-radioactive TMG. After 40 min at 25°C samples were diluted in 200 vol. of mineral medium containing sodium azide and a small amount of [^{14}C]TMG of high specific activity. Ordinates, counts per min × 10^3. Top insert, growth curves showing the times of addition of inducer: IPTG $2 \cdot 10^{-4}$ M to samples A, B and C. At the arrow D all four samples were supplemented with chloramphenicol. Lower insert, initial velocity of overshoot measured by the uptake in 1 min 15 s, as a function of percent induction calculated from the ratio of the increase of A_{600} in the presence of inducer to the total A_{600}.

tration gradient. The energy necessary for this active transport clearly comes from the loss of chemical potential of the preloaded substrate, but this energy is geared to the inward flux by the transport machinery, more specifically by a possible shuttling carrier. The comparison of the overshoot curve with the uptake kinetics in the non-preloaded control of Fig. 1 strongly suggests a *trans* acceleration instead of a mere inhibition of exit.

This is illustrated in a quantitative fashion in Fig. 2, where cell suspensions, in which synthesis of *lac* permease was induced to different extents, have been submitted to the procedure of overshoot. The inhibitor used was 40 mM sodium azide, here playing essentially the role of an uncoupler. Although the different samples have been preloaded to the same level, the initial velocity and the peak of the overshoot both varied in direct relation with the amount of permease present. It is worth noting that the less-induced samples have a broader time course together with a lower peak. Similar results have been reported by Wong and Wilson through partial chemical inactivation of the permease [17].

In contrast, temperature differences influence the time constants of the overshoot curves without strongly altering the hight of the peak as shown by Fig. 3. The height of the peak can be viewed as a reflection of the frequency of shuttles of the substrate loaded carrier. The one to one stoichiometry of the countertransport was approached in several experiments (Figs. 1 and 3). In other experiments (e.g., Fig. 2) there was a further departure from the value of one to one and the mechanism of this leak or sliding is central to the problems raised here. With the model of an H^+-β-galactoside symporter, the effect of an H^+ leak on the yield of the overshoot was explored.

Ionophores for H^+ have been used to deenergize lactose permease in order to produce countertransport [1—2]. Fig. 4 shows what happens when the concen-

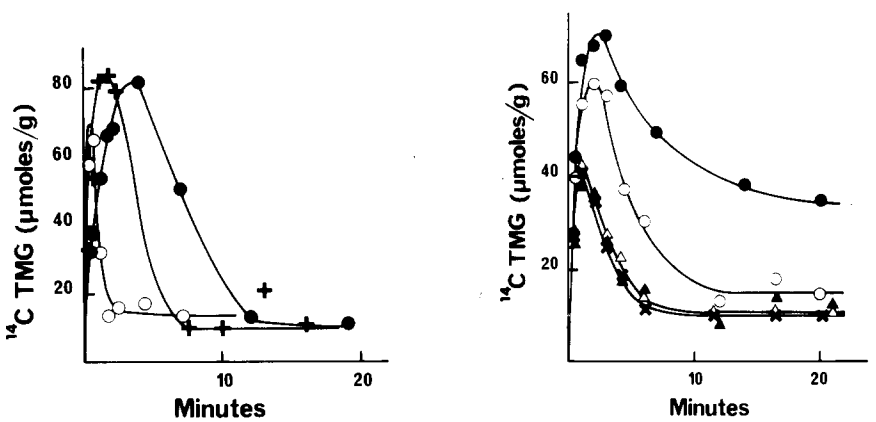

Fig. 3. Overshoot experiments at different temperatures. ML308 cells were poisoned with 2 mM azide plus 2 mM KCN in the presence of 0.1 M TMG at 14° C (●———●), 22° C (+———+) and 37° C (○———○) for 40 min, and then diluted in 200 vol. of medium 63 at the same temperature in the presence of 0.5 mM [^{14}C]TMG (final concentration).

Fig. 4. Overshoot experiments with increasing concentrations of CCCP. CCCP was present during preloading of the cells (strain ML308) with 0.1 M TMG and in the dilution medium at the following concentrations: 2 μM (●———●), 5 μM (○———○), 10 μM (△———△), 50 μM (X———X), 100 μM (▲———▲). [^{14}C]TMG was added at a final concentration of 0.5 mM in the dilution medium.

tration of CCCP is progressively increased: the extent of the overshoot is first decreased, then it tends to a limiting value which cannot be counteracted by further doses of CCCP.

In Fig. 5 increasing amounts of CCCP were applied during overshoot experiments where the deenergization was first obtained by a combination of cyanide and azide; CCCP again caused a moderate decrease of countertransport.

Fig. 6 illustrates a parallel situation, where in the course of an overshoot experiment under cyanide plus azide inhibition a penetrating cation, $TPMP^+$, was added in order to collapse the transmembrane electrical potential. This again allowed a moderate decrease in the yield of energy transfer from efflux to influx but was incapable of achieving complete flux uncoupling.

In contrast, the uncoupler had no effect when the overshoot was made under fluoride inhibition at pH 6 (Fig. 7). The overshoot was not diminished by a CCCP concentration as high as 20 μM. Since fluoride has no inhibitory effect at pH above 6.5 [22], the question arose of whether the difference of behavior was due to the pH or to the energy inhibitor used. The pH dependence of the overshoot phenomenon altogether and of the depressing effect of proton-conducting uncouplers thereupon had to be explored. As a preliminary to his exploration, the pH dependence of the active transport of TMG in normally energized cells, i.e. aerated cell suspensions with no added inhibitors and the

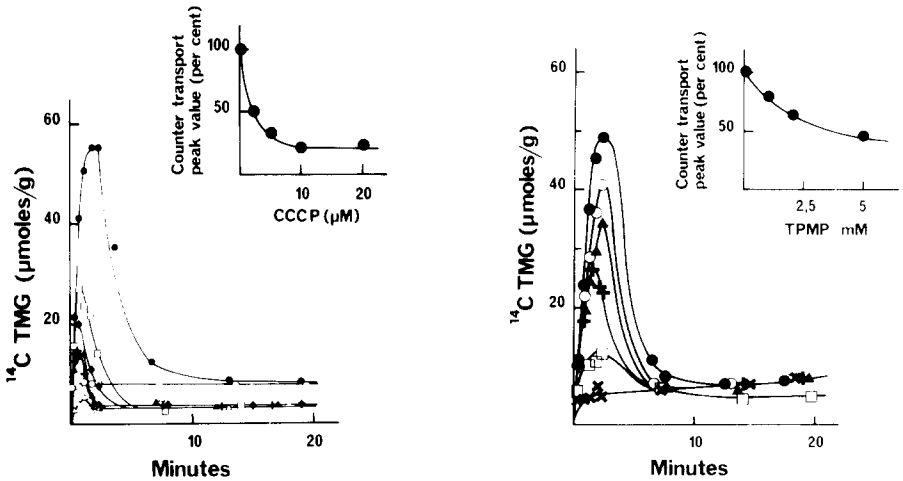

Fig. 5. Effects of increasing concentrations of CCCP during overshoot experiments conducted with 2 mM azide plus 2 mM KCN. ML308 cells were exposed to 2 mM azide plus 2 mM KCN and 0.1 M TMG (or no sugar) for 40 min. Samples were then diluted in 200 vol. of mineral medium containing 0.5 mM [^{14}C]-TMG (final concentration), 2 mM azide, 2 mM KCN and the following concentrations of CCCP. 0, (●——●); 2 μM, (□——□); 5 μM, (◆——◆); 10 μM, (+——+); 20 μM, (▲——▲). Cells not preloaded with TMG: 0, (○——○); 20 μM, (△——△). Insert: CCCP concentration dependence of the peak value.

Fig. 6. Effect of increasing concentrations of $TPMP^+$ bromide during overshoot experiments conducted with 2 mM azide plus 2 mM KCN. Details of the experiment are the same as for Fig. 5. except that $TPMP^+$ was added in the dilution medium instead of CCCP. $TPMP^+$ concentrations are 0, (●——●); 1 mM, (○——○); 2 mM, (▲——▲); 5 mM, (+——+). In additional experiment, 5 mM $TPMP^+$ and 10 μM CCCP were added together (□——□). X——X, cells not preloaded with TMG and diluted in the presence of 2 mM azide plus 2 mM KCN without $TPMP^+$. Insert: $TPMP^+$ concentration dependence of the peak value.

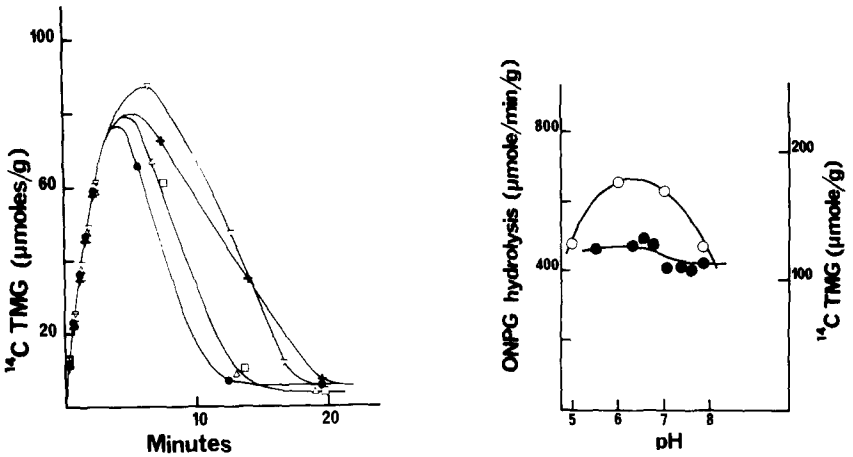

Fig. 7. Effect of increasing concentrations of CCCP during overshoot experiment performed with NaF at pH 6. ML308 cells washed and resuspended with medium 63 adjusted at pH 6, then exposed to 50 mM NaF and 0.1 M TMG for 40 min. Samples were successively diluted in 200 vol. of medium, pH 6, containing 0.5 mM [^{14}C]TMG, 50 mM NaF and the following concentration of CCCP: 0, (●———●); 2 µM, (△———△); 5 µM, (□———□); 10 µM, (+———+); 20 µM, (▽———▽).

Fig. 8. pH Dependence of the active transport of TMG and of the downhill transport of ONPG in normally energized cells. *E. coli* K12 cells (strain 3300) were centrifuged and resuspended in 0.1 M phosphate buffer adjusted at pH 5.0 to 8.0. Steady state level of TMG accumulation was measured 15 min after addition of 0.2 mM [^{14}C]TMG (○———○), and ONPG hydrolysis measured for 20 min at 30°C with 2 mM ONPG (●———●). (ONPG hydrolysis resistant to 5 mM TDG has been subtracted).

pH dependence of the downhill uptake of ONPG, measured by its rate of hydrolysis were first reviewed as shown in Fig. 8. Both these processes have a moderate sensitivity to pH between 5 and 8. This is in sharp contrast with the pH dependence of the countertransport.

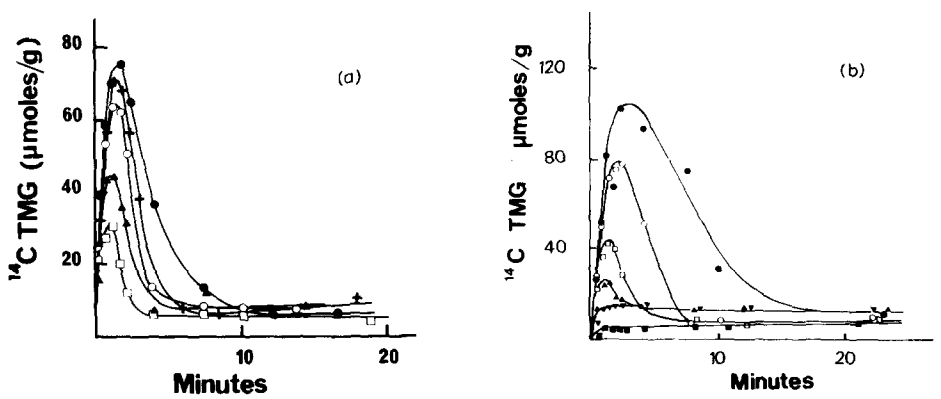

Fig. 9. a. Overshoot experiments with 2 mM azide 2 mM KCN at different pH values. AR13 cells were washed and resuspended with medium 63 adjusted to the appropriate pH with HCl and KOH respectively. They were preloaded with 0.1 M TMG in the presence of 2 mM azide and 2 mM KCN for 40 min, and then diluted in 200 vol. of medium 63 at the same pH, containing 0.5 mM [^{14}C]TMG (final concentration), 2 mM azide and 2 mM KCN. ●———●, pH 6.0; +———+, pH 6.5; ○———○, pH 7.0; ▲———▲, pH 7.4, □———□, pH 7.8. b. Overshoot experiments with 40 mM azide at different pH values. ML308 cells were treated as indicated in a except that 40 mM azide was used instead of 2 mM azide and 2 mM KCN. ●———●, pH 5.0; ○———○, pH 6.0; □———□, pH 7.0; ▲———▲, pH 8.0. Cells not preloaded: ■———■, pH 5.0; ▼———▼, pH 8.0.

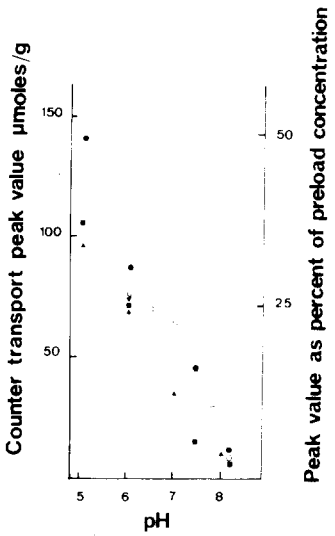

Fig. 10. General pH dependence of the countertransport. ML308 cells were washed, resuspended and preloaded at different pH values with 2 mM azide, 2 mM KCN, 0.1 M TMG as described in Fig. 9. Medium of dilution contained 0.5 mM [^{14}C]TMG (final concentration)/2 mM azide/2 mM KCN (●); or 2 mM azide/2 mM KCN/5 mM $TPMP^+$ (○); or 2 mM azide/2 mM KCN/10 µM CCCP (■). Results from the experiment of Fig. 7 with 50 mM NaF (▼) or 50 mM NaF plus 10 µM CCCP (▽), and of Fig. 9b with 40 mM azide (▲), □, Data from the experiment of Fig. 9a with strain AR13.

It can be seen on Figs. 9a and 9b that the yield of flux coupling approached 1 on the acid side and dropped to nearly zero on the alkaline side of this pH range with either the combination of azide and cyanide or with sodium azide at uncoupling concentration. Fig. 10 summarizes the results of Figs. 9a and 9b and a number of experiments with additional inhibitors at different pH values. The peak of the overshoot taken here as the measure of countertransport efficiency showed a nearly linear drop, when pH was raised from 5.5 to 8. The effect of 10 µM CCCP on the size of the overshoot is such that the peak value is lowered by approximately the same amount at each pH value, but the relative inhibition is increased with pH from 20 to 30% at pH 5.0 to nearly 100% at pH 8. A similar but smaller inhibitory effect was observed with 5 mM $TPMP^+$.

Discussion

The coupling of TMG fluxes of opposite directions is clearly a function of the *lac y* gene product as shown by its proportion to gene expression through induced synthesis [2], its correlaiton with gene activity in structural and regulatory mutants, its proportional decrease with inactivation by thiol reagents and its substrate specificity identical to that of active transport [17].

The exploration of this counterflux in the form of an overshoot is particularly unambiguous, since an uphill flux is observed instead of a downhill transport, in the absence of metabolic energy. This stresses the methodological importance of the energy inhibitors used. The combination of cyanide, a respiratory inhibitor and azide, an ATPase inhibitor [20] used here have three advantages: (1), the inhibition is immediate and easily reversible, (2), it is not

strongly pH-dependent and (3) it does not rapidly dissipate H^+ gradients.

This last quality is probably the reason for the yields of overshoot reported here being higher than observed previously [2,17] using proton-conducting uncouplers as energy inhibitors. Nevertheless, the overshoot pehenomenon is essentially similar whatever the energy inhibitor used; the efficiency of the inhibition is checked by the final equilibrium level and by the absence of overshoot in the non-preloaded control.

Most previous countertransport experiments were performed at pH values close to 7 and results were found somewhat variable from one experiment to the next and yields of coupling between opposite fluxes were rather poor.

Results reported here show at pH 6 flux coupling ratios of 0.5—0.6 with external TMG concentrations of 5.10^{-4} M, close to the K_m. Knowing the concentration dependence of the overshoot, these results strongly suggest that at saturation at pH 6 flux coupling would be approaching one to one.

Every deviation from this one to one ratio means a net efflux of TMG, and if this occurs via the permease, the lactose-proton symporter hypothesis would predict a concomitant flux of H^+, and this should be at least electrically compensated. The limited effect of the proton-conducting uncouplers in decreasing the coupling ratio between the two substrate fluxes is in conformity with this model. Flux ratio is diminished, however, when pH is increased from 6 to 8, even without added uncoupler, and the question is raised of whether the concomitant H^+ flux can be compensated by an inward leak or whether the obligate inward or outward symport can suffer exceptions.

In the present discussion we are using the height of the peak of overshoot as the index of flux coupling, although it is obviously a composite parameter, which results from the rate of influx and the net efflux. Initial rate of influx would be theoretically more adequate, but it is subject to larger error due to the short duration of the linear part and because of the sudden change during dilution of many environmental conditions, the influence of which is difficult to evaluate.

The time course of the efflux of the preloaded substrate is an important parameter, since this is the driving flux. It is partly reflected in the descending part of the overshoot curve. It can be noticed in Fig. 9a that the time course is shorter as the pH increases from 6 to 8, so that the shorter duration of the driving force can partly explain the smaller overshoot. However, Fig. 3 shows that the ratio of influx rate vs. efflux rate is more important; longer and shorter time courses can give the same final yield.

It was verified in separate experiments (not shown) that the preload under energy inhibitors permitted equilibration of external and internal substrate independently from the pH of the medium.

Thus, the pH-dependent decrease of the countertransport is not due to a significant lowering of the driving efflux. It could, therefore, be related either to an increase of the proportion of the permease-independent efflux, the existence of which has been described [2] or to an increase of the frequency of non-productive cycles of the carrier: outward crossing substrate loaded, inward crossing unloaded. At saturating external substrate and low pH these events are infrequent, flux coupling approaches one to one. It was shown previously that the flux ratio decreases with non-saturating substrate concentrations [2]. If

increased pH caused a decreased carrier-substrate affinity, this could also contribute to the drop of flux ratio.

It was found (unpublished results), that the permease-independent leak increased slightly when pH was raised from 7 to 8 and the K_m of the permease for TMG also increased by a factor of approx. 2 in the same pH interval when measured in the active transport assay, and a little more when measured in the countertransport assay. No significant variation of these two parameters was observed in the interval from pH 6 to 7 where the countertransport already significantly decreased.

The sum of the two contributions, increase in the permease-independent leak and decrease of the saturation of the carrier, does not seem to be sufficient to account for the virtual abolition of countertransport at pH 8.

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