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On the Rate Limiting Step in Downhill Transport Via the LacY Permease of Escherichia coli

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Strains of Escherichia coli K12 were constructed for the specific purpose of evaluating the inducibility of the influx mechanism controlled by the *lacY* gene. These strains are heteromerodiploids characterized by a high and relatively constant level of β-D-galactosidase which is not affected significantly by induction of the Lac operon. These properties were obtained by introducing episomal $lacI^+, O^c, Z^+, Y^-$ genes into the cells. In these merodiploids the rate of o-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis of extracted cells is 50-times that of intact cells. This difference indicates that the rate limiting step in the ONPG hydrolysis by intact cells is influx.

Using a set of merodiploids with and without the LacY transport system, we were able to demonstrate a specific induction of ONPG influx. However, the increase in influx due to induction was only 3.5-fold as compared to the 40-fold increase observed when the LacY permease was measured by intracellular accumulation of [^{14}C]TMG.

Key words: transport, induction of influx, LacY permease, β-D-galactosidase, facilitated diffusion

Measurements of carrier-mediated transport across cell membranes may be readily interpreted when nonmetabolizable radioactive substrates are used, since the intracellular radioactivity is a direct function of the difference between influx and efflux (1). Under these conditions, measurements of influx independent of efflux are meaningful if made during the initial substrate uptake when efflux has negligible values. On the other hand, the use of metabolizable substrates for measuring influx offers convenient features while at the same time having serious limitations. This communication is concerned with these limitations and how to circumvent them.

The system chosen for this study is the LacY permease of Escherichia coli which is often measured by the transport of the metabolizable substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG). It has been traditionally assumed that the influx of ONPG via the LacY permease is the rate limiting step in the rate of ONPG hydrolysis by the intact cell (2–4). The hypothesis underlying this assumption is that the excess of β-D-galactosidase present in the cytoplasm assures that practically every molecule of ONPG transported by the permease is immediately hydrolyzed. However, experiments which involve induction of the permease may not fulfill this condition because of the low level of enzyme present at the onset of induction. For this reason the inducibility of the LacY permease measured

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by influx of ONPG must be tested in a system which provides a constant elevated level of β-D-galactosidase under all conditions.

We have fulfilled this criterion by constructing heteromerodiploids of E. coli with $lacI^+,O^c,Z^+,Y^-$ genes in the episomes and $lacI^+,O^+,Z_{del},Y^+$ in the chromosomes. While the LacY permease of these merodiploids may be varied experimentally by induction, the level of β-D-galactosidase is constant since the $lacZ^+$ gene is controlled by the constitutive $lacO^c$ operator in the cis position. In addition, by introducing the $lacI^+,O^c,Z^+,Y^-$ episome in bacteria containing a deletion of all the chromosomal *lac* genes, it was possible to determine the extent of influx due to passive diffusion or to transport systems other than the LacY permease.

METHODS

Organisms

The bacterial strains used in this study are listed in Table I. Episomal transfers were done according to the methods of Miller (5).

Chemicals

Methyl-1-thio-β-D-[1-^{14}C]galactopyranoside (TMG), 1.85 mC/mmole, was purchased from New England Nuclear Corporation (Boston, Massachusetts), and was purified by paper chromatography (6). Isopropyl-1-thio-β-D-galactopyranoside (IPTG) and o-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Nortok Associates (Lexington, Massachusetts).

Growth Conditions.

Cells were grown at 37°C in Davis minimal medium (7) supplemented with 0.4% sodium lactate (Fisher Scientific Company, Pittsburgh, Pennsylvania), and 0.5 μg/ml vitamin B_1. Cultures were aerated on a gyratory shaker. Prior to the experiments, overnight cultures were diluted in fresh, warm medium and allowed to divide for at least 2 generations. For induction, cells were grown in the presence of 5×10^{-4} M IPTG.

Transport Assay

Intracellular accumulation of [^{14}C]TMG was measured using cells harvested during exponential growth. Chloramphenicol at a final concentration of 30 μg/ml was added to the cultures and the cells were centrifuged and washed 3 times with cold growth medium without lactate. The assay mixtures (3 ml final volume) contained 5×10^8 cells suspended in growth medium, 100 μg of chloramphenicol, and 1.17 μM [^{14}C]TMG (10^4 cpm). After

TABLE I. Strains of E. coli K12 Used

Strain No.	Genetic markers[a]
W4680	$lacZ39_{del},strA$
W4980	$lac(Z,Y,A)_{del},strA$
W4980/F'$lacO^c$	$lac(Z,Y,A)_{del},strA$/F'$lacO^c$
S170	$lacZ39_{del},strA$/F'$lacO^c$,$lacY$
S172	$lac(Z,Y,A)_{del},strA$/F'$lacO^c$,$lacY$

[a]Genotype abbreviations are given according to Bachmann et al. (13).

incubation at 37°C with shaking, the mixtures were filtered through HA millipore membranes. The radioactivity of the dried membranes was determined by liquid scintillation counting with 68% efficiency. Blank values were obtained using formaldehyde-treated cells in the assay (8).

For measurements of ONPG hydrolysis in vivo, the washed cells were diluted with buffer B to a density of about 2×10^8 cells per ml. This buffer consists of 10 mM $MgCl_2$, 100 mM NaCl, and 50 mM 2-mercaptoethanol, adjusted to pH 7.45 (23°C) using acetic acid. The mercaptoethanol was added daily to the buffer. The cell suspension was allowed to equilibrate at 37°C for about 5 min, and then 0.2 ml of 3×10^{-2} M ONPG (in water) was added to start the reaction. After development of suitable color, 3 ml of 0.2 M Na_2CO_3 was added to stop the reaction and the mixture was immediately filtered through an HA millipore membrane. The supernatant was collected and its absorbance was measured at 420 nm. A molar extinction coefficient of 4,700 was used to convert absorbance readings to o-nitrophenol concentration.

The β-D-galactosidase activity of disrupted cells was measured using bacterial suspensions treated with deoxycholate and toluene (9, 11). For this assay, 1 ml of a cell suspension was mixed with 0.05 ml of 2% sodium deoxycholate and 3 drops of toluene. The mixture was left at room temperature for 10 min with occassional shaking. At the end of this period, 0.1 ml of the mixture was diluted 1:18 with buffer B and assayed for enzymatic activity as described above.

An enzyme unit for β-D-galactosidase is defined as the amount of enzyme liberating 1 nmole of substrate in 1 min under the indicated conditions.

RESULTS

Construction of a *lacI*$^+$, O^c, Z^+, Y^- Episome

A number of spontaneous Lac$^-$ mutants of W4980/F'*lacI*$^+O^cZ^+Y^+$ were obtained by penicillin selection in the presence of lactose followed by screening on EMB lactose agar (10). Among these mutants we chose 26 which had high levels of β-D-galactosidase while failing to accumulate [^{14}C] TMG intracellularly. The episome of each presumptive *lacY* mutant was transfered to W4680 for individual quantitative tests and one of them, termed S170, was selected for further studies. Subsequently the episome of S170 was transfered back to W4980 to yield S172, a strain with a deleted *lac* operon in the chromosome and the *lacI*$^+O^cZ^+Y^-$ episome. As shown in Table II, our postulation of the genotypes was confirmed by the phenotypes of the resulting heteromerodiploid strains. Both S170 and S172 had comparable levels of enzyme which were not affected significantly by induction. Strain S170 exhibited an inducible LacY transport system (measured by uptake of [^{14}C] TMG) while S172 was unable to accumulate TMG.

Inducibility of the LacY Transport System in the Merodiploids

The merodiploid S170 was compared to W4680, the parental haploid strain, in terms of inducibility of the LacY transport system. Exponentially growing cultures of each strain were induced in the presence of 5×10^{-4} M IPTG. At intervals cells were harvested, washed, and assayed for intracellular accumulation of [^{14}C] TMG. As shown in Fig. 1, S170 and W4680 exhibited similar patterns of inducibility. In contrast, the uptake of [^{14}C] TMG by either normal or induced cells of strain S172 was indistinguishable from

TABLE II. Phenotype of Parental and Derived Bacterial Strains

Strain	β-D-galactosidase of disrupted cells[a]		Transport	
	Noninduced	Induced	Noninduced	Induced
W4680	0	0	14	681
W4980	0	0	0	0
S170	2,800	3,300	32	502
S172	2,800	2,700	0	0

[a] The β-D-galactosidase activity of cells disrupted by treatment with deoxycholate and toluene was measured. The data is given in enzyme units per 10^9 bacteria. Transport was measured by the intracellular accumulation of [^{14}C] TMG and is expressed in counts per min per 10^9 cells. These values are corrected for a blank of 175–180 cpm; this was obtained when formaldehyde-treated cells were used in the permease assay (8).

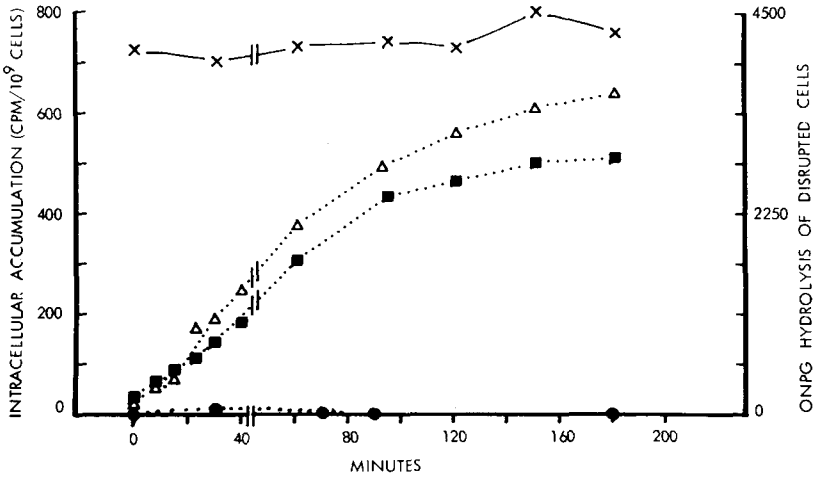

Fig. 1. Induction of the LacY transport system. At zero time, IPTG was added to exponentially growing bacterial cultures. Samples were withdrawn from the cultures at the indicated intervals and the level of LacY permease was determined by the intracellular accumulation of [^{14}C] TMG as indicated in the text. The results are expressed in cpm per 10^9 cells. A blank of 179 cpm was subtracted from each value. Accumulation of W4680 (△••△); S170 (■•••■); S172 (●•••●). The amount of β-D-galactosidase of S170 (X—X) was measured using cells disrupted by treatment with sodium deoxycholate and toluene (9, 11).

that of negative controls (formaldehyde-treated cells). These experiments also demonstrated that the level of β-D-galactosidase present in either S170 or S172 was not significantly affected by induction since we observed less than a 6% increase in extractable enzymatic activity at the end of a 3.5-h induction period (upper curve of Fig. 1).

Transport Measured by In Vivo Hydrolysis of ONPG

Cells from the same cultures used for determinations of uptake of [^{14}C] TMG were assayed for their ability to transport ONPG using the ONPG hydrolysis of intact cells as the parameter. Noninduced S172 cells (genetically defective in the *lacY* gene) showed

about half the rate of ONPG hydrolysis of noninduced cells of S170. Upon induction, the rate of S172 remained relatively constant (less than 20% increase) while that of S170 increased about 3.5-fold (Fig. 2). In both strains intact cells hydrolyzed considerably less than disrupted cells. The ratio of the hydrolysis rates of disrupted cells over that of intact cells was 25 for S170. This ratio decreased by a factor of 10 following an induction period of 70 min. In contrast, S172 exhibited a ratio of 50 which remained constant during induction.

Specificity of Induction of the LacY Transport System Measured by In Vivo Hydrolysis of ONPG

Cultures of S170 were grown in the presence of 10^{-3} M D-galactose, 10^{-3} M D-fucose, 10^{-3} M melibiose, or 5×10^{-4} M IPTG. The hydrolysis of both intact and disrupted cells of each culture was measured after 14.5 h of induction. The results of these experiments (Table III) indicated that among the sugars tested only IPTG is an inducer. We also observed that the level of enzyme in disrupted cells was reduced when cultures were grown in the presence of inducers other than IPTG. This was most noticeable when cells were grown with D-galactose.

DISCUSSION

The inducibility of the LacY transport system has been well documented through studies involving measurements of intracellular accumulation of radioactive, non-metabolizable substrates, i.e., measurements of the difference between influx and efflux. However, no direct evidence has been presented concerning the inducibility of the entry mechanism itself. Previous studies of the LacY permease used ONPG hydrolysis as a measure of influx, relying on the assumption that β-D-galactosidase is always present in

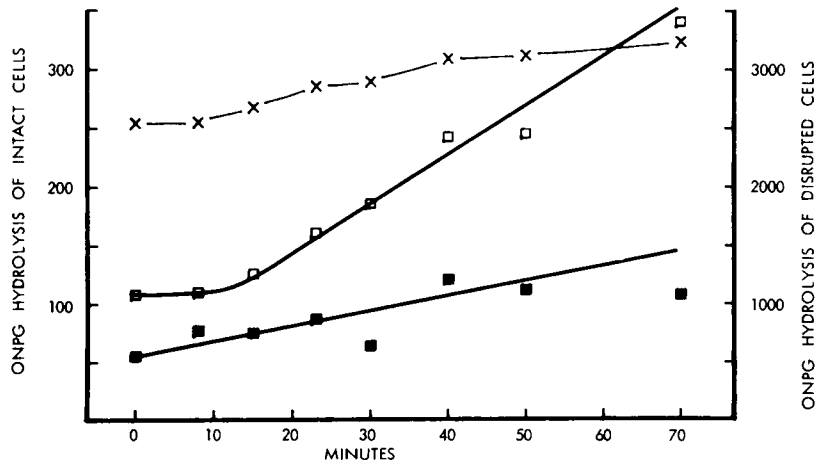

Fig. 2. Induction of the LacY transport system measured by the rate of ONPG hydrolysis of intact cells. We used the same cell suspensions previously assayed for intracellular accumulation of [^{14}C]-TMG in the experiment shown in Fig. 1. Hydrolysis rate of S170 (□–□); S172 (■–■). The rate of hydrolysis of disrupted cells of S170 is shown (X—X).

TABLE III. Induction of the LacY Transport System

Inducer[a]	β-D-galactosidase activity	
	Intact cells	Disrupted cells
IPTG	1,136	4,343
D-fucose	188	2,698
Melibiose	157	2,470
D-galactose	114	1,870
None	108	3,160

[a]Cells were induced overnight. The concentration of inducer was 5×10^{-4} M for IPTG, and 10^{-3} M for the other inducers. The results are given in enzyme units per 10^9 cells.

sufficient excess so that the influx is the rate limiting step. Although there are experimental data supporting this assumption, they are only valid for either constitutive or fully induced bacteria since these were the organisms used in the experiments. For conditions involving induction of the LacY transport system, it is necessary to have a relatively high level of β-D-galactosidase, a condition which is not usually fulfilled in E. coli because the uninduced cell contains only a few molecules of enzyme (11). We have presented here a system specially designed for experiments requiring induction of the LacY transport mechanism. The basis of this system is the use of bacteria containing an episomal *lac* operon with a constitutive operator gene and a defective *Y* gene. These bacteria produce high levels of β-D-galactosidase which are affected only minimally by induction. Because of the *lacY* mutation in the episome, it is possible to study the inducibility of the permease as controlled by the normal chromosomal Y^+ gene.

Using this system, we observed that the rate of ONPG hydrolysis in vivo increases with induction but that the maximum achieved is considerably less than that anticipated on the basis of induction of entry of TMG. Considering that induction causes an increase of 1,000-fold in the β-D-galactosidase level and an increase of 40-fold in the level of TMG accumulation, our finding that there is less than a fourfold increase in ONPG influx is surprising. The possibility that the episome somehow causes a defect in the expression of the normal *lacY* gene of the bacteria is unlikely, since S170 exhibited a normal LacY permease as measured by the intracellular accumulation of radioactive TMG (Fig. 1).

These paradoxical results may be explained by postulating that either ONPG can enter the cell through a system independent of the LacY permease or that induction causes a decrease in the exit rate of TMG. The former idea seems more likely for 2 reasons: i) our results using S172 (a strain with a deletion of the chromosomal *lacY* gene) show that these bacteria exhibit substantial transport of ONPG under either induced or noninduced conditions (Fig. 2); ii) results of previous experiments indicate that the exit mechanism of the LacY permease is induced by IPTG (8, 12).

We also tested the specificity of induction of the LacY influx mechanism using inducers of other transport systems. The results indicate that only inducers of the LacY permease increase the influx.

The heteromerodiploids described here can be of value for future studies on conditions affecting the LacY transport system. A possible application of the merodiploids would be in reexamination of the role of lipid biosynthesis where the use of strains with an inducible β-D-galactosidase may be responsible for the conflicting reports.

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