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METABOLIC CONTROL OF LACTOSE ENTRY IN *ESCHERICHIA COLI*

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Summary

A general method has been developed for determining the rate of entry of lactose into cells of *Escherichia coli* that contain β-galactosidase. Lactose entry is measured by either the glucose or galactose released after lactose hydrolysis. Since lactose is hydrolyzed by β-galactosidase as soon as it enters the cell, this assay measures the activity of the lactose transport system with respect to the translocation step. Using assays of glucose release, lactose entry was studied in strain GN2, which does not phosphorylate glucose. Lactose entry was stimulated 3-fold when cells were also presented with readily metabolizable substrates. Entry of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was only slightly elevated (1.5-fold) under the same conditions. The effects of arsenate treatment and anaerobiosis suggest that lactose entry may be limited by the need for reextrusion of protons which enter during H^+/sugar cotransport. Entry of *o*-nitrophenyl-β-D-galactopyranoside is less dependent on the need for proton reextrusion, probably because the stoichiometry of H^+/substrate cotransport is greater for lactose than for ONPG.

Introduction

In *Escherichia coli* the lactose transport system is induced coordinately with β-galactosidase. This has allowed two independent tests of the activity of this transport system [1]. In one case, transport is measured by the accumulation of non-metabolizable thio-galactosides, such as methyl-thio-β-D-galactopyranoside (TMG). Alternatively, the entry step is studied apart from accumulation by measurement of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) entry

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Abbreviations: TMG, methyl-thio-β-D-galactopyranoside; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; TDG, D-galactopyranosyl-thio-β-D-galactopyranoside; *p*CMB, *p*-chloromercuribenzoate; FCCP, carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone; IPTG, isopropyl-thio-β-D-galactopyranoside.

from the appearance of its hydrolysis product, *o*-nitrophenol. While use of these synthetic galactosides has proven extremely important in studies of this transport system, it is desirable to develop methods that employ the natural substrate, lactose. Although lactose accumulation may be studied with cells lacking β-galactosidase, it has not yet been possible to examine lactose entry in the absence of such accumulation.

This paper describes a method for the assay of lactose entry into cells containing β-galactosidase. Cells which possess both the lactose transport system and β-galactosidase, but which do not utilize glucose or galactose (or both), will excrete the unused hexose(s) when exposed to lactose. In this way, the appearance of either glucose or galactose provides a sensitive assay for the entry of lactose itself. One may use either normal cells, grown in media that do not induce the enzymes required for glucose of galactose utilization, or mutants defective in the metabolism of either hexose. This report presents studies with one such mutant, strain GN2, a cell that does not phosphorylate glucose [2].

The assay of lactose entry using strain GN2 provided a simple experimental system to test the effects of fermentable or oxidizable substrates on the activity of the lactose membrane carrier. These studies show that oxidative metabolism can elevate lactose entry 3-fold. However, entry of the analogue, ONPG, was enhanced only 1.2- to 1.5-fold. It is suggested that entry of the natural substrate, lactose, is limited by the need for proton extrusion, while entry of ONPG is not subject to such constraint. To account for this difference, it is postulated that the stoichiometry of H^+/substrate cotransport is greater for lactose than for ONPG.

Materials and Methods

Bacterial strains. The *E. coli* K12 strains used included strain 3300 (from the Pasteur Institute) and its derivative GN2, isolated by Fraenkel, Falcoz-Kelly and Horecker [2]. Strain X71 [3] was also used. These strains are constitutive for the products of the *lac* operon due to mutations in *lacI* (repressor). Strain GN2 also carries mutations which render it defective in glucokinase [2], and Enzyme I [4] of the phosphotransferase system [5]. GN2 was derived from MM6 (Enzyme I-negative) which had been isolated from 3300 [6]. Two strains inducible for the *lac* operon were used. Strain X9003 [7] has a deletion in *lacZ* (β-galactosidase); strain DF2000 [8] carries a normal *lac* operon, but is defective in both phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. A few experiments employed GN2-3, a lactose-negative derivative of GN2, selected by the method of Müller-Hill et al. [9] after nitrosoguanidine mutagenesis. GN2-3 has the parental level of β-galactosidase, but less than 2% of the parental level of the lactose transport system, as assayed by either ONPG entry or the accumulation of TMG.

Media and growth conditions. Mineral medium 63 [10] was used, with 1% (w/v) Difco-Bacto Tryptone as carbon source unless indicated otherwise; B_1 was present at 1 μg/ml. Vitamin-free casamino acids were obtained from Difco Laboratories. Cultures were shaken at 37°C in sidearm flasks, and growth was monitored turbidimetrically with a Klett-Summerson colorimeter (No. 42 filter). A Klett reading of 100 units corresponds to 810 μg wet wt. cells/ml; dry

weight was taken as 27% of wet weight [11]. For induction of the *lac* operon, isopropyl-thio-β-D-galactopyranoside (IPTG) was present at 0.5 mM during growth. D-Fucose was present at 10 mM during growth to induce maximal levels of the *gal* operon. For induction of the uptake system for hexose phosphates (*uhp*), cells were grown in the presence of 0.5 mM glucose-6-phosphate during the last generation before harvesting [12].

Chemicals. ONPG, IPTG and D-galactopyranosyl-thio-β-D-galactopyranoside (TDG) were purchased from Schwarz/Mann Co. Sigma Chemical Co. was the source of the following: lactose (substantially glucose-free), glucose, glucose-1-phosphate, glucose-6-phosphate, fructose-1-phosphate, fructose-6-phosphate, chloramphenicol, *N*-ethylmaleimide, *p*-chloromercuribenzoate (*p*CMB), dithiothreitol and adenosine 3′ : 5′ cyclic monophosphate (cyclicAMP). Pentachlorophenol was obtained from Eastman Kodak. Tetrachlorosalicylanilide was a gift of F.M. Harold (National Jewish Hospital and Research Center, Denver Colo.), and carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift of E.P. Kennedy (Harvard Medical School, Boston, Mass.). [^{14}C]Lactose and [^{14}C]TMG were purchased from New England Nuclear Corp.; the labelled lactose was purified by paper chromatography prior to use.

Assays. For routine assays, exponentially growing cells were treated with chloramphenicol (50 μg/ml) and chilled on ice. Cells were harvested by centrifugation at 4°C, washed once with cold medium 63 containing chloramphenicol and resuspended in this same medium at 4°C as a concentrated stock.

To measure lactose entry, 0.1 ml of stock cells was added to 2.9 ml of an incubation mixture (pH 7.0) prepared as follows: medium 63 with 50 mM sodium chloride (1.8 ml)/1 mg/ml chloramphenicol (0.3 ml)/100 mM lactose (0.3 ml)/water (0.5 ml). Final cell density was 30–60 μg dry weight of cells/ml. After incubation at either 26 or 37°C, the reaction was stopped by addition of 0.025 ml 12 mM *p*CMB (0.1 mM final concentration), followed by 0.9 ml of 0.3 M barium hydroxide. Samples were then chilled, after which 0.9 ml 5% zinc sulfate was added, and the precipitate removed by centrifugation. Glucose in the supernatant was determined with glucose oxidase (Glucostat, Worthington Biochemicals); galactose was measured with galactose oxidase (Galactostat, Worthington Biochemicals) or galactose dehydrogenase (Boehringer Mannheim Biochemicals) [13]. To determine lactose in the presence of glucose and galactose, the procedure outlined by Asensio et al. [6] was followed. The sample was first reduced by borohydride, and the remaining D-galactopyranosyl-β-D-glucitol was measured by the anthrone method [14]. To extimate lactose entry not mediated by the lactose transport system, parallel tubes contained 0.1 mM *p*CMB. When β-galactosidase activity was determined with lactose as substrate, the procedure was the same as for lactose entry except that cells were pretreated with toluene and deoxycholate [15].

ONPG entry was determined by incubating intact cells with this sugar and measuring the appearance of *o*-nitrophenol. Washed cells were incubated at pH 7.0 in the following medium [16]: 100 mM sodium phosphate/10 mM potassium chloride/1 mM magnesium sulfate/50 μg/ml chloramphenicol/1 mM β-mercaptoethanol/1 mM ONPG. After incubation at 37°C for suitable time, three vols. of 0.6 M sodium carbonate were added and the concentration of

o-nitrophenol determined with a Klett-Summerson colorimeter (No. 42 filter). To correct for the non-carrier-mediated entry of ONPG, parallel tubes contained 5 mM TDG. β-Galactosidase was routinely assayed using ONPG as substrate. The procedure was the same as that given above except that cells were pretreated with toluene and deoxycholate. Under these conditions, distruption of the cell membrane by toluene and deoxycholate increased ONPG hydrolysis by an average of 16-fold (9 experiments).

Assays of the accumulation of lactose or TMG were performed using published methods [1]. Assays of intracellular adenosine-5′-triphosphate (ATP) using firefly lantern extract (FLE-50, Sigma Chemical Co.) have been described [17].

Results

Assay for lactose entry. When strain 3300 was grown with Tryptone as carbon source, incubation of washed cells with lactose led to excretion of most of the glucose derived from lactose hydrolysis. Glucose appearing in the medium accounted for about 80% of the lactose lost (Fig. 1a). When the glucose-negative mutant GN2, derived from 3300, was tested under similar conditions, glucose was excreted quantitatively into the medium (Fig. 1b). For both strains, the galactose derived from lactose was not metabolized, and was recovered in the medium (not shown in the figure).

GN2 was studied further to determine if the quantitative excretion of glucose from lactose could serve as an assay for lactose transport. Glucose release proceeded linearly with time at a rate that was directly proportional to the cell density used. Glucose release was blocked 95% in the presence of 0.1 mM *p*CMB (a concentration that does not inhibit intracellular β-galactosidase). In addition, it was shown that glucose release by washed cells was the same as

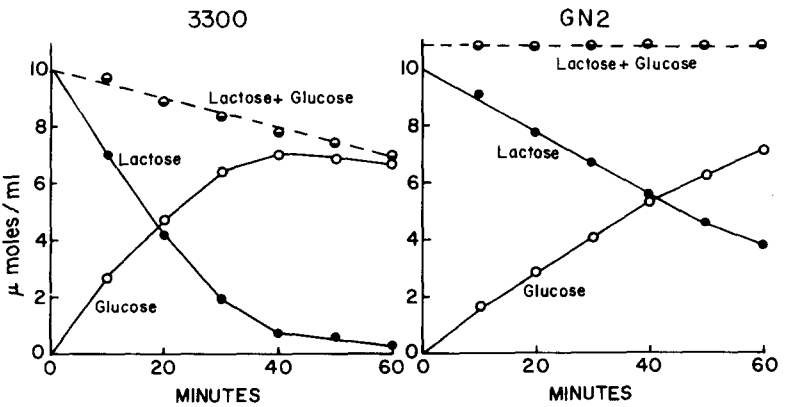

Fig. 1. Lactose utilization and glucose release by strains 3300 and GN2. Washed cells of 3300 or GN2 were shaken at 37°C in 5 ml of medium 63 containing 50 mM sodium chloride, 100 μg/ml chloramphenicol and 10 mM lactose. Final cell density was 1.1 mg dry wt. cells/ml. At the indicated times 0.5 ml samples were withdrawn for assays of lactose, glucose and galactose in cell-free supernatants, as described in Materials and Methods. Symbols: ●———●, lactose; ○———○, glucose; ⊖- - - - -⊖, lactose plus glucose.

that found in a growing culture. Cells were grown with lactose as carbon source, along with 0.1% casamino acids. As expected [2], appearance of glucose paralleled cell growth. From measurements of glucose in the medium and from the doubling time of cell mass (94 min), the in vivo rate of glucose release was calculated at 130 μmol/min per g dry wt. of cells. These same cells were harvested, washed and tested at 37°C. Glucose release from lactose by washed cells was 139 μmol/min per g dry wt. of cells.

If glucose release by GN2 is to be used as an assay for lactose transport, one must also show that transport rather than hydrolysis of lactose is rate limiting for glucose appearance. Two lines of evidence suggest that this is the case. For example, lactose hydrolysis was increased 3.5-fold (mean of 3 experiments) when the permeability barrier between external medium and intracellular β-galactosidase was removed with toluene. Additional evidence that transport is rate limiting was provided by experiments with *N*-ethylmaleimide, which inhibits transport but not β-galactosidase [18,19]. The experiment given in Fig. 2 shows that lactose hydrolysis by toluenized cells was unaltered by *N*-ethylmaleimide. However, lactose hydrolysis by intact cells was inhibited. The kinetics of that inactivation indicate exponential decay beginning from zero time. If β-galactosidase were rate limiting for hydrolysis of lactose by intact cells, one would expect a significant lag before glucose release decays. This is because external lactose (10 mM) was significantly higher than the K_m for either lactose transport (1 mM [11]) or β-galactosidase (approx. 1 mM [20]). Thus, entry and subsequent hydrolysis of lactose should be limited by either the number of membrane carriers or the quantity of internal enzyme.

Since strain GN2 was derived in two steps from its ancestor, 3300, it was

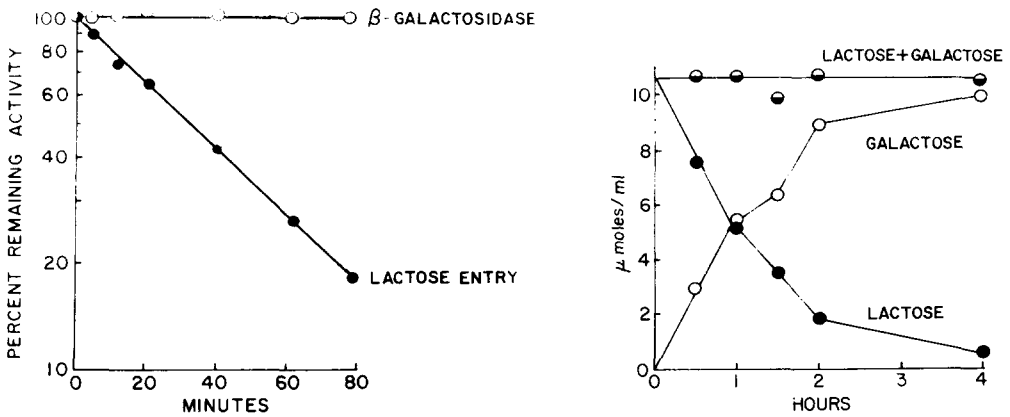

Fig. 2. Inhibition of lactose entry by *N*-ethylmaleimide in strain GN2. Washed cells (1.1 gm dry wt./ml) were placed at 0°C with 0.2 mM *N*-ethylmaleimide [18]. At the indicated times samples were removed and dithiothreitol (1 mM) added to inactivate *N*-ethylmaleimide. After 15 min at 0°C with dithiothreitol, a portion of each sample was assayed for lactose entry (26°C) (●———●) as described in Materials and Methods, except that final *p*CMB concentration was 0.25 mM. A second portion was used for assay of β-galactosidase with lactose as substrate (○———○). Activities at zero time were 55 and 250 μmol glucose formed/min per g dry wt. of cells, respectively, for intact and toluenized cells.

Fig. 3. Lactose utilization and galactose release by strain X71. Experimental details are given in Fig. 1. Symbols: ●———●, lactose; ○———○, galactose; ◒———◒, lactose plus galactose.

also important to show that a normal lactose membrane carrier had been preserved during mutagenesis and selection. For this reason, carrier activity in the two strains was tested with the conventional ONPG entry assay. The data were normalized to β-galactosidase content, since constitutive expression of the *lac* operon is lower in GN2 than in 3300 *. In nine comparisons the ratio of ONGP entry in GN2 and 3300 was 1.00 ± 0.04 (mean ± S.E.).

The data given above indicate that glucose excretion by GN2 may be used to assay entry of lactose. However, glucose excretion will not reflect lactose transport in normal cells where the capacity to phosphorylate glucose may be expressed at a high level. Under these conditions one may measure the galactose derived from lactose, using Tryptone-grown cells (which do not carry out significant galactose metabolism). As shown by Fig. 3, lactose entry could be estimated for one such strain, X71. Galactose derived from lactose was excreted quantitatively, while only 10% of the glucose from lactose was recovered in the medium (glucose data not shown).

Factors altering the activity of the lactose transport system. The assay of lactose entry using strain GN2 provided an experimental system to test whether cellular energy supplies were limiting for the transport of lactose. In initial experiments, cells were exposed to D-fucose during growth to induce the *gal* operon, allowing metabolism of the galactose derived from lactose. In this series of experiments, assays of ONPG entry showed only minor increases in D-fucose-induced cells (1.2-fold enhancement). However, carrier activity measured by lactose entry was increased 2-fold (Table I). Similar 2-fold increases in lactose entry were seen when assays were performed with the sodium-based medium used to test ONPG entry. Several observations indicate that such stimulation of lactose entry was not due to the appearance of a new transport system for lactose in D-fucose-induced cells. For example, no increment in lactose entry was found for the lactose-negative (*lacY*) derivative, GN2-3, when pregrown with D-fucose. Furthermore, TMG accumulation by GN2 was unaltered by prior growth with D-fucose. In addition, an experiment using [^{14}C]lactose labelled in the glucose moiety showed that during lactose entry similar levels of glucose (80—110 mM) were maintained by cells uninduced or induced for the *gal* operon. Thus, elevated entry of lactose in the induced cells appeared to be a result of galactose metabolism.

Several explanations were considered for the observed stimulation of lactose entry which accompanied induction of the *gal* operon. For example, stimulation of entry might occur as a result of specific interactions between the transport protein (or possibly β-galactosidase) and either galactose or one of its metabolites. Alternatively, there may be an enhanced carrier activity as a response to an increased rate of "energy" metabolism. To distinguish between these different possibilities, several kinds of experiments were performed.

In one series of experiments strain DF2000 was compared to strain GN2, since genetic blocks in DF2000 prevent metabolism beyond glucose-6-phos-

* *Lac* operon expression in GN2 is about 65% of that found in 3300. This is presumably related to defects in cyclic AMP metabolism introduced by the Enzyme I mutation [41]. When GN2 was grown with 1 mM cyclic AMP, *lac* operon expression was elevated. Cyclic AMP had no effect on the entry of either lactose or ONPG in cells grown in the presence or absence of the nucleotide.

TABLE I

EFFECTS OF METABOLIZABLE SUBSTRATES ON THE ACTIVITY OF THE LACTOSE TRANSPORT SYSTEM

Data expressed relative to values found for controls (cells uninduced for *gal*; cells without hexose phosphate or D-lactate). Mean ratios ± the standard errors of the means are shown, for the number of experiments indicated in parentheses. For strain GN2, hexose phosphate was present at saturating concentration (0.1 mM or above, see Fig. 5 and text). For strain DF2000, hexose phosphate was present at 0.1 mM. D-Lactate was present at 10 mM.

Strain	Prior induction	Added energy source	Carrier assay (relative to control)	
			ONPG entry	Lactose entry *
GN2	*gal* operon	None	1.19 ± 0.02 (15)	2.04 ± 0.07 (15)
	None	Glucose-6-phosphate	1.06 ± 0.02 (14)	1.03 ± 0.03 (14)
	uhp transport	Glucose-6-phosphate	1.42 ± 0.05 (12)	2.53 ± 0.14 (15)
	None	Lactate	1.47 ± 0.08 (4)	2.78 ± 0.22 (6)
DF2000	None	Fructose-6-phosphate	1.04 ± 0.04 (6)	
	uhp transport	Fructose-6-phosphate	1.56 ± 0.07 (6)	

* Since relative stimulation of lactose entry was the same at 37 and 26°C, data from assays at both temperatures was combined for *gal*-induced and uhp-induced cells. Lactose entry in D-lactate-treated cells was tested at 26°C.

phate. For this strain, in contrast to strain GN2, no increase in lactose or ONPG entry was found for cells induced with D-fucose (data not shown). This suggested that metabolism beyond the level of glucose-6-phosphate was required to "activate" the carrier. Experiments supporting this idea examined the effects of exogenous hexose phosphates, using cells induced for the uptake system for hexose phosphates (*uhp*) [21,22]. As shown in Fig. 4, addition of glucose-6-phosphate to DF2000 gave no change in carrier activity (ONPG entry). However, fructose-6-phosphate gave significant stimulation of carrier activity in *uhp*-induced cells. At these concentrations (less than 0.1 mM) glucose-1- and fructose-1-phosphate were ineffective, and higher levels of hexose-1- or -6-phosphate gave inhibition of carrier activity. For example, in an experiment like that of Fig. 4, increasing fructose-6-phosphate to 1 mM lowered OPNG entry to 60% of the control. Such inhibition probably reflected the inhibitory interaction between the lactose transport and phosphotransferase systems [23,24] due to low levels of free sugar contaminating hexose phosphates. For this reason lactose entry was not routinely tested in DF2000. However, a few experiments did show that lactose entry in DF2000 (galactose or glucose excretion) was elevated about 2-fold by 0.1 mM fructose-6-phosphate, but not glucose-6-phosphate.

Similar experiments were performed with GN2, using both lactose and ONPG as transport substrates. For GN2, glucose-6-phosphate and fructose-6-phosphate were equally effective in enhancing carrier activity, giving maximal stimulation of lactose or ONGP entry at 0.1 mM or above; no inhibition of

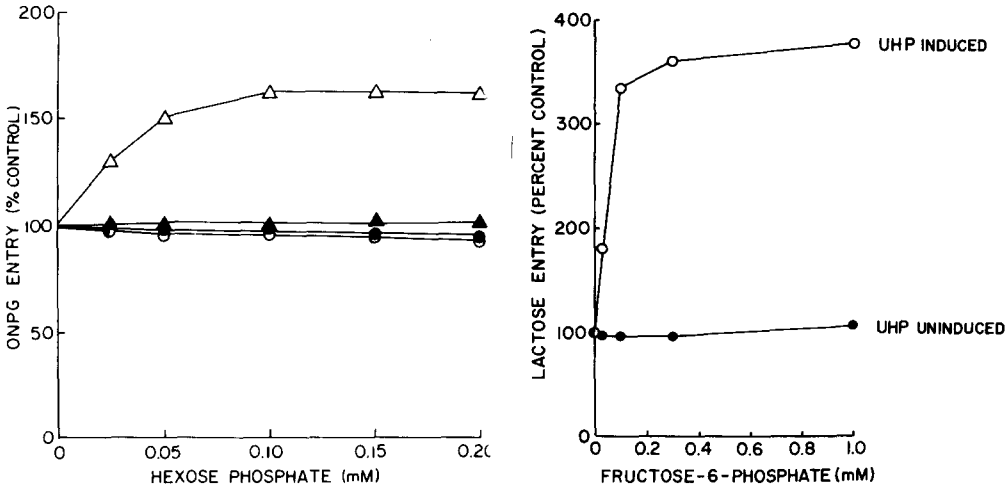

Fig. 4. Effect of hexose phosphates on ONPG entry in strain DF2000. ONPG entry was performed as described in Materials and Methods, using cells either induced (open symbols) or uninduced (closed symbols) for hexose phosphate transport. Assays were done in the presence of the indicated levels of glucose-6-phosphate (○, ●) or fructose-6-phosphate (△, ▲). ONPG entry is expressed relative to values found for cells tested in the absence of added hexose phosphate.

Fig. 5. Effect of fructose-6-phosphate on lactose entry in strain GN2. Lactose entry (26°C) was performed as described in Materials and Methods, in the presence of the indicated concentrations of fructose-6-phosphate, using cells induced (○———○) or uninduced (●———●) for hexose phosphate transport. Lactose entry is expressed relative to values found for cells tested without added fructose-6-phosphate.

carrier activity was found when either hexose-6-phosphate was as high as 10 mM. Glucose-1- or fructose-1-phosphate also stimulated carrier activity, but maximal effects required higher levels (1 mM) than for hexose-6-phosphates (0.1 mM). Fig. 5 shows the effect of fructose-6-phosphate on lactose entry in GN2; the effects of glucose-6-phosphate on both lactose and ONPG entry are summarized in Table I. Such stimulation of lactose entry was 2- to 3-fold in GN2 (Fig. 5, Table I), but maximal stimulation of ONPG entry was only 1.5-fold, for both GN2 and DF2000 (Fig. 4, Table I).

The effect of hexose phosphates was not confined to the entry step. Experiments with strain X9003 (β-galactosidase-negative) showed that for *uhp*-induced cells, glucose-6-phosphate also increased lactose accumulation by about 3-fold (Fig. 6).

D-Lactate, an oxidizable substrate, also elevated carrier activity in GN2, and once again, it was found that assays of lactose entry were more susceptible to stimulation than assays of ONPG entry (Table I). The experiments given in Table II show that no stimulation of lactose entry was observed under anaerobic conditions, when either D-lactate or glucose-6-phosphate (*uhp*-induced cells) was present. D-Fucose-induced cells also failed to show elevated lactose entry when tested under anaerobic conditions (not shown). Thus, oxidative metabolism appears necessary for enhancement of carrier activity. However, net synthesis of ATP during oxidative phosphorylation is not required, for lactose entry was elevated 3-fold when glucose-6-phosphate-

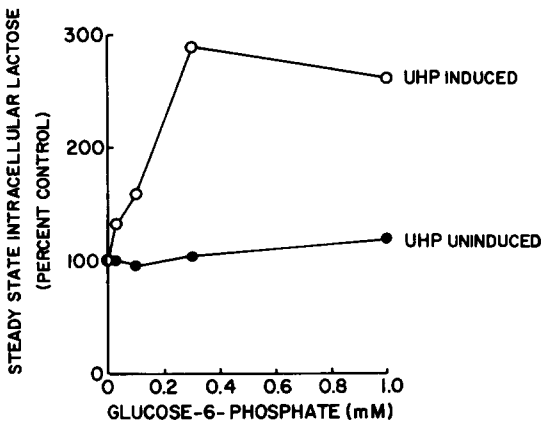

Fig. 6. Effect of glucose-6-phosphate on lactose accumulation by strain X9003. Cells induced (○———○) or uninduced (●———●) for hexose phosphate transport were incubated for 15 min at 26° C with 0.1 mM lactose (0.5 mCi/mmol) together with the indicated levels of glucose-6-phosphate. Lactose accumulation is expressed relative to that found in controls with no added glucose-6-phosphate. For these controls, internal lactose was 130 times higher than medium concentration.

treated cells were tested in the presence of arsenate (30 mM, in phosphate-free medium). Under these conditions, exposure of control and glucose-6-phosphate treated cells to arsenate (1 mM or above) lowered internal ATP by 85 to 90% as determined by the luciferase assay.

The effect of proton conductors was also examined. For the three proton conductors tested (FCCP, pentachlorophenol, and tetrasalicylanilide), potent inhibition (85—95%) of lactose entry was observed at inhibitor levels between 20 and 50 μM final concentration. Such high levels of inhibition were observed whether or not cells had been treated with D-lactate.

TABLE II

EFFECT OF ANAEROBIOSIS ON LACTOSE ENTRY IN STRAIN GN2

In each experiment, data are expressed relative to the value found for cells tested aerobically without further additions. For cells tested aerobically, lactose entry (26°C) was performed as described in Methods, using cells uninduced (I) or induced (II) for the hexose phosphate transport system. For anaerobic assays, the diluted cell suspensions were placed in Thunberg tubes, the sidearms containing either lactose or lactose plus additive. Thunberg tubes were subjected to three cycles of deaeration and flushing with nitrogen before equilibration at 26°C. The reaction was initiated by mixing the contents of the sidearms and tubes.

Experiment	Additions to assay	Lactose entry	
		Aerobic	Anaerobic
I	None	100	60
	D-Lactate *	330	65
II	None	100	55
	Glucose-6-phosphate *	280	63

* D-Lactate was present at a final concentration of 10 mM; glucose-6-phosphate was used at 0.2 mM final concentration.

Discussion

One objective of this study has been to develop a general method for studying lactose entry in cells containing β-galactosidase. Under the appropriate conditions lactose entry may be estimated by the excretion of either the glucose or the galactose derived from the hydrolysis of the disaccharide. Assays of galactose release may be more suitable for general use, for strains vary in their basal capacity of phosphorylate glucose (ref. 25; compare 3300 and X71). Use of these assays requires that the transport system, rather than β-galactosidase, is rate limiting for hydrolysis of lactose. This was shown by two kinds of experiments. First, lactose hydrolysis increased when the membrane permeability barrier was disrupted. Second, exposure to *N*-ethylmaleimide led to simple exponential decay of lactose hydrolysis by intact cells (Fig. 2).

The experiments reported here also show that the activity of the lactose membrane carrier can be significantly elevated if cells are allowed to metabolize other substrates during sugar entry. This phenomenon is distinct from the modulations of carrier activity described previously. During "exchange diffusion" efflux of a preloaded substrate accelerates entry of external substrate [26]. Thus, exchange diffusion may complicate the interpretation of experiments comparing lactose entry in cells induced or uninduced for the *gal* operon, since galactose efflux on the lactose carrier may be reduced when cells metabolize galactose [27]. Interpretation of such experiments is complicated further by the possibility that when cells are unable to metabolize galactose, an increased level of internal galactose might alter β-galactosidase activity. However, these criticisms are not appropriate for the experiments showing elevated substrate entry in cells exposed to hexose phosphates or D-lactate.

The mechanism responsible for the marked stimulation of lactose entry is not completely understood, but the effects of anaerobiosis and arsenate treatment suggest that such stimulation may result from increased respiratory-driven proton extrusion. Since the lactose transport system catalyzes H^+/substrate cotransport [28], initiation of sugar entry would bring protons into the cell and diminish the difference in electrochemical potential for H^+ ($\Delta\tilde{\mu}_{H^+}$) present across the membrane [29]. This would establish a new and lower steady state value for $\Delta\tilde{\mu}_{H^+}$, reducing the driving force for later sugar entry. The presence of additional metabolizable substrates should increase the capacity of the cell to reextrude protons entering with sugar, and elevate $\Delta\tilde{\mu}_{H^+}$. If one takes the reasonable view that the value of $\Delta\tilde{\mu}_{H^+}$ determines the fraction of carriers in a form capable of binding and transporting sugar [30,31], then increased proton reextrusion would be reflected by an increased maximal velocity of sugar entry. Thus, the observed stimulations of lactose entry (Table I) are understood if sugar entry is limited by the need to reextrude protons entering during cotransport.

This explanation for the observed stimulations of lactose is supported by the findings of Schuldiner and Kaback [32] and Ramos and Kaback [33], who showed that lactose accumulation by membrane vesicles reduced both the electrical and pH gradients across the vesicle membrane. In addition, Koch [34] has shown that carrier-mediated ONPG entry is absent in cells starved of endogenous energy reserves. This has been attributed to a failutre to reextrude

protons which entered with ONPG [35]. The experiment of Fig. 6 supports the idea that added metabolizable substrate can elevate $\Delta\tilde{\mu}_{H^+}$, since glucose-6-phosphate increased lactose accumulation by strain X9003 from 130- to 400-fold above medium level. If lactose accumulation is in equilibrium with $\Delta\tilde{\mu}_{H^+}$ then the chemical potential for lactose measures $\Delta\tilde{\mu}_{H^+}$ indirectly. This has been verified for TMG accumulation in *Streptococcus lactis* [36] and *E. coli* [37]. Thus, the data in Fig. 6 indicate that metabolism of glucose-6-phosphate can elevate $\Delta\tilde{\mu}_{H^+}$ from 127 to 155 mV if H^+/lactose stoichiometry is 1.

The idea that sugar entry is limited by the need to reextrude protons may appear inconsistent with the finding that proton conductors block lactose entry, since Cecchini and Koch [35] report that some proton conductors stimulate ONPG entry in starved cells. However, in addition to providing low resistance pathways for H^+ movements, proton conductors will also reduce the value of $\Delta\tilde{\mu}_{H^+}$, thus lowering the fraction of carriers in a form capable of accepting substrate. Under conditions where $\Delta\tilde{\mu}_{H^+}$ is zero, one presumes that this "active" fraction of the carriers is determined by the absolute value of external pH and the pK of the protonatable group functioning during transport. Such reasoning is suggested by recent work of Amanuma et al. [38] which showed pH-dependent binding of proline to its carrier in *E. coli* vesicles. Since there may be strain variation in the pK of the critical protonatable group, there is no necessary conflict between the finding that proton conductors inhibit lactose entry and the idea that sugar entry is limited by a requirement for proton reextrusion in the absence of proton conductors. (If, as suggested below, more than one moiety is protonated during lactose transport, then inhibition by proton conductors is expected to be more potent for lactose entry than ONPG entry.)

In absolute amount, the basal rate of sugar entry was greater for ONPG than for lactose (296 ± 11 and 178 ± 7 μmole translocated/min per g dry w. (mean ± S.E.), respectively, for 17 comparisons at 37°C). Thus, it is not readily apparent why increased proton extrusion markedly elevates lactose entry (3-fold), whereas ONPG entry shows only a modest 1.5-fold increase. This paradox is resolved if one postulates that the stoichiometry of H^+/substrate cotransport may be different for ONPG and lactose. For assays performed at pH 7, the calculations of Purdy and Koch [39] indicate that the ratio H^+/ONPG is 1. Recent experiments of Ramos and Kaback [40] suggest that the ratio H^+/lactose is pH dependent, rising from 1 at pH 5.5 to 2 at pH 7.5. Since both ONPG and lactose entry show some dependence on proton reextrusion (Table I), it is reasonable to assume that under basal conditions, transport of each of these substrates reflects equal numbers of protons entering the cell. Thus, the data reported here suggest that for strain GN2 at pH 7, H^+/lactose cotransport has a value of about 1.7. Clearly, the hypothesis that the stoichiometry of H^+/ONPG is less than H^+/lactose is subject to direct tests, and further experiments will be required to substantiate the idea that stoichiometry of cotransport may vary according to the substrate employed.

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