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COUNTERFLOW OF GALACTOSIDES IN *ESCHERICHIA COLI*

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SUMMARY

Counterflow of β-galactosides in metabolically poisoned cells of *Escherichia coli* was systematically examined. In one type of experiment ("entrance counterflow"), azide-treated cells were preloaded with a galactoside, cells separated from the medium and finally resuspended in thio[^{14}C]methyl-β-D-galactopyranoside ([^{14}C]TMG). The concentration of radioactive sugar within the cell rose to levels well above that in the medium (due to the inhibition of efflux) and subsequently fell to the concentration of the external medium (as the preloaded sugar was lost from the cell). Preloading the cell to different levels of non-radioactive sugar resulted in different heights of the counterflow curve, but the time to attain the peak of each was the same. Reduction in the number of membrane carriers resulted in a proportionate reduction in the initial rate of rise of the counterflow curve and also a time delay in the peak of the counterflow curve. The effect of the following factors on counterflow was investigated: various concentrations of [^{14}C]TMG for uptake, various substrates, temperature and *p*-chloromercuribenzoate.

In a second type of experiment ("exit counterflow"), poisoned cells were first equilibrated with [^{14}C]TMG and then a high concentration of galactoside added to the external medium. Inhibition of influx without a corresponding effect on efflux results in a net loss of [^{14}C]TMG from the cell. The effects of different substrates and different levels of carrier were investigated.

INTRODUCTION

Membrane transport systems possess specific affinities for their substrates and competition between two different substrates for a common carrier is a characteristic feature of these systems. In a facilitated-diffusion system, where affinities for entrance and exit for a specific substrate are equal and no energy is required for movement, a special case of competitive inhibition exists in which the addition of Substrate A to one side of the membrane results in a net movement of Substrate B across the membrane in the opposite direction against a concentration gradient. This special case, designated "counterflow", was first predicted by WIDDAS[1] in 1952 from theoretical considerations and demonstrated experimentally 4 years later for the sugar

Abbreviations: TMG, thiomethyl-β-D-galactopyranoside; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; IPTG, thioisopropyl-β-D-galactopyranoside; PCMB, *p*-chloromercuribenzoate.

transport system of erythrocytes[2,3] and later for the sugar transport system of yeast[4,5] and other biological systems (see review by STEIN[6]).

The first experimental demonstration of this phenomenon was carried out with the sugar transport system of the erythrocyte, a membrane carrier-mediated system which facilitates the equilibration of external and internal concentrations of sugars. This process is not coupled with energy-yielding processes and thus the cell cannot actively transport against a concentration gradient under normal circumstances. Red blood cells were exposed to a solution of xylose (a non-metabolizable sugar) and sufficient time allowed for the internal concentration to equilibrate with that in the medium[2]. A high concentration of glucose was then suddenly added to the external solution and a net exit of xylose from the cells was observed. This resulted from a competitive inhibition of influx of xylose with a continued efflux from the cell. This net movement of the sugar out of the cell against a concentration gradient due to competitive inhibition of unidirectional flux has been termed counterflow[3].

This phenomenon is of interest both for its own sake and also because it may be used as a tool for the "assay" for membrane carriers in cells whose carrier detection is difficult because of loss of the energy coupling mechanisms or energy uncoupling. This communication describes experiments on counterflow of β-galactosides in energy uncoupled cells of *Escherichia coli*.

MATERIALS AND METHODS

Bacteria

The strains of *E. coli* used in this study were: ML-308 ($i^-z^+y^+a^+$) which is constitutive for β-galactosidase (EC 3.2.1.23) and β-galactoside transport and thiogalactoside transacetylase; ML-308-225 ($i^-z^-y^+a^+$) (ref. 7) possesses the same transport activity as ML-308 but possesses no β-galactosidase; ML-308-831 ($i^-z^+y^+a^-$) (ref. 8) which differs from ML-308 in lacking thiogalactoside transacetylase; ML-35 ($i^-z^+y^-a^+$) which possesses both enzymes, but lacks the β-galactoside transport system; ML-32400 ($i^+z^+y^+a^+$; K^-) which is inducible for the lactose operon and is galactokinase-negative. ML-308, ML-35 and ML-32400 were isolated in the laboratory of Dr. J. Monod.

ML-308-1320, ML-308-1337 and ML-308-1710 were obtained by mutagenizing ML-308 with ethylmethanesulfonate[9], growing in 0.2 % glucose to remove auxotrophs, and finally growing in lactose in the presence of penicillin G (Lilly)[10,11]. The penicillin treatment was repeated a second time. Surviving cells were grown to stationary stage 5 times in 0.2 % lactose. Cells were then plated on agar plates containing amino acids, tetrazolium dye and thio[^{14}C]methyl-β-D-galactopyranoside ([^{14}C]TMG). Sterile filter paper was pressed on the surface of the clones and a portion of each clone was removed onto the filter paper as a red spot. After drying, the papers were exposed to X-ray film for several days as described previously[8]. TMG transport capacity was estimated by the degree of darkening of the film.

Chemicals

[^{14}C]TMG was obtained from New England Nuclear Corp.; TMG, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), thio-β-D-digalactopyranoside, thioisopropyl-β-D-galactopyranoside (IPTG), D-fucose and 2,4-dinitrophenol from Mann Research Labo-

ratories; thiophenyl-β-D-galactopyranoside, *p*-chloromercuribenzoate (PCMB) and *N*-ethylmaleimide from Calbiochemical Corp.; D-galactose and α-D-lactose from Sigma Chemical Co.; thio-*o*-nitrophenyl-β-D-galactopyranoside from Cyclo Chemical Corp.; KN_3 from Eastman Kodak; casein tryptone digest from Difco Laboratories. Chloramphenicol was a gift of the Parke-Davis Co. and methyl-β-D-galactopyranoside was a gift from Corn Products.

Preparation of cell suspensions

The mineral medium used was Medium 63 (ref. 12) *plus* NaCl. It contains (in g) KH_2PO_4, 13.6; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7\ H_2O$, 0.2; $FeSO_4 \cdot 7\ H_2O$, 0.005; NaCl, 2.9; and 1 l of water adjusted to pH 7.0 with KOH.

Bacteria were grown aerobically at 37° in the mineral medium supplemented with 1% casein tryptone digest. To obtain logarithmically growing cells, 1.0 ml of an overnight cell suspension was transferred to a side-arm flask containing 30 ml of fresh growth medium and incubated on a rotary shaker at 37° until late logarithmic growth phase was attained. The cells were then centrifuged at 4°, washed with mineral medium, again centrifuged and finally resuspended at a suitable density for subsequent experiments in the mineral medium containing chloramphenicol (0.1 mg/ml).

"Entrance counterflow"

Washed cells were exposed to azide (30 mM) for 30 min prior to preloading (30 min), or poisoned and preloaded simultaneously. In the latter procedure (essentially that of WINKLER AND WILSON[7]) 4 ml of cells (absorbance on Klett = 200 (No. 42 filter); 0.22 mg dry wt./ml) were simultaneously metabolically poisoned with azide (30 mM) and preloaded with the appropriate sugar for 30 min at 25°. In some experiments cells were poisoned without preloading. Cells were then centrifuged in 50-ml plastic tubes at 39000 × *g* for 10 min at 4° in a Servall refrigerated centrifuge. The supernatant fluid was decanted and the tube was wiped free of adhering droplets with absorbant paper (or with the aid of a Pasteur pipette). Contamination of the cells with extracellular fluid under these conditions was 14.5 ± 1.9 μl (S.D.) ($n = 6$). These tubes were kept in ice until needed. No leakage of preloaded sugar was possible during storage as the cells were not washed and remained exposed to the same external medium containing the sugar.

At zero time, 4 ml of Medium 63 (at 25°) containing 30 mM azide and ^{14}C-labeled sugar (0.2 μC/ml) were pipetted directly onto the small cell pellet in the centrifuge tube and mixed quickly on a Vortex mixer. At various time intervals 0.5-ml samples were withdrawn and filtered through a Millipore filter (0.65 μ pore size) which had been presoaked with Medium 63. Cells were then washed on the filter with 5 ml of mineral medium and the Millipore filter containing cells placed in a liquid-scintillation vial. A 15-ml volume of Bray's liquid scintillation fluid[13] was added, the vials shaken vigorously and counted in a Nuclear-Chicago liquid-scintillation counter. At the end of the experiment a 0.1-ml sample of cell suspension was placed in a counting vial for radioactivity determination. Another 0.2 ml of cell suspension was diluted with 1 ml of Medium 63 and its absorbance determined on a Gilford spectrophotometer (420 mμ). 100 Klett units (No. 42 filter) equal an absorbance of 1.050 on the Gilford spectrophotometer at 420 mμ. This final step was to determine directly the absorbance in the incubation medium. In the calculation of intracellular concentrations the

volume of 0.6 μl of cell water per ml of cell suspension of an absorbance of 100 Klett units (No. 42 filter) was used[7].

"Exit counterflow"

Washed cells at a cell density of 0.22 mg dry wt./ml were poisoned with azide (30 mM) for 30 min at 25°. At zero time, a suitable volume of [^{14}C]TMG (1 μC/ml) was added to the poisoned cell suspension to give a final concentration of 0.5 mM. At various time intervals 0.5-ml samples were withdrawn and filtered as described above. When the [^{14}C]TMG concentration in the poisoned cells had equilibrated with that in the medium (usually after a 10-min incubation period), a small volume of 1 M "inhibitor" sugar was added to give a final concentration of 10 mM. In one experiment (Fig. 16) 0.1 vol. of 100 mM inhibitor sugar was added. This slight dilution may be evaluated by comparison with a control in which the same volume of mineral medium without sugar was added. Samples were removed for analysis of intracellular [^{14}C]TMG as before.

Assay for membrane carriers

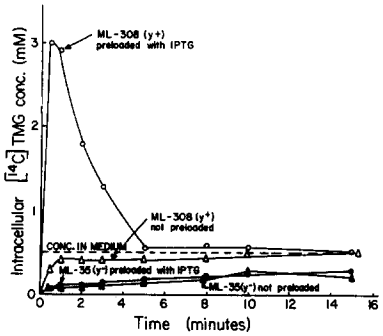
In experiments described in Figs. 4 and 5 the number of membrane carriers was assayed with two methods. The 15-sec uptake of [^{14}C]TMG (0.5 mM) at 25° was measured and compared with the parental organism, ML-308. In the second method the rate of ONPG (2 mM) hydrolysis by washed cells was assayed (by continuous colorimetric measurement) in the presence and absence of 10 mM thiodigalactoside. The thiodigalactoside-inhibited rate was compared with that for ML-308. These two methods agreed to within 5 %.

RESULTS

Counterflow by preloading cells (entrance counterflow)

Description of the phenomenon. The active transport of β-galactosides by *E. coli* can be virtually completely inhibited by several metabolic inhibitors including KN_3 (30 mM) and dinitrophenol (1 mM). Fig. 1 shows that when such poisoned cells (ML-308) were exposed to [^{14}C]TMG the sugar rapidly entered the cell until the internal and external concentrations were equal; no active transport occurred. When transport-negative cells (ML-35) were treated similarly, the entry rate of TMG was extremely slow. Little is known concerning the precise mechanism by which TMG enters these y^- cells except that the entry rate is proportional to the external concentration up to 20 mM. This experiment was repeated following the preloading of both types of cells with non-radioactive IPTG. After exposing the poisoned cells to IPTG for 30 min, they were centrifuged, the supernatant fluid decanted and the tube carefully wiped. A solution containing [^{14}C]TMG was then suddenly squirted onto the pellet, mixed vigorously and samples filtered and washed on a Millipore filter to determine the intracellular TMG concentration. Fig. 1 shows that in the ML-308 the intracellular TMG concentration rose to a level 6 times higher than that in the external medium, followed by a fall to the equilibration level. This phenomenon of counterflow was completely absent in the cell lacking the β-galactoside transport system. Similar results were obtained with 1 mM dinitrophenol as the metabolic inhibitor rather than azide.

Effect of preloading with different concentrations. Cells of ML-308-831 ($i^-z^+y^+a^-$) were preloaded to different levels of non-radioactive TMG, separated from the external medium and the pellets resuspended in 0.5 mM [^{14}C]TMG. Fig. 2 shows that such an experiment results in a family of curves, each showing a peak intracellular concentration at about 30 sec followed by a fall to approximately the level in the external medium. The higher the level of preloading, the higher the peak of the counterflow curve. Several other experiments gave similar results.



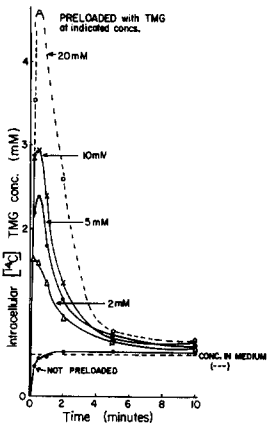


Fig. 1. TMG uptake in poisoned cells with or without preloading with IPTG. Cells were exposed to 30 mM KN_3 *plus* 20 mM IPTG (or no sugar) for 30 min at 25°. They were then centrifuged for 10 min at 4° to form a firm pellet. The supernatant fluid was decanted and the tube carefully wiped. The pellet was then resuspended in Medium 63 containing 30 mM KN_3 and 0.5 mM [^{14}C]TMG (0.2 μC/ml) at 25°. Samples were taken at 30 sec, 1, 2, 3, 5, 8, 10 and 15 min.

Fig. 2. Effect of preloading with different concentrations on entrance counterflow. Washed cells of ML-308-831 were exposed to 30 mM KN_3 in the presence of 0, 2, 5, 10 or 20 mM TMG for 30 min at 25°. Following centrifugation each pellet was resuspended in medium containing 30 mM KN_3 *plus* 0.5 mM [^{14}C]TMG (0.2 μC/ml) at 25°. Samples of 0.5 ml were filtered on Millipore filters.

Effect of varying external concentration. Cells of ML-308-225 ($i^-z^-y^+a^+$) were preloaded with non-radioactive lactose (20 mM), separated from the medium and the pellet resuspended with a medium containing either 0.5 mM [^{14}C]TMG or 0.05 mM [^{14}C]TMG (Fig. 3). The intracellular concentration rose higher and attained a maximum concentration earlier in the case of 0.5 mM TMG. On the other hand, the ratio of the concentrations inside to outside was about 12 with the higher external concentration and 40 in the case of the lower concentration.

Effect of varying the number of carriers. To reduce the number of carrier sites, cells of ML-308 were exposed to *N*-ethylmaleimide for various periods of time (under conditions described by FOX AND KENNEDY[14]), samples were removed and excess *N*-ethylmaleimide neutralized by addition of mercaptoethanol. In this manner cells were obtained with a variable percentage of membrane carriers still active, as assayed with TMG uptake and ONPG hydrolysis *in vivo*. All cells were poisoned with 30 mM azide and preloaded with 20 mM TMG for 30 min, centrifuged and resuspended in [^{14}C]TMG. The pattern of the counterflow curves is shown in Fig. 4. The initial rate of uptake is in direct proportion to the number of carriers. In addition, the fewer the membrane carriers, the more displaced to the right is the peak internal concen-

tration. In other similar experiments cells possessing only 1 % of the normal carriers showed a rise in internal concentration well above the external concentration under the conditions of these counterflow experiments (*e.g.*, poisoned cells preloaded with 20 mM TMG).

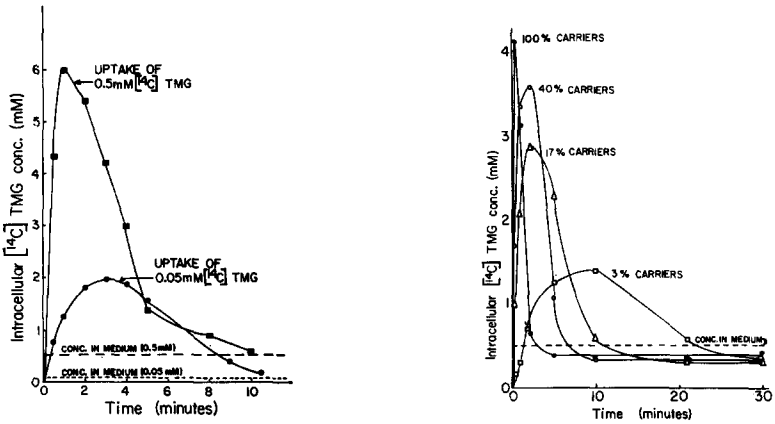


Fig. 3. Effect of varying external concentration of TMG in ML-308-225. Washed cells were poisoned with 6 ml of 30 mM KN_3 in the presence of 20 mM lactose for 30 min at 25°. In this experiment the cells were washed free of medium after incubation since the contamination of the centrifuged pellet by extracellular fluid would inhibit uptake of 0.05 mM [^{14}C]TMG. Accordingly, the cells were separated from the medium by filtration on a Millipore filter (2 inch diameter; 0.65 μ pore size) and were washed quickly on the filter with 10 ml cold Medium 63. The filter was transferred into a 50-ml centrifuge tube. At zero time, 6 ml of Medium 63 containing 30 mM KN_3 *plus* either 0.5 or 0.05 mM [^{14}C]TMG (0.2 μC/ml) were squirted onto the filter at 25°. The tube was mixed vigorously to remove cells from the filter and samples were withdrawn at intervals as described in MATERIALS AND METHODS.

Fig. 4. The effect of reducing the number of carriers with *N*-ethylmaleimide on counterflow. Cells of ML-308 were grown in tryptone amino acid mixture to log phase. Washed cells (absorbance = 1000 Klett units) were suspended in 0.1 M phosphate buffer (pH 7.0) *plus* chloramphenicol (100 μg/ml) and 1 mM *N*-ethylmaleimide at 0° (ref. 14). At 2-, 4- and 10-min intervals samples were withdrawn and mercaptoethanol added to give a final concentration of 5 mM. Hydrolysis *in vivo* of ONPG and 15 sec TMG uptake were measured on these cells for assay of carriers (as given in MATERIALS AND METHODS). For counterflow experiments aliquots of the *N*-ethylmaleimide-treated cells were exposed to TMG (20 mM) and NaN_3 (30 mM) for 30 min at 25°. Following centrifugation Medium 63 containing NaN_3 (30 mM) and [^{14}C]TMG (0.5 mM; 0.7 μC/ml) was squirted rapidly onto the small cell pellet at the bottom of the centrifuge tube and stirred briefly on a Vortex mixer. Samples of 0.5 ml were removed at 15 sec, 1, 5, 10, 20 and 30 min, filtered and washed with 10 ml Medium 63.

An alternative method of obtaining cells with variable transport activity was by mutagenesis followed by penicillin selection and identification of defective transport by a radioautographic technique (see MATERIALS AND METHODS). These mutants when poisoned with azide showed a similar pattern of curves to that of *N*-ethylmaleimide-treated cells (Fig. 5).

Effect of preloading with different substrates. The effect of preloading induced and uninduced cells with three different compounds was tested with ML-32400 ($i^+z^+y^+$; K^-). This strain was selected as it cannot metabolize D-galactose, a sugar to be tested in these experiments. The rate of entry of [^{14}C]TMG into the uninduced poisoned cell was very slow (Fig. 6). Preloading with D-fucose or D-galactose had

little effect and TMG appeared to stimulate slightly. Three additional experiments showed consistent elevations of the TMG preloaded curves compared with those of the non-preloaded controls. This is interpreted to indicate the presence of a small number of membrane carriers (less than 1 % of normal) present in the uninduced cells. On the other hand, cells with fully induced galactoside transport systems showed marked counterflow following preloading with both TMG and D-galactose; a very slight effect was noted with D-fucose (Fig. 7). This indicates that galactose, and to a much lesser extent D-fucose, is a substrate for the galactoside transport system.

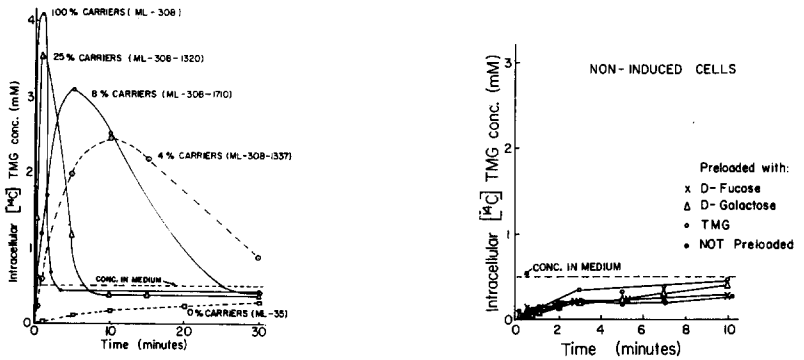


Fig. 5. TMG counterflow in mutants possessing different quantities of membrane carrier. ML-35 (0% carriers), ML-308-1337 (4% carriers), ML-308-1710 (8% carriers), ML-308-1320 (25% carriers) and ML-308 (100% carriers) were grown into log phase in casein hydrolysate, centrifuged, washed once in mineral Medium 63 and resuspended in mineral medium containing chloramphenicol (50 μg/ml). Cells were then incubated in 4 ml of Medium 63 containing NaN_3 (30 mM) and TMG (20 mM) for 30 min at 25°. Cells were then centrifuged, and the supernatant fluid poured off. 4 ml of Medium 63 containing NaN_3 (30 mM) and [^{14}C]TMG (0.5 mM; 0.7 μC/ml) were squirted rapidly onto the small cell pellet at the bottom of the centrifuge tube and stirred briefly on a Vortex mixer. Samples of 0.5 ml were removed at 15 sec, 1, 5, 10, 15 and 30 min.

Fig. 6. Effect of preloading with different substrates on the subsequent uptake of TMG into uninduced cells of ML-32400. Cells were grown in tryptone amino acid mixture to log phase without inducer. They were washed and then poisoned with 30 mM KN_3 in the presence of 20 mM TMG, D-galactose, D-fucose or no sugar for 30 min at 25°. The cells were centrifuged, the supernatant fluid was decanted and the tube carefully wiped. The pellet was resuspended with a medium containing 0.5 mM [^{14}C]TMG and 30 mM KN_3. Samples were withdrawn as indicated.

Figs. 8, 9 and 10 show the counterflow curves obtained with ML-308-225 ($i^-z^-y^+$) preloaded with a variety of sugars. Preloading cells with TMG, IPTG, thiophenyl-β-D-galactopyranoside and ONPG produced similar counterflow curves with [^{14}C]TMG as the substrate. Preloading with lactose produced the highest level in the counterflow curves. Thio-β-D-digalactopyranoside and thio-*o*-nitrophenyl-β-D-galactopyranoside behaved rather differently from other sugars giving a slow entry rate, a low delayed peak and a slow return to normal. These two counterflow curves are consistent with the hypothesis of ROBBIE AND WILSON[15] that certain sugars (especially thio-β-D-digalactopyranoside) result in "negative exchange diffusion".

Effect of temperature. Attempts were made to alter the shape of the counterflow curve by reducing the temperature during uptake of the substrate. According to a simple model one would expect that while the radioactive substrate would enter the cell slowly at a low temperature, it would be retained for a long period due to the

blockage of exit by the non-radioactive sugar which in turn would be delayed in leaving the cell *via* the carrier. In Fig. 11 is shown an experiment in which two aliquots of poisoned cells were preloaded with non-radioactive TMG for 30 min at 25°. One aliquot was exposed to [^{14}C]TMG at 25° and the other exposed to substrate at 4°. The peak of the 25° counterflow curve was 30 sec while that for the 4° experiment was 10 min. A striking feature of the 4° curve (not shown in the figure) was its slow fall, being 8 times the concentration of the medium at 30 min and 5 times the concentration of the medium at 60 min. Other experiments gave entirely similar curves,

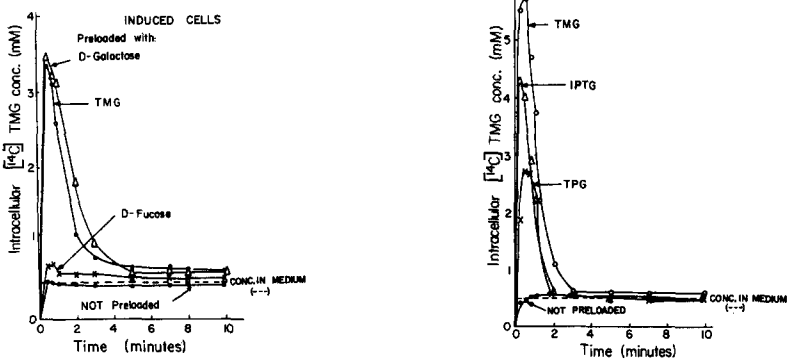


Fig. 7. Effect of preloading induced cells with different substrates on the subsequent uptake of TMG. Cells of ML-32400 were induced by growing in tryptone amino acid mixture in the presence of 0.5 mM IPTG as inducer. See legend of Fig. 6 for other experimental details.

Fig. 8. Effect of preloading ML-308-225 with different galactosides on the uptake of [^{14}C]TMG. Washed cells were incubated in the presence of 20 mM galactoside *plus* 30 mM KN_3 for 30 min at 25°. Cells were then centrifuged and the pellet was resuspended in 0.5 mM [^{14}C]TMG *plus* 30 mM azide at 25°. Samples of 0.5 ml were removed at various time intervals and filtered on Millipore filters. The galactosides tested were thiomethylgalactoside (TMG), thioisopropylgalactoside (IPTG) and thiophenylgalactoside (TPG).

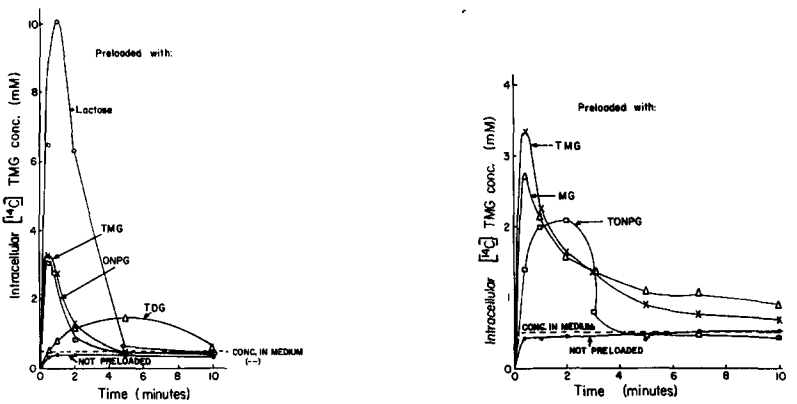
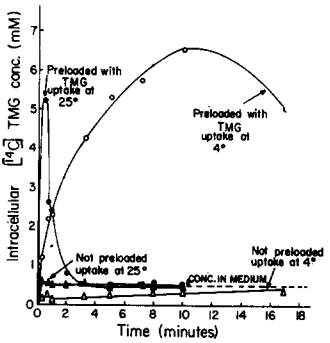


Fig. 9. Effect of preloading ML-308-225 with different galactosides on the uptake of [^{14}C]TMG. See legend of Fig. 8 for experimental details. The galactosides tested were lactose, thiomethylgalactoside (TMG), *o*-nitrophenylgalactoside (ONPG) and thiodigalactoside (TDG).

Fig. 10. Effect of preloading ML-308-225 with different galactosides on the uptake of [^{14}C]TMG. See legend of Fig. 8 for experimental details. The galactosides tested were thiomethylgalactoside (TMG), methylgalactoside (MG) and thio-*o*-nitrophenylgalactoside (TONPG).

with intermediate temperatures giving curves intermediate between the two shown in Fig. 11. A similar experiment was performed by preloading with non-radioactive lactose followed by uptake of [^{14}C]lactose. The results were entirely analogous to those reported in Fig. 11 for TMG.



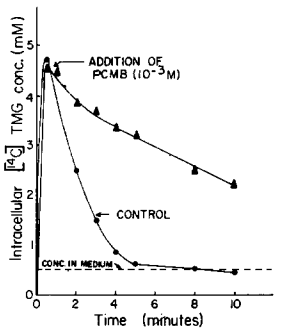


Fig. 11. Effect of temperature on TMG counterflow in poisoned ML-308-225. Four aliquots of cells were poisoned with 30 mM KN_3 and either preloaded or not preloaded with 20 mM TMG for 30 min at 25°. They were centrifuged, the supernatant fluids were poured off and the tubes carefully wiped. Two aliquots of the cell pellets were exposed to 0.5 mM [^{14}C]TMG and 30 mM KN_3 at 25° while the other two aliquots treated at 4°. Samples of 0.5 ml were withdrawn at different time intervals.

Fig. 12. Effect of PCMB on TMG counterflow in poisoned ML-308-225. Washed cells of ML-308-225 were preloaded with 20 mM TMG in the presence of 30 mM KN_3 for 30 min at 25°. Following centrifugation the pellet was resuspended in Medium 63 containing 30 mM KN_3 *plus* 0.5 mM [^{14}C]TMG. At 30 sec PCMB was added to one aliquot of cell suspension to a final concentration of 1 mM (an equal volume of Medium 63 was added to the other cell suspension). Samples were withdrawn at intervals.

Effect of PCMB. It was considered of interest whether one could block the exit of [^{14}C]TMG once it had entered by the counterflow procedure. Consequently poisoned cells were preloaded with non-radioactive TMG, separated from the medium, and [^{14}C]TMG added. At 30 sec, after the [^{14}C]TMG had accumulated, PCMB was added in an attempt to block exit. The exit of [^{14}C]TMG from these cells was greatly slowed by the addition of the inhibitor, although not blocked completely (Fig. 12).

Correlation between internal and external concentrations of sugars during counterflow. It was of interest to correlate the exit of the preloaded sugar with the entry of the radioactive sugar under conditions when counterflow was occurring. Experiments were carried out in parallel, in the first case cells were preloaded with 20 mM labeled TMG and then, following centrifugation, allowed to exit into 0.5 mM non-radioactive sugar. The usual counterflow experiment was carried out in parallel. In Fig. 13 are shown the results of experiments with cells possessing 100 % of the carriers and with other cells possessing only 8 % of the carrier activity. At the peak of the counterflow curve, the concentration of the "preloaded" sugar is approximately equal to that of the "test" sugar. The rate of exit of the preloaded sugar is slower from the cells possessing fewer membrane carriers, thus accounting for the later peak of its counterflow curve.

Counterflow by substrate addition to equilibrated cells (exit counterflow)

Effect of different substrates. The second type of counterflow results from the competitive inhibition of influx with efflux unaffected. The experimental procedure

was to expose poisoned cells to a radioactive sugar until the concentration inside was equilibrated with that outside. Then, a high concentration of non-radioactive sugar was added to the external medium and the concentration of the internal radioactive sugar was measured. Such an experiment is shown in Fig. 14. Addition of

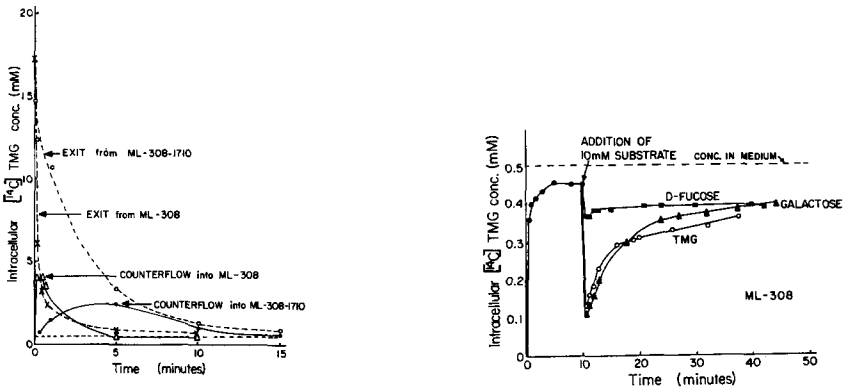


Fig. 13. Correlation between exit of preloaded sugar and entrance of sugar. Two experiments were run in parallel on the same batch of cells. (A) Cells were preloaded with 20 mM [^{14}C]TMG and exit into 0.5 mM cold TMG measured and (B) cells were preloaded with 20 mM cold TMG and the uptake of 0.5 mM [^{14}C]TMG measured. In the first type of experiment cells were exposed to azide (30 mM) for 15 min at 25° and then [^{14}C]TMG added to give a final concentration of 20 mM. After 30 min 0.5-ml samples were taken (initial intracellular concentration). Cells were centrifuged, supernatant poured off, and pellet resuspended in Medium 63 containing 0.5 mM cold TMG and 30 mM azide. The rate of exit was followed at various time intervals. In the second type of experiment cells were exposed to azide (30 mM) for 15 min and then cold TMG was added to give a final concentration of 20 mM. After 30 min cells were centrifuged, supernatant fluid poured off and the pellet was resuspended in Medium 63 containing 0.5 mM [^{14}C]TMG *plus* 30 mM azide. The uptake of radioactivity was followed at various time intervals. The horizontal dotted line at 0.5 mM indicates the concentration in the medium during the counterflow experiment. ML-308 (100% carriers) and ML-308-1710 (8% carriers) were used in these experiments.

Fig. 14. Effect of adding different substrates to poisoned ML-308 on exit counterflow of [^{14}C]TMG. Cells were poisoned with 30 mM KN_3 for 30 min at 25°. [^{14}C]TMG at a final concentration of 0.5 mM, 1 μC/ml was added to the cell suspension. Samples were withdrawn at intervals. At 10 min 1 M unlabeled substrate was added to give a final concentration of 10 mM and exit counterflow of [^{14}C]TMG was measured at different time intervals.

galactose and of TMG dramatically reduced the internal concentration of [^{14}C]TMG followed by a return toward the previous level. D-Fucose produced a small effect which is consistent with data in Fig. 7 suggesting that this sugar has some affinity for this transport system. That the counterflow requires the presence of the specific β-galactoside transport system was indicated by the lack of measurable counterflow in cells lacking "y", the gene controlling the synthesis of the transport protein (Fig. 15). In Fig. 16 are shown additional curves showing the effects of IPTG and lactose. The latter is of interest as its addition resulted in a permanent depression of TMG levels within the cell. This behavior is to be expected if lactose has affinity for the transport system but is metabolized within the cell, its intracellular concentration remaining very low. It might be noted that the shape of the curve for galactose (Fig. 14) is similar to the curves for non-metabolizable sugars and unlike that of the metabolizable sugar lactose. This suggests that under these conditions galactose accu-

mulates within the cell, while neither lactose nor its split product galactose accumulates sufficiently to inhibit efflux of TMG.

Effect of varying the number of carriers. "Exit counterflow" was tested with three organisms, one with 100 % carriers, one with 4 % carriers and one with 0 % carriers. In the cells with 4 % carriers the [^{14}C]TMG entered quite slowly but approached the plateau (equilibration level) at 30 min. Addition of 10 mM non-radioactive TMG to these cells resulted in a slow fall, the minimum being much later than that in the ML-308. After the minimum of the curve was reached, there was a very slow rise

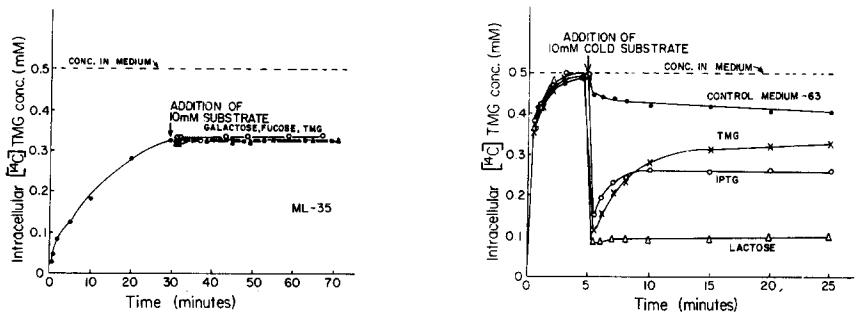


Fig. 15. Effect of adding different substrates to poisoned ML-35 on exit counterflow of [^{14}C]TMG. Washed cells of ML-35 were poisoned with 30 mM KN_3 for 30 min at 25°. At zero time, a suitable volume of [^{14}C]TMG (1 μC/ml) was added to the poisoned cell suspension to give a final concentration of 0.5 mM. Samples of 0.5 ml were withdrawn and filtered at different time intervals. At 30 min 1 M unlabeled substrate was added to the cell suspension to give a final concentration of 10 mM and exit counterflow of [^{14}C]TMG was measured at intervals. The apparent failure of the internal concentration of [^{14}C]TMG to reach the concentration in the external medium may be an artifact due to the use of an incorrect value for calculating the internal cell water from absorbance measurements.

Fig. 16. Effect of adding different substrates to poisoned ML-308 on exit counterflow of [^{14}C]TMG. Cells were poisoned with 30 mM KN_3 for 30 min at 25°. [^{14}C]TMG at a final concentration of 0.5 mM (1 μC/ml) was added to the poisoned cell suspension. When the [^{14}C]TMG concentration in the poisoned cells had equilibrated with that in the medium (after a 5-min incubation period), 0.1 vol. of 100 mM inhibitor sugar was added. This slight dilution may be evaluated by comparison with a control in which the same volume of Medium 63 (no sugar) was added. Samples of 0.5 ml were removed for analysis of intracellular [^{14}C]TMG as before.

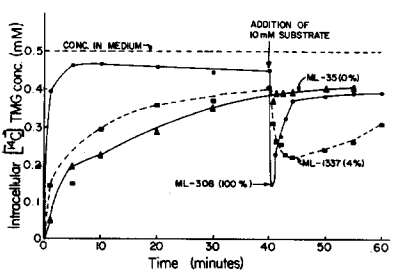


Fig. 17. Effect of varying the number of membrane carriers on exit counterflow of [^{14}C]TMG. Washed cells of ML-308 (100% carriers), ML-308-1337 (4% carriers) and ML-35 (0% carriers) were poisoned with 30 mM KN_3 for 30 min at 25°. [^{14}C]TMG at a final concentration of 0.5 mM (1 μC/ml) was added. Samples of 0.5 ml were withdrawn at intervals. After 40-min incubation, 1 M of unlabeled TMG was added to the cell suspension to give a final concentration of 10 mM. Samples of 0.5 ml were again withdrawn to determine the exit counterflow of [^{14}C]TMG from the cells.

back toward the equilibrium level. Cells with no membrane carriers showed slow entry and no counterflow. A delay in the time to reach the minimum was observed with other mutants with reduced transport activity. It was felt, however, that this method of assaying carriers was rather less sensitive than the "entrance counterflow" method previously described.

DISCUSSION

Counterflow as an example of active transport

Counterflow is a type of active transport in which the energy is not provided by the products of metabolism but by the investigator. Energy for counterflow is provided to the system in the form of the addition of a competitive inhibitor by the experimenter. This "inhibitor" blocks the unidirectional movement of the substrate from one side of the membrane while allowing movement in the opposite direction across the membrane. Movement of substrate in one direction with restricted movement in the opposite direction results ultimately in net movement against a chemical gradient. The two types of experiment are shown in Fig. 18. In the "entrance counterflow" situation accumulation of substrate continues until the inhibitor concentration falls to low levels (as a result of exit of the inhibitor). While this process normally occurs very rapidly in *E. coli* special cases can be found, such as lowering the temperature (Fig. 11) or adding PCMB after accumulation (Fig. 12), in which the accumulation may be prolonged. The maximum intracellular concentration attained in these experiments was 40 times that of the external medium (Fig. 3).

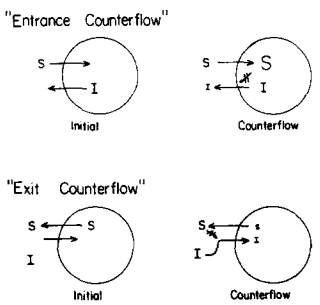


Fig. 18. Model for counterflow. *S* is the substrate and *I* the inhibitor.

"Exit counterflow" also results in active transport, in this case due to addition of inhibitor to the outside of the cell (Fig. 18). While extrusion from the cell is normally transient, if the inhibitor is metabolizable, it may never reach an internal concentration sufficient to inhibit exit and consequently result in a permanently low internal concentration of the substrate (Fig. 16).

Counterflow as an assay for membrane carriers

One of the major interests in this work was the development of an assay for membrane carriers uncoupled from the energy yielding processes of the cell either by the presence of metabolic inhibitors or by an alteration of the membrane protein by mutation. The measurement of the initial rate of uptake of [^{14}C]TMG into un-

coupled cells is extremely difficult due to the rapidity of equilibration and the low levels of radioactivity within the cell. With preloaded cells, on the other hand, high levels of ^{14}C-labeled substrate will enter the cell. In addition the number of carriers may be estimated both by the initial rate of uptake of the counterflow curve and also by the time taken to reach the peak of the curve.

Quantitative aspects of counterflow

Assuming a simplified model for carrier-mediated transport (or facilitated diffusion) it is possible to derive a relationship between several parameters at the time of the peak of the counterflow curve (see APPENDIX). When the v_{max} value is adjusted for the known degrees of exchange diffusion the equation predicts at least semi-quantitatively the internal concentration of [^{14}C]TMG.

APPENDIX

The most commonly considered carrier hypothesis for facilitated diffusion assumes specific binding sites on a carrier which alternately faces inside and outside the cell (either by actual mobility or by some type of allosteric change). It assumes that chemical interaction at the inner or outer surface of the membrane is much faster than the "migration" of the carrier. Several authors[1,3,16] have developed equations which describe such models. One relationship for the entry counterflow is the following:

$$\frac{dS_i}{dt} = \text{rate of entry} - \text{rate of exit}$$

$$\frac{dS_i}{dt} = v_{\max(in)} \frac{S_o}{K_t + S_o} - v_{\max(out)} \frac{S_i}{K_t + S_i + I_i} \quad (1)$$

where S_i and S_0 = substrate concentrations inside and outside the cells, I_i = inhibitor concentration inside the cell (the preloaded sugar in the entrance counterflow experiments), $v_{max\ (in)}$ = maximal rate of transport into the cell, $v_{max\ (out)}$ = maximal rate of transport out of the cell, K_t = concentration of substrate at which one half $v_{max\ (in)}$ is reached (analogous to K_m of the enzyme reaction).

Two simple relationships may be derived from Eqn. 1:

(1) Initial rate of entry

$$= v_{max\ (in)} \frac{S_o}{K_t + S_o} \quad (2)$$

(2) At the peak of the counterflow curve

$$\frac{dS_i}{dt} = 0 \text{ and}$$

$$v_{max\ (in)} \frac{S_o}{K_t + S_o} = v_{max\ (out)} \frac{S_i}{K_t + S_i + I_i} \quad (3)$$

An attempt was made to test the validity of Eqn. 3 by measuring several parameters and comparing them with calculated values. Measurements were made of the rate of exit of the preloaded or inhibitor TMG and, in parallel experiments, the rate of entry of TMG into preloaded cells was estimated. Values for I_i and for S_i

were taken at the time of the peak of the counterflow curve. The K_t for entrance is assumed to equal the K_t for exit under the conditions of these experiments[7]. The K_t (1 mM) is the same for entry into preloaded and non-preloaded cells (J. P. ROBBIE AND T. H. WILSON, unpublished results). It is assumed that the affinity of I for the carrier is the same as that of S. The v_{max} is affected by the "*trans*" concentration of the substrate[15]. The v_{max} into non-preloaded cells is 72 μmoles/ml per min while maximal preloading gives a v_{max} of 131. The v_{max} values used in these calculations are estimated by taking half saturation for exchange diffusion at 1 mM (the K_t). Thus, with a "*trans*" concentration of 1 mM, v_{max} would be 72 + 29 = 101.

TABLE I

CALCULATION OF INTERNAL CONCENTRATION OF [^{14}C]TMG AT THE PEAK OF THE COUNTERFLOW CURVE KNOWING OTHER PARAMETERS

For ML-308 v_{max} into non-preloaded cells = 72 μmoles/ml per min cell water; v_{max} into preloaded cells = 131 μmoles/ml cell water per min[15]. For ML-308-1710 the corresponding v_{max} values are 8% of those for ML-308. The calculation of the v_{max} values in the table is explained in the text. K_t for uptake into preloaded cells is the same as the K_t for uptake into non-preloaded cells (J. P. ROBBIE AND T. H. WILSON, unpublished results). Expt. 3 is the same as that shown in Fig. 13.

Expt. No.	*E. coli strain*	S_o (*mM*)	K_t (*mM*)	$v_{max\ (in)}$ (*μmoles/ml per min*)	$v_{max\ (out)}$ (*μmoles/ml per min*)	I (*mM*)	S_i (*mM*)	
							Exptl.	*Calc.*
1	ML-308	0.5	1	126	92	4.4	5.2	4.5
	ML-308-1710	0.5	1	9.86	7.33	3.2	3.5	3.4
2	ML-308	0.5	1	120	92	2.0	3.5	2.3
	ML-308-1710	0.5	1	9 41	7.33	1.5	1.9	1.9
3	ML-308	0.5	1	125	92	4.2	4.2	4.3
	ML-308-1710	0.5	1	9.78	7.33	3.4	2.4	3.5

With the measured values for S_0 and I *plus* the previously determined v_{max} values, calculations of S_i were made utilizing the Eqn. 3 (Table I). Cells with both a normal complement of carriers (ML-308) and ones with 8% carriers (ML-308-1710) were utilized. It can be seen from Table I that the calculated values were of the same order of magnitude as the experimental values. It is of interest that when a factor for "diffusion" was added to entry and exit components of the equation the value for S_i was affected by only a few percent (diffusion coefficient for TMG in ML-308 is 0.05 μmole/ml cell water per min per 1 mM concentration difference). Thus, the equation may be useful in predicting certain parameters at the time of the peak of the counterflow curve.

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