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INHIBITION OF GROWTH OF *ESCHERICHIA COLI* BY LACTOSE AND OTHER GALACTOSIDES

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A study has been made of the inhibition of growth caused by the addition of lactose or other galactosides to *lac* constitutive *Escherichia coli* growing in glycerol minimal medium. The effect was greater at pH 5.9 and pH 7.9 than at pH 7.0. Inhibition of growth by lactose was observed also in the case of a β-galactosidase negative mutant. However, a *lacY* mutant, which has a defect in the entry of protons normally coupled with galactoside transport, showed only slight inhibition of growth on the addition of galactosides. In the case of the parental strain the addition of lactose resulted in a sharp fall in ΔpH across the cell membrane and a reduction in intracellular ATP, and the recovery was slow. Under the same conditions the *lacY* mutant showed a smaller and only transient effect. It is postulated that the sudden entry of protons associated with lactose uptake lowers the protonmotive force, reducing the ATP levels and inhibiting growth of the cells. This hypothesis would account also for the selection of *lacY* mutants found when *E. coli* is grown in the presence of isopropyl-β-D-thiogalactoside.

Introduction

The expression of lactose transport of *Escherichia coli* is carefully regulated in cells growing in this disaccharide. When growing normally on lactose only about one third of the maximal expression of the lac-operon is observed. If fully induced or constitutive cells growing on a different substrate are suddenly exposed to the disaccharide, a severe inhibition of growth is seen. In 1961 Von Hofsten [1] showed that the addition of lactose to *lacI E. coli* growing in succinate liquid medium caused an abrupt cessation of growth. Following several hours of stasis, growth gradually resumed and finally returned to normal in several more hours. Inducible cells show this effect only when previously induced with isopropyl thio-galactoside. The addition of raffinose to *lacI* cells growing in glycerol resulted in a similar inhibition of growth [2]. A particularly striking effect of lactose was observed by Dykhuisen and Hartl [3] who showed that after growth for many generations in a lactose-limited chemostat a large percentage of cells was killed when placed on lactose minimal agar plates. They found correlation between the number of constitutive cells resulting from the chemostat growth and the number of cells killed by lactose. Cells with multiple copies of the *lac* operon are particularly sensitive to inhibition by lactose [4].

This communication describes experiments which investigate the mechanism by which lactose inhibits growth of cells with a fully induced *lac* operon. Some preliminary experiments have been reported in a previous paper [5]. The data are consistent with the

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Abbreviations TMG, methylthio-β-D-galactopyranoside, IPTG, isopropylthio-β-D-galactopyranoside; TDG, D-galactopyranosylthio-β-D-galactopyranoside; TONPG, *o*-nitrophenylthio-β-D-galactopyranoside; βONPG, *o*-nitrophenyl-β-D-galactopyranoside; XG, 5-bromo-3-indolyl-β-D-galactopyranoside; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid. *lacY*^{un} designates the gene which codes for an abnormal lactose carrier protein which transports lactose without protons and shows a defect in accumulation [6].

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view that the protons which enter the cell with lactose on the carrier partially collapse the protonmotive force across the membrane which results in a lowered ATP concentration and inhibition of growth.

Materials and Methods

Bacterial strains. *Escherichia coli* ML308 (*lacIZ*$^+$*Y*$^+$*A*$^+$) was obtained from J. Monod at the Pasteur Institute. The following mutants of ML308 were isolated in this laboratory: ML308-22 (*lacIZ*$^+$*Y*^{un}*A*$^+$) [6], ML308-225 (*lacIZY*$^+$*A*$^+$) [7]; and ML308-831 (*lacIZ*$^+$*Y*$^+$*A*) [8]. The K_{12} strains WP1-M6 (*lacI*$^+$*Z*$^+$*Y*$^+$ *mel AB*) and WP1-M6-4 (WP1-M6/F'*lacI*$^+$*Z*$^+$*Y*$^+$) were constructed. Strain A324-5 (*lacIZ*$^+$*Y*$^+$ *proAB*/F' *lacIZ*$^+$*Y*$^+$ *proAB*$^+$) was kindly provided by Dr. E.P. Kennedy, Strain HP6R, constitutive for the glucose-6-phosphate transport system [9], was a gift of Dr. Herbert Winkler.

Growth conditions. Cells were grown in side-arm flasks with rotary shaking at 37°C. Growth was carried out in medium No. 63 [10] with the addition of 0.4% or 1% glycerol. The medium was adjusted to the pH indicated. Growth was followed turbidimetrically with a Klett-Summerson colorimeter (No. 42 filter).

Measure of ΔpH. Internal pH was calculated from the observed distribution of the weak acid, [^{14}C]-benzoic acid, between the intracellular space and the external medium, using [^{3}H]inulin as a marker for external water [11]. A parallel experiment with [^{14}C]inulin and ^3H_2O gave the intracellular volume. Subtraction of the periplasmic space volume was made, based on the figures obtained by the sucrose space of the *lacY* cell [12]. Samples (1 ml) were taken in duplicate at various intervals and layered on top of 0.5 ml of silicone oil mixture (75% 550 fluid plus 25% 510 fluid; Dow-Corning, Lansing, Mich.) in 1.5-ml plastic microfuge tubes. After centrifugation for 1 min, the supernatant aqueous medium and most of the silicone oil were carefully removed, and the tip of the tube containing the cell pellet was cut off with a razor blade, placed in a scintillation vial containing 1 ml of 1 N NaOH, and incubated at room temperature with occasional shaking for about 30 min to suspend the cells. After neutralization with 2 N HCl, 9 ml of scintillation fluid (7.2 g of 2,5-diphenyloxazole (PPO), 1200 ml of toluene, and 600 ml of Triton X-100 [13]) were added.

Intracellular ATP. ATP was extracted from the cells with 3 M perchloric acid, neutralized with M KOH and then assayed with firefly lantern extract (FLE-50 Sigma Chemical Co.) by the method of Cole et al. [14].

Chemicals. Methyl-β-D-thiogalactoside (TMG), isopropyl-β-D-thiogalactoside (IPTG), D-galactosyl-β-D-thiogalactoside (TDG), D-raffinose were from Schwartz-Mann. *o*-Nitrophenyl-β-D-galactoside (βONPG), α-lactose (substantially glucose-free) and 3-(*N*-morpholino)propane sulfonic acid (Mops) were from Sigma. *o*-Nitrophenyl-β-D-thiogalactoside (TONPG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XG) were from Cyclo. [^{14}C]Benzoic acid, [^{3}H]inulin, [^{14}C]inulin and ^3H_2O were from New England Nuclear. Inulin was dialyzed to remove traces of free fructose.

Results

The addition of lactose inhibited the growth of lactose-constitutive (*lacI*) strains of *E. coli* which were growing on glycerol (Fig. 1). This inhibitory effect was more pronounced at pH 5.9 and at pH 7.9 than at pH 7. Addition of the disaccharide to cells growing on glycerol at pH 7 resulted in a cessation of growth for only 15–45 min followed by a rapid recovery, but when the external pH was 5.9 or 7.9, growth was stopped for 1–4 h and recovery was quite slow. Many of the subsequent experiments were carried out at the lower pH, at which the inhibition was most marked.

The inhibitory effect of lactose on the *lacI* cells was reduced or abolished when cells were grown on amino acids or glycerol plus amino acids. With added amino acids, the cells grew more rapidly than when growing on glycerol alone. Conversely cells grow more slowly on succinate and show very severe inhibition by lactose, as originally observed by Von Hofsten [1].

Addition of a variety of sugars that enter through the lactose carrier resulted in inhibitory effects on growth (Table I). Sucrose, a non-metabolizable substrate for the lactose transport system [12], enters more slowly than lactose and had very little effect. Thiomethylgalactoside (TMG) and thioisopropylgalactoside (IPTG) has weak effects while thio-*o*-nitrophenylgalactoside (TONPG) showed a moderately

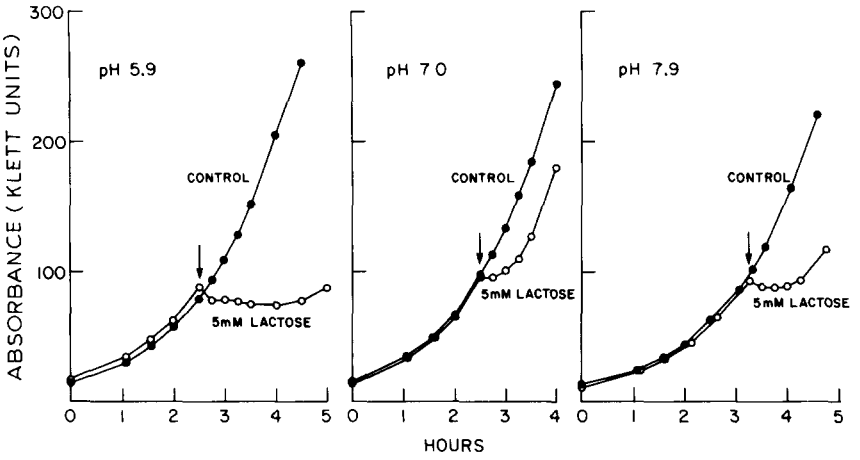

Fig. 1. Effect of pH of the growth medium on the response of ML308 to sudden lactose addition. ML308 was grown with 1% glycerol in Medium 63 at pH 5.9, 7.0 or 7.9. Lactose was added at the time indicated by the arrow, no sugar was added to the control.

strong inhibition of cells growing on glycerol.

The inhibitory effect of certain galactosides was observed also in cells lacking β-galactosidase (*lacZ*). Fig. 2 shows that lactose causes a complete cessation of growth in ML 308-225 growing on glycerol. This finding is of importance since it shows that the inhibition by lactose cannot be due to metabolic products formed. βONPG caused a 50% inhibition of the growth rate, while thiodigalactoside, a substrate with a slow transport rate but very high affinity for the carrier, had no effect alone but completely blocked the inhibition resulting from addition of lactose or other galactosides.

TABLE I

EFFECT OF ADDITION OF SUGARS ON ML308-381 GROWING IN GLYCEROL pH 5.8

Sugar addition		Growth rate during the first 2 h after sugar addition (% of control)
None		100
Glucose	(5 mM)	100
Lactose	(5 mM)	0
TONPG	(5 mM)	32
Raffinose	(5 mM)	45
Sucrose	(100 mM)	81
TMG	(25 mM)	79
ITPG	(25 mM)	86

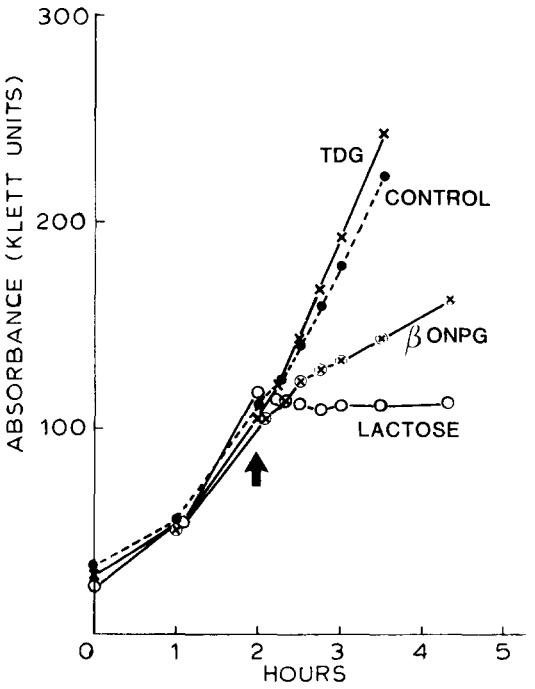

Fig. 2. Inhibition of growth of ML308–225 (*lacZ*) by the addition of galactosides. Lactose, βONPG or TDG (final concentration of 5 mM) was added to cells of ML308-225 growing in glycerol at pH 6.0.

One hypothesis to explain these observations is that the protons entering the cell with galactosides on the membrane carrier partially collapse the protonmotive force leading to inhibition of oxidative phosphorylation, a fall of ATP and cessation of growth. It is known that lactose entry can cause partial collapse of membrane potential [15] and/or pH gradient [16]. A test of this hypothesis was a study of a lactose transport mutant which shows a defect in the normal cation-sugar coupling and transports galactosides with only about 10% of the normal entry of protons. This mutant possesses membrane carriers that recognize lactose and growth on 25 mM of this disaccharide is normal. It fails to grow, however, in 0.25 mM lactose and is unable to accumulate non-metabolizable galactosides. Fig. 3 shows that lactose produced no inhibition of growth in the mutant ML-308-22 at pH 7 while the usual inhibition was observed in the parental strain. At pH 5.8 a mild inhibition by lactose was observed in the mutant but far less than that found in the parent.

A further comparison between parent and mutant was in a study of the effect of lactose addition on the membrane pH gradient which is the major component of the protonmotive force at pH 5.8. At this pH the normal respiratory proton pumps generate a ΔpH of 2 units, inside alkaline. If protons enter the cell with lactose at a sufficiently rapid rate the pH gradient would be reduced. To test this possibility parent and mutant cells were grown in glycerol and the ΔpH was measured. An initial pH gradient of about 2 units was observed in each of the two cells (Fig. 4). On the addition of lactose to ML308-831 the ΔpH fell to zero and did not recover during the 150 min of the experiment. Although a distinct fall in ΔpH was observed in the mutant, it was less pronounced than that in the parental strain and returned to normal after an hour.

If the protonmotive force were reduced below 210 mV, oxidative phosphorylation would be prevented [17] and ATP levels would be expected to fall in aerobically growing *E. coli*. Therefore the levels of ATP were measured in mutant and parental strains. Addition of lactose to the parental strain resulted in a fall of ATP from an initial value of about 2 mM to a

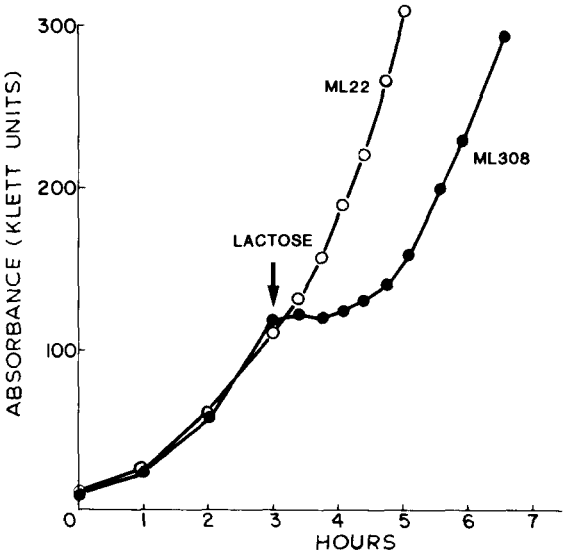

Fig. 3. Effect of lactose on growth of ML308 and ML308-22. Cells were growing on glycerol at pH 7.

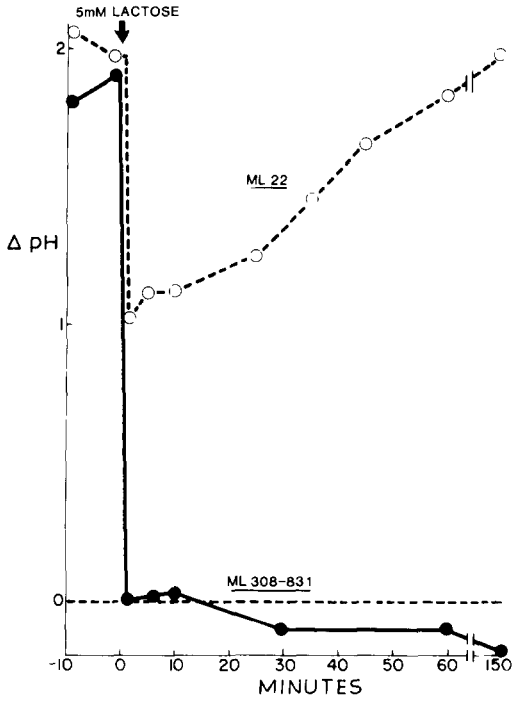

Fig. 4. The effect of lactose on the ΔpH of ML308-831 or ML308-22 growing in glycerol at pH 5.8. [^{14}C]Benzoic acid (7.4 kBq/ml, 2 μg/ml) and $^{3}H_2O$ (159 kBq/ml) were added to growing cells. One ml samples were centrifuged through silicone oil. A parallel flask had $^{3}H_2O$ and [^{14}C]inulin to determine the intracellular space.

value of 0.9 mM and this remained low for 2 h during the period of growth stasis (Fig. 5). The ATP returned toward normal at 3–4 h and the cell began to grow again. When the comparable experiment was carried out with the mutant, only a transient fall in ATP was observed followed by a quick return to normal levels.

If rapid proton uptake were the cause of lactose inhibition of growth the effect should be duplicated by other substrates which are cotransported with protons. Ramos and Kaback [16] have shown proton-glucose-6-phosphate contransport and demonstrated that addition of glucose-6-*P* partially collapses the protonmotive force across the membrane. Experiments were carried out with a mutant (HP6R) constitutive for the hexose phosphate transport system. This cell was grown on glycerol and either glucose or glucose 6-phosphate was suddenly added. Glucose (5 mM) stimulated the growth while glucose 6-phosphate (5 mM) strongly inhibited. Since glucose utilization involves glucose 6-phosphate as an intermediate the presence of this phosphorylated sugar in the cell was presumably not the cause of the inhibition. This experiment shows that other transport processes associated with rapid entry of protons can result in growth inhibition.

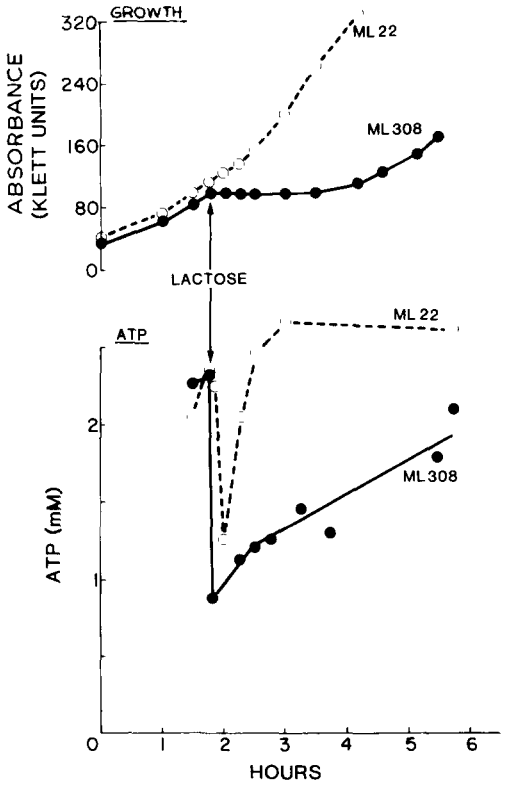

Fig. 5. Effect of lactose on the intracellular ATP level of ML-308 and ML308-22 growing in glycerol at pH 5.7.

Lactose inhibition of growth on agar plates

A striking inhibitory effect of lactose was observed when cells constitutive for the normal *lac* operon were placed on agar plates containing minimal medium plus lactose. In three experiments ML 308 was grown to mid-logarithmic phase in glycerol minimal medium, then was diluted and placed on minimal plates containing either glucose, glycerol or lactose. Only 2% of the number of colonies that grew on the glucose on glycerol plates were found on the lactose plates even after many days of incubation. In contrast, the mutant ML 308-22 shows very little inhibition by lactose, with 93% of the control number of colonies appearing on the lactose plates. Wild-type inducible cells show no inhibition by lactose.

Selection of lacY mutants by IPTG

The lactose analog TONPG has been shown to inhibit the growth of induced cells and to select for *lacY* mutants [18]. It was believed that TONPG was accumulated within the cell where it had some type of toxic effect, perhaps involving precipitation of the sugar. However, a similar type of selection for *lacY* mutants can be obtained with IPTG which is very water soluble and without any previously described toxic effects. Table II shows that after approximately 15 generations in succinate in the presence of 1 mM IPTG many of the surviving cells are *lacY* mutants. This was found both with haploid and diploid cells. There was some variation in the percentage of *lacY* mutants from experiment to experiment but large numbers of *lacY* mutants were consistently found.

Possible osmotic effects

An alternative hypothesis for the lactose inhibition was the possibility that the cellular accumulation of the disaccharide plus the hydrolytic products (glucose and galactose) increased the internal osmotic pressure resulting in water uptake, cell swelling and loss of critical intracellular metabolites. This possibility was tested in several types of experiments. Attempts were

TABLE II

THE EFFECT OF ITPG ON THE SELECTION FOR *lacY* MUTANTS

Cells were grown in medium 63 plus 0.4% succinate plus 0.3% citrate with or without 1 mM ITPG for 15 generations prior to plating. Determination of cell type was carried out with a combination of techniques XG plates to assay presence of β-galactosidase, MacConkey lactose indicator plates to determine lactose fermentation and MacConkey melibiose plates incubated at 42°C to determine the presence of lactose transport. As confirmation, a few clones were cultured and both β-galactosidase and transport of TMG were determined.

Cell	Genotype	IPTG added	Cell type after growth for 15 generations		
			Z^+Y^+	Z^+Y^-	Z^-Y^-
WP1-M6	$lacI^+Z^+Y^+$	–	100%	0	0
		+	19	61	20
WP1-M6-4	$lacI^+Z^+Y^+/F'\ lacI^+Z^+Y^+$	–	100	0	0
		+	47	49	4
A324-5	$lacIZ^+Y^+/F'\ lacIZ^+Y^+$	–	98	0	2
		+	9	30	61

made to overcome the possible osmotic effect by addition of non-diffusible substances to the external medium at the time of lactose addition. NaCl or Mops were added at various concentrations before, during or after lactose addition. Addition of NaCl concentrations up to 250 mM had no effect on growth in the absence of lactose and did not prevent inhibition by lactose. Higher concentrations (300–500 mM) of NaCl inhibited growth of glycerol grown cells but partially prevented lactose inhibition.

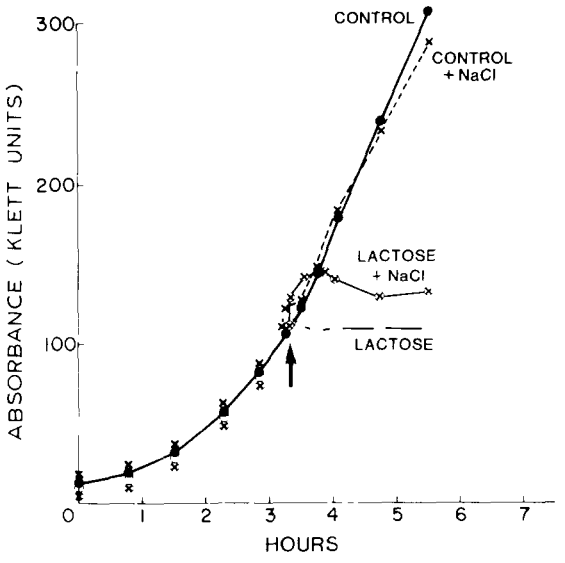

Fig. 6. Effect of addition of 5 mM lactose and 250 mosM NaCl at the same time to ML308-225 growing in glycerol at pH 7. The intracellular concentration of lactose was determined with [^{14}C]lactose in the culture in which lactose alone was added, and was found to be 252 mM.

Several quantitative experiments were carried out with β-galactosidase-negative cells in which lactose accumulation was measured and an equivalent osmotic 'balance' of NaCl or Mops added to the external medium. One such experiment is shown in Fig. 6. Addition of NaCl caused a rapid increase in absorbance, presumably due to shrinkage of the cell resulting from raising the external osmotic pressure. This was followed by a fall in absorbance during the subsequent 30 min (probably due to osmotic adjustment by K^+ pumping [19]). The addition of NaCl, however, did not prevent the lactose inhibition of growth.

Discussion

Under normal circumstances there is an exact balance between the number of protons extruded from the cell via the respiratory chain and the inward movement of protons via the ATPase and a variety of membrane carriers. Many of these carriers place only a minor drain on the energy stored as a protonmotive force while others, such as proton-cotransport of the major carbon source, utilize a significant fraction of the energy.

If the cell is to grow normally in the latter case, it is essential to increase the outward pumping of protons via the respiratory chain in order to balance the drain in $\Delta\bar{\mu}_{H^+}$. Indeed, it has been shown that the addition of lactose [20], or TMG [21], to energy-depleted cells does, in fact, cause stimulation in the rate of respiration. The observation that cells growing rapidly in a rich medium are far less susceptible to lactose inhibition, may perhaps be explained by a more active respiratory chain.

The experiments presented in this paper have focused on the ΔpH aspect of the protonmotive force because of the relative ease of this measurement compared with estimations of membrane potential ($\Delta\psi$) in growing cells. For this reason many of the experiments were carried out a pH 5.8 where large ΔpH values are normally observed across the membrane of the cell. The lactose inhibition, however, is observed at external pH values of 7.9 where the protonmotive force consists of a large $\Delta\psi$ but no ΔpH [22,23]. Under these conditions proton entry associated with lactose transport presumably reduces the $\Delta\psi$ as has been reported by Schuldiner and Kaback for membrane vesicles. A similar fall in membrane potential (measured with tetraphenylphosphonium) can be demonstrated after addition of lactose to EDTA-treated resting cells of glycerol-grown ML-308 at pH 7.8 (unpublished results). Thus it is reasonable to suppose that under the conditions of these experiments proton entry with sugar reduces the total protonmotive force (either $\Delta\psi$, ΔpH or both).

The process of recovery from the growth inhibition by lactose is not completely understood. The initial step that permits the cell to escape from inhibition could be an increase in activity of the respiratory proton pumps [20,21], so that extrusion exceeds uptake and the normal protonmotive force is restored. When the cells begin to grow *lac* operon expression is suppressed, since β-galactosidase is reduced in a population of cells that have fully recovered from lactose-induced stasis. Consistent with this idea is the observation [5] that the presence of added cyclic AMP considerably prolongs or prevents recovery.

The inhibition of growth in the presence of certain thiogalactosides is so severe that these sugars may be used in the selection of lactose transport mutants. In 1968 Müller-Hill et al. [18] reported that TONPG could be used to select for *lacY* mutants due to its strong inhibition of growth of fully induced cells growing in glycerol. To assure full induction of inducible strains IPTG was included in the growth medium. Smith and Sadler [24] later showed that somewhat stronger selective pressure could be achieved by growth on succinate rather than glycerol as carbon source, presumably because cells grow very slowly in succinate. It is interesting to note that the *lacY*^{un} mutant ML308-22 used in this study was isolated with the TONPG selection procedure. This mutant fails to take up protons with galactosides. A similar type of selection for *lacY* mutants can be obtained by growing cells on succinate plus 1 mM IPTG in liquid medium (Table II). The inhibition of growth of succinate grown cells by thiogalactosides is consistent with the hypothesis of growth stasis due to excessive proton entry.

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