CONTROL OF LACTOSE TRANSPORT IN *ESCHERICHIA COLI*

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1. Introduction

It is essential for optimal growth rate on a carbon source such as lactose that cells carefully regulate the transport and the first step in metabolism. Inducibility as a mode of regulation of catabolic pathways is physiologically economical since specific proteins are produced only when needed by the cell. In the case of the *lac* operon of *Escherichia coli*, 1000-fold variation in the levels of transport and β-galactosidase is possible. It is interesting to note that lactose itself does not induce this operon but is converted by β-galactosidase into another disaccharide, allolactose, which is the true physiological inducer [1]. Normal induction requires the proper relationship between the levels of β-galactosidase and transport. According to Mieschendahl et al. [2] the absence of β-galactosidase (*lacZ*) leads to partial (10%) constitutivity of lactose transport and the transacetylase. They propose that this phenomenon is due to the accumulation of an endogenous inducer which is normally destroyed by β-galactosidase in the uninduced cell. An interesting type of imbalance of an opposite nature is seen in cells which possess an abnormally high level of β-galactosidase compared with the level of transport. When a *lacZ* gene is introduced (via an episome) into a cell with low levels of *lac* expression the cell becomes lactose negative. The lactose negative phenotype may be due to the failure of the cell to accumulate sufficiently high levels of allolactose [3] or to the destruction of an endogenous inducer [2].

An important further control is that exerted by glucose. Early studies of Monod [4] indicated that when cells of *E. coli* were placed in a medium containing glucose and lactose there was preferential utilization of glucose; induction of the *lac* operon and subsequent metabolism of lactose occurred only after all of the glucose had been exhausted. Such a physiological control system spares the cell synthesis of proteins not essential for growth.

Glucose control of catabolic systems has been the subject of intensive study for many years. In 1965 Makman and Sutherland [5] discovered that glucose added to *E. coli* reduced the intracellular level of adenosine 3′,5′-cyclic monophosphate. It was then shown by Perlman and Pastan [6] and by Ullman and Monod [7] that the addition of cAMP reversed the glucose catabolite repression of systems including the *lac* operon. This cyclic nucleotide was shown to be necessary for the transcription of the *lac* operon.

In addition to its effect in catabolite repression glucose exerts a second effect at the level of the membrane carrier. Glucose or α-methyl-glucoside strongly inhibits lactose transport in glucose-grown lactose-constitutive cells. The inhibition occurs within a few seconds of addition of glucose [8] and is not affected by the addition of cyclic AMP. This effect is due to an element in the PEP-dependent phosphotransferase (PT) system [9–11]. Saier has developed the hypothesis [9] that the metabolism of glucose or α-methyl-glucoside reduces the level of phosphorylated intermediates in the PT-system. A dephosphorylated membrane protein interacts directly with the lactose-transport protein within the membrane and lowers the activity. The term 'inducer exclusion' has been used for this phenomenon [see preceding paper in this issue].

Although considerable work has been done on glucose repression very little attention has been paid to the repression resulting from growth in lactose itself. It has been known for many years that cells grown on lactose possess only 20–40% of the β-galactosidase and transport levels that are found in constitutive or fully-induced cells. This lactose repression of its own operon (and other catabolic systems such as trypto-

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Dedicated to Professor Sir Hans Krebs, FRS, on his eightieth birthday

phanase) was originally assumed to involve glucose catabolite repression [12]. However, Jobe and Bourgeois [13] found that it occurred in a *lacZ* cell and that cyclic AMP did not affect the phenomenon. They showed that high concentrations of lactose could act as an anti-inducer and postulated that this was the mechanism of its repression. More recently, Peterkofsky and Gazdar [14] showed that the addition of lactose or non-metabolizable analogues to *lac*-constitutive cells lowered the adenyl cyclase activity. These apparently contradictory results stimulated the present investigation.

This paper presents additional experiments on lactose repression in growing cells. The use of several types of mutants has helped to clarify the mechanism of this inhibition. Evidence will be presented to support the view that lactose repression is partly due to glucose spillage and glucose catabolite repression, and partly to another phenomenon which does not involve glucose as suggested by Peterkofsky and Gazdar [14]. Data will also be presented to show that *lac*-constitutive cells growing on glycerol, free of regulation, are vulnerable to growth inhibition by the sudden addition of lactose. Evidence is presented supporting the hypothesis that this inhibition is due to a partial collapse of the membrane potential from the rapid proton entry with lactose on the carrier.

2. Materials and methods

The following *Escherichia coli* strains were used: X71-15 ($lacI^+Z^+Y^+A^-$) and 54-41 ($lacI^+Z^+Y^{UN}A^-$) [15], ML308-831 ($lacI^-Z^+Y^+A^-$) [16], 3300 ($lacI^-Z^+Y^+A^+$) from the Pasteur Institute, ZSC17 (glk^-) and ZSC112 (*glk, ptsG, ptsM*) isolated by Curtis and Epstein [17] provided by A. Peterkofsky, UV5 (promoter mutant resistant to catabolite repression) from Jon Beckwith, and HVT 291 constitutive for tryptophanase [18] from M. D. Yudkin. Cells were grown in medium 63 [19] plus 1% tryptone (Difco) or other carbon sources indicated. Concentration of cells was monitored with a Klett–Summerson colorimeter (no. 42 filter) (100 Klett units = 0.82 mg wet wt/ml).

Tryptophanase was assayed by the method in [20], β-galactosidase by the method in [21], glycerol kinase by the method in [22] and α-glycerophosphate dehydrogenase by the method in [23]. The method for centrifugation of cells through silicone oil was that in [24]. The ΔpH measurement was given in [25]. ATP was determined by the method in [26].

Lactose, galactose, maltose, melibiose, isopropyl-β-D-thiogalactopyranoside (IPTG), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), adenosine 3′,5′-cyclic monophosphate (cAMP) and tryptophan were from Sigma. Methyl-β-D-galactopyranoside was purchased from Corn Products Co. Thio-*o*-nitrophenyl-β-D-galactopyranoside (TONPG) was from Cyclo Chemical Corp.

3. Results

3.1. *Lactose repression*

The phenomenon of lactose repression is illustrated in fig.1. Cells of strain X71-15 were grown in lactose or other carbon sources in the presence of IPTG to induce β-galactosidase and L-tryptophan to induce tryptophanase. Cells grown on either glucose or lactose showed about 60% inhibition of the β-galactosidase and about 97% inhibition of the tryptophanase compared with glycerol-grown cells. Cyclic AMP prevented the glucose repression of the *lac* operon and most of the glucose repression of tryptophanase. On the other hand, the nucleotide had little or no effect on the lactose-grown cells. The effect of lactose is not on the process of induction as entirely similar inhibitory effects are obtained with several *lac*-constitutive strains. Growth on galactose resulted in a smaller degree of repression and again was unaffected by

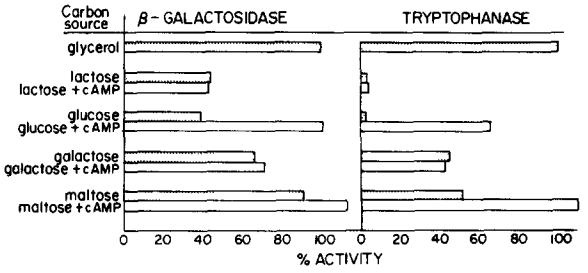

Fig.1. β-Galactosidase and tryptophanase activities in X71-15. At the start of the experiment, cultures had been growing logarithmically for 10 doublings in medium 63 (pH 7.0) containing L-tryptophan (3 mM) IPTG (1 mM) and one of the following carbon sources: glycerol (0.4%); lactose (10 mM); glucose (0.4%); galactose (10 mM); maltose (10 mM). The cells were diluted into fresh media with or without cAMP (5 mM) and grown for 4–5 more doublings. The data are mean values of 4 separate expt.

cAMP (fig.1). Cells grown on melibiose or β-methyl-galactoside showed little or no repression of *lac* operon expression (not shown).

Additional experiments were done on two other catabolic enzymes, glycerol kinase and β-glycerophosphate dehydrogenase. Growth on either glucose or lactose resulted in severe repression (compared with that observed in cells grown on glycerol). Cyclic AMP abolishes most of the glucose repression and only part of the lactose repression.

Under certain conditions cAMP exerts a significant curing effect on lactose repression. When the tryptophanase-constitutive strain (HVT 291) was grown in the presence of lactose, cAMP prevented much of the repression of the enzyme (table 1). The nucleotide was most effective at the lower pH, perhaps due to faster uptake of cAMP when in the more protonated form.

It is possible that some of the glucose resulting from lactose hydrolysis spills out of the cell and is subsequently taken up by the PT-system giving glucose catabolite repression. Apparently intracellular glucose is ineffective, as growth on maltose (fig.1) and melibiose (unpublished) has little inhibitory effect on the two enzymes. Although growth of cells on lactose does not result in detectable quantities of glucose and galactose in the growth medium [27] it is possible that a small amount of glucose appears in the periplasmic space and is 'recaptured' by the PT-system. If this were the sole method of repression a PT-mutant should be insensitive to lactose. An experiment on such a mutant is shown in table 2. Lactose produces repression of tryptophanase and β-galactosidase synthesis in a cell lacking the enzymes II for glucose and mannose whereas glucose produces no effect in this mutant. Thus, lactose can repress tryptophanase and β-galactosidase by a mechanism independent of glucose.

Table 2
Lactose repression in glucose-negative mutant[a]

Cell	Sugar addition	Specific activity (% control)[b]	
		β-Galactosidase	Tryptophanase
Parent	Glucose	53	31
Mutant	Glucose	103	106
Parent	Lactose	59	18
Mutant	Lactose	61	54

[a] The glucose-negative mutant (ZSC 112) and its parent (ZSC 17) were grown for 4 generations in tryptone alone (pH 7) or with the addition of glucose or lactose. Tryptophan (3 mM) was included in the growth medium, to ensure induction of tryptophanase. IPTG (1 mM) and thiamine (0.5 μg/ml) were added to all cultures

[b] The expression of enzyme by cells grown in tryptone alone was taken as the control (100%)

An experiment was carried out with a *lac* promoter mutant (UV5) to determine whether lactose repression of the *lac* operon was mediated via the promoter (table 3). No lactose repression (or glucose catabolite repression) was observed on β-galactosidase in this mutant although >90% inhibition of tryptophanase was observed with each sugar. Thus, the effects of

Table 1
Partial relief by cAMP of lactose repression of tryptophanase

Conditions of growth[a]	Specific activity of tryptophanase % control (tryptone grown)	
	pH 7	pH 5
Tryptone	100[b]	100[b]
Tryptone + cAMP	108	120
Tryptone + lactose	19	11
Tryptone + lactose + cAMP	60	70

[a] Strain HVT291 (constitutive for tryptophanase) was grown overnight in medium 63 plus 1% tryptone, 50 μg/ml methionine and 0.5 μg thiamine/ml, at pH 7 and at pH 5. Cells were diluted 1/50 into fresh tryptone medium with or without lactose (10 mM), and were grown for 4–5 doublings. Cyclic AMP (10 mM) was added 2 h prior to harvesting

[b] The absolute level of tryptophanase for the tryptone grown cells was the same in the cells grown at the two pH values

Table 3
Lactose repression in *lac* promoter mutant (UV5)[a]

Carbohydrate addition[b]		Specific activity (% control)[c]	
		β-Galactosidase	Tryptophanase
Glycerol	0.2%	100	100
Glucose	0.2%	97	8
Lactose	0.2%	99	9

[a] UV5 is a *lac* promoter mutant that shows normal *lac* operon expression but is resistant to glucose catabolite repression
[b] Cells were grown in tryptone (pH 7) with the addition of 1 mM IPTG and the carbohydrate listed
[c] Cells grown in tryptone plus glycerol were taken as the control

lactose probably are mediated via the promoter (presumably via cAMP regulation).

One possible glucose-independent mechanism for lactose repression is the rapid entry of protons (with lactose) on the lactose carrier reducing the protonmotive force. Such lactose-induced proton entry is known to cause partial collapse of the membrane potential [28]. Peterkofsky and Gazdar [14] have shown that reduction in protonmotive force leads to a fall in cAMP. In order to test this possibility experiments were carried out with a *lacY* mutant [15] in which lactose enters the cell without the normal proton entry. The lactose carrier in this mutant transports lactose at 85% of the rate of the parent and ONPG at a rate somewhat faster than the parent. Although the carrier in the mutant is capable of transfer of these substrates down a concentration gradient into the cell it fails to accumulate the substrates due to inability to cotransport protons with the sugar [29]. Table 4 indicates that when this mutant is grown on lactose it shows much less repression of β-galactosidase or transport than shown by the parent cell. Note that the carrier activity for ONPG is not defective in the mutant but actually elevated compared with the parental strain X71-15. When the mutant strain 54-41 was grown on lactose in the presence of tryptophan, it showed only 50% repression of tryptophanase while the parental cell showed 95% repression (av. 3 expt). A similar *lacY* mutant in a constitutive *lac* operon (ML308-22) shows less lactose repression of β-galactosidase than its parent (ML-308) (unpublished).

Another example of an effect that may be mediated via changes in protonmotive force is the repression caused by the non-metabolizable thiogalactoside TONPG. Table 5 shows an experiment in which the addition of thiogalactoside resulted in inhibition of growth and repression of both β-galactosidase and tryptophanase. Other thiogalactosides, such as IPTG and thiomethyl-β-D-galactoside, show very weak inhibitory effects in *lac*-constitutive cells.

3.2. *Inhibition of growth by the addition of lactose*

The importance of regulation of the *lac* operon is demonstrated by the following experiments. In 1965

Table 4
β-Galactosidase and membrane carrier activity in X71-15 and 54-41 grown on lactose

Growth medium	ONPG hydrolysis (μmol . min^{-1} . g wet wt^{-1})	
	X71-15 (parent)	54-41 (mutant)
I. β-Galactosidase activity		
Glycerol	1834 ± 26 (5)	1834 ± 133 (5)
Lactose	765 ± 44 (8)	1477 ± 63 (8)
Lactose + cAMP	1010 ± 92 (6)	1856 ± 32 (5)
II. Carrier mediated entry		
Glycerol	83 ± 4 (5)	133 ± 9 (5)
Lactose	38 ± 3 (9)	99 ± 11 (9)
Lactose + cAMP	54 ± 10 (6)	137 ± 28 (5)

Cells were grown to mid-exponential phase in medium 63 (pH 5.8) containing the indicated additions plus IPTG (0.5 mM). Carrier mediated entry was determined by exposing washed cells to 2 mM ONPG and measuring *o*-nitrophenol released. The data are expressed as the mean values ± SEM. The number of experiments performed are given in parentheses

Table 5
Repression of β-galactosidase and tryptophanase by thio-o-nitrophenylgalactoside

Addition	Growth rate (% control)	Level of constitutive β-galactosidase (% control)	Induction of tryptophanase (% control)
Control	100	100	100
TONPG	37	42	2.3

ML 308-831 (*lacI*$^-$*Z*$^+$*Y*$^+$*A*$^-$) was grown in medium 63 (pH 5.8) containing 1% glycerol. L-Tryptophan (5 mM) was added to induce tryptophanase. The specific activities of β-galactosidase and tryptophanase were measured after 1 generation of growth following the addition of 5 mM TONPG

Hofsten [30] found that the sudden addition of lactose to *lac*-constitutive (*lacI*) cells growing in succinate immediately stopped the growth of the cells for a period of 1–2 h followed by a gradual resumption of growth. Fig.2 shows an example of this phenomenon. X71-15 was grown on glycerol at pH 5.8 in the presence of IPTG to induce the *lac* operon. When 5 mM lactose was added, there was complete cessation of growth for about 30 min. This was followed by a return of the growth rate to almost control levels. A similar experiment was carried out except that the cells were grown on glycerol plus 0.2% amino acids. The addition of lactose had little or no effect (not shown). It has been consistently observed that when cells are grown on carbon sources such as amino acids and sugars supporting moderate to rapid growth, the lactose inhibition is not observed.

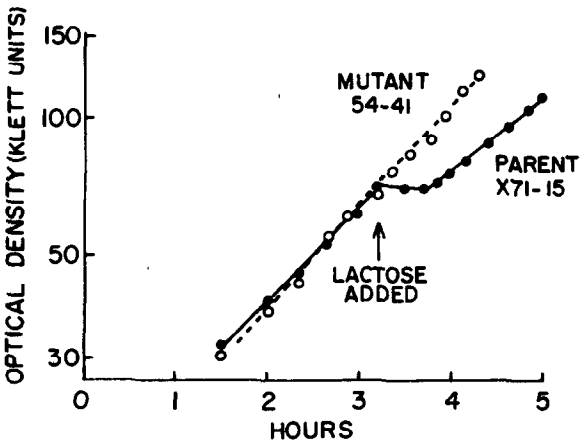

Fig.2. Effect of lactose on X71-15 and 54-41 growing in glycerol plus IPTG. Cells were grown for many generations in medium 63 (pH 5.8) containing glycerol (0.4%) and IPTG (0.5 mM). At the beginning of the experiment the cells were transferred to fresh medium and the growth rate followed. At the indicated time lactose was added to each flask to 10 mM final conc. and the growth rate was followed.

Several non-metabolizable substrates of the lactose carrier show growth inhibition. Hofsten found that thiomethyl-β-galactoside and IPTG produced a moderate growth inhibition of constitutive cells (ML308) growing on succinate. Holms [31] found that raffinose (a non-metabolizable trisaccharide) likewise inhibited. Rather severe growth inhibition was observed in cells growing on glycerol to which TONPG was added. Thus metabolism of the galactoside is not essential for the inhibitory effect on growth.

Because of the suspicion that the inhibitory effect of lactose in the fully constitutive cell might be due to the rapid entry of protons (with the lactose) and partial collapse of the protonmotive force, experiments were done with a mutant cell (54-41) which possesses a defect in the proton uptake with lactose. Lactose addition to the mutant had relatively little effect (fig.2). This experiment provides support for the view that it is the sudden inrush of protons with lactose that initiates a series of reactions leading to inhibition of cell growth. An entirely similar result has been obtained in an independently isolated mutant (ML-308-22) of this same type [32].

A second related phenomenon is the failure of *lac*-constitutive cells to grow on lactose minimal plates if they have been pre-grown in glycerol medium. Strain 3300 was grown in glycerol to logarithmic phase. It was then diluted and placed on agar plates containing glycerol, glucose or lactose. The cells grew well on glycerol and glucose plates but 99% failed to grow on lactose even when such plates were incubated for many days (table 6). A similar failure of growth on lactose plates was observed by Dykhuisen and Hartl [33] for cells previously grown in a lactose-limited chemostat. A severe inhibition of growth has

Table 6
Lactose inhibition of growth on agar plates

Carbon source on plate[a]	Growth of cells (%)[b]
Glucose	100
Glycerol	97
Lactose	0.7

[a] Strain 3300 (*lacI*) was grown in medium 63 (pH 5.8) plus glycerol into logarithmic phase, diluted and plated on agar plates containing medium 63 plus glucose, glycerol or lactose (0.2%) as carbon source

[b] Growth on glucose plates taken as 100%; values represent the mean of 3 expt

been observed also in cells growing on succinate or glycerol in the presence of two lactose analogues: TONPG [34] and IPTG (Putzrath, unpublished). These two non-metabolizable lactose analogues are such potent inhibitors under these conditions that they can be used for the selection of *lacY* mutants.

A direct measurement of the protonmotive force would be helpful in confirming the hypothesis that the lactose inhibition of growth is due to inrush of protons. It was decided to measure the pH gradient which, at pH 5.6, comprises a major fraction of the total protonmotive force. No attempt was made to measure the membrane potential in these particular experiments because such measurements require the use of EDTA which would interfere with growth of the cells. Cells were grown in glycerol in the usual manner except that a low concentration of radioactive benzoic acid was added. During the control period a pH gradient of 2 pH units was maintained across the membrane (inside alkaline). Immediately after the addition of 5 mM lactose to the cells the pH gradient fell precipitously to low levels (fig.3). In other experiments it was found that when the cells began to grow once again, the pH gradient returned towards the normal values. A similar fall in ΔpH (although less striking) was also observed when lactose was added to β-galactosidase negative (*lacZ*) cells under conditions where there was also an inhibition of growth. When the non-metabolizable lactose analogue TONPG was added instead of lactose, there was a significant fall in ΔpH under conditions where there was a slowing of the growth rate.

Among all the functions of the cell which are dependent upon protonmotive force ATP synthesis would be most sensitive since it requires an extremely high driving force (of the order of 220 mV) [35]. It was predicted that the cell would be unable to maintain its steady state level of intracellular ATP (about 2 mM) in the face of falling protonmotive force. It was observed that within seconds of the addition of lactose to strain 3300, the intracellular concentration of ATP fell from its normal level of 2.6 mM to 0.6 mM. The ATP levels remained low until the time when cells began to grow once again.

One important alternative explanation for growth inhibition was seriously considered, namely, that lactose entering the cell was converted to glucose and galactose which accumulated within the cell to such high concentrations that water entered the cell osmotically and damaged the cell membrane; but our data argues against such an osmotic explanation. medium by 400 mosM did not relieve the inhibition.

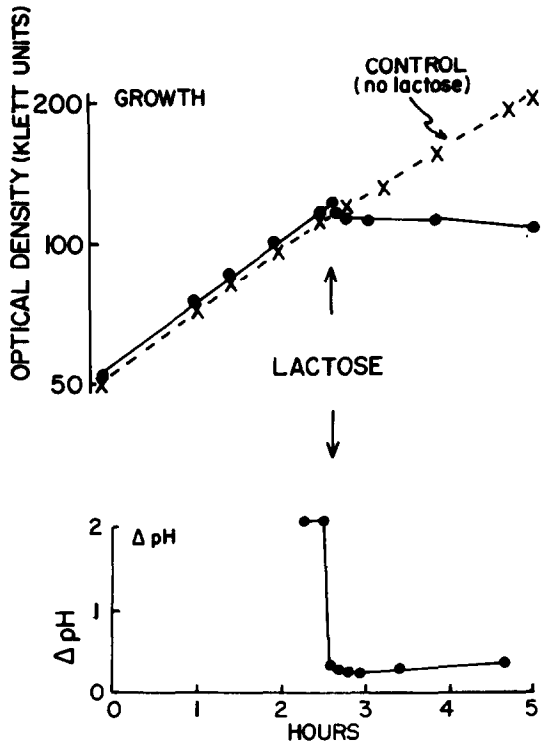

Fig.3. Effect of lactose on growth and ΔpH. Cells of strain 3300 were grown on medium 63 (pH 5.6) with glycerol as carbon source; 15 min before lactose addition [^{14}C]benzoic acid was added to 2 μM final conc. At various time intervals cells were centrifuged through silicone oil [22] and radioactive benzoic acid determined in both the supernatant and the pellet. The ΔpH was estimated before and after lactose addition. Cell growth is given in the upper panel and ΔpH in the lower panel.

of growth by lactose, although the accumulation of sugars within the cell would be expected to be similar to that found in glycerol-grown cells. Additions of NaCl to raise the osmotic strength of the incubation medium by 400 mosM did not relieve the inhibition. In a different type of experiment it was possible to generate an osmotic gradient which would tend to cause swelling of the cell of the same order of magnitude as that expected from lactose or hexose accumulation within the cell. In a series of experiments growing cells were diluted with distilled water to establish an osmotic gradient for the inward movement of water. Even when the external medium had been reduced in osmotic pressure by 200–300 mosM, there was only a very slight inhibitory effect on growth of the cells.

4. Discussion

It is remarkable how many factors are involved with the regulation of the activity of lactose-transport system. In addition to the normal induction process there is also the important and well-documented phenomenon of glucose catabolite repression. Glucose has its effect through varying the level of cAMP which exerts its effect at the transcriptional level in both inducible and constitutive cells. In addition, the uptake of sugars or polyhydric alcohols via the phosphotransferase system can inhibit the activity of the lactose carrier perhaps by way of a direct effect of a membrane component of the PT-system on the membrane carrier [see preceding paper in this issue].

A type of regulation that is still poorly understood is the repression of catabolic systems by growth of cells on lactose. Physiologically lactose appears to regulate the expression of the *lac* operon so that potentially toxic effects of excessive transport and metabolism can be prevented. Because one of the hydrolytic products of lactose is glucose it is possible that part of the phenomenon could be due to glucose catabolite repression. Although there is no gross spillage of glucose or galactose from cells adapted to growth on lactose it is conceivable that low concentrations of glucose do spill into the periplasmic space which are recaptured via the PT-system. The reduced lactose effect in the PT-negative mutant supports the view that part of the effect is indeed due to glucose catabolite repression. On the other hand, the fact that there is still considerable repression in this mutant indicates that there is an additional pathway that does not involve glucose. This second aspect of the repression by lactose may involve a reduction in membrane potential due to proton entry in cotransport with lactose with a reduction in protonmotive force and lowering of adenyl cyclase activity [14]. Studies with the proton-defective transport mutant 54-41 are consistent with this hypothesis. Thus, lactose repression appears to involve both glucose catabolite repression and a glucose-independent process which probably involves a fall in protonmotive force. Both of these factors inhibit adenyl cyclase, reducing the intracellular levels of cAMP and resulting in depressed synthesis of β-galactosidase, tryptophanase and other catabolic enzymes.

It is assumed that the failure of cAMP to cure the lactose repression completely is simply due to the inability of the nucleotide to enter the cell sufficiently rapidly when the cell has been grown in lactose. There is considerable variation between strains in the effect of cAMP in alleviating even the classical glucose repression. ML 308 for example shows very little curing of glucose repression of tryptophanase in the presence of 10 mM cAMP whereas the K12 strain 3300 shows 75% curing (not shown).

The failure to regulate the lactose-transport system may lead to serious physiological consequences for the cell. Cells with maximal *lac* expression (either constitutive or induced with IPTG) which are suddenly exposed to lactose abruptly stop their growth. This is not due to glucose spilling with subsequent reduction of cAMP since the addition of glucose stimulates growth rather than inhibits [30] and the 'Hofsten effect' occurs in cells unable to take up glucose (glucose enzyme II mutants). Furthermore the presence of added cAMP either at the time of lactose addition or throughout the entire growth of the cell results in a more severe inhibition by lactose (unpublished). Data presented by Peterkofsky and Gazdar [14] and those in this communication suggest that the protonmotive force of the cell is precipitously reduced by the sudden surge of protons into the cell in cotransport with lactose. This is associated with a fall in the ATP level within the cell. Presumably the entry rate of protons via the lactose-transport system exceeds the rate at which protons can be extruded through the respiratory chain. Perhaps the reason for the rapid recovery from this condition in the presence of amino acid supplements is because alternative sources of energy are available which permit a more rapid rate of respira-

tion. Cells are most vulnerable to this inhibitory effect when they are grown in succinate, a substrate which is utilized at a relatively low rate and provides only limited substrate for the respiratory chain. Recovery from the inhibition of growth is directly associated with the rise of ATP toward normal levels, presumably because the rate of proton pumping from the respiratory chain exceeds the rate of uptake through the lactose carrier and other modes of proton entry. These inhibitory effects of lactose on constitutive cells vividly illustrate the importance of regulatory mechanisms on membrane transport processes.

Acknowledgements

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