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Lag in Adaptation to Lactose as a Probe to the Timing of Permease Incorporation into the Cell Membrane

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If bacteria are incapable of forming and incorporating proteins into the cytoplasmic membranes in all phases of the cell cycle, then not all cells from an asynchronous culture should be capable of growth when switched to a new carbon and energy source whose metabolism requires new membrane function. The transfer of an inducible culture to low lactose provides such a situation since the cells cannot grow unless galactoside permease can function to concentrate the lactose internally. From such experiments, it was concluded that the *Y* gene product of the *lac* operon is synthesized, incorporated, and can start functioning in active transport, at any time throughout the bulk of the cell cycle. Not only were the lags before growth re-ensued much shorter than would be expected if the membrane transport capability could only be developed in a small portion of the cycle, but brief pulses of a gratuitous inducer shortened the lags much further. Three types of *Escherichia coli* ML 30 culture were studied: cells that had exhausted the limiting glucose; cells taken directly from glucose-limited chemostats; and a washed suspension of highly catabolite repressed cells from cultures grown in high levels of glucose and gluconate. The growth studies reported here were performed on-line with a minicomputer. They represent at least an order of magnitude increase in accuracy in estimating growth parameters over previous instrumentation.

The evidence is overwhelming that synthesis of many specific proteins takes place throughout the cell cycle in prokaryotes (24). This is clearly the case for both constitutive and induced synthesis of β-galactosidase in *Escherichia coli*, where the doubling of the rate of synthesis has been observed to coincide with the presumptive duplication of the *lac* region deoxyribonucleic acid (8). Since it is also well established that first and last products of the *lac* operon are translated from a polycistronic message (6), it follows that the product of the middle *Y* gene, galactoside permease (TMG-I), like β-galactosidase, should be produced continuously throughout the cell cycle. After induction, the *Y* gene product should start to appear with a lag of 3 to 5 min intermediate between the β-galactosidase and transacetylase (1, 3, 11, 20).

When and if this newly formed *Y* gene product functions is another matter. In contrast to β-galactosidase and transacetylase, this material cannot function alone but needs to be incorporated into a complex intact membrane structure to register activity according to any known methods of detection. Fox (7) has suggested that membrane growth may be required for the permease to become functional, but this has recently been denied by Nunn and Cronan (28). Whether the *Y* gene product is incorporated into the membrane at all stages of the cell cycle and becomes functional is the question under study in the present work.

The cell envelope of gram-negative rods grows throughout most (or all) of the cell cycle. This was first observed by Bayne-Jones and Adolph in 1933 (2) and has been repeatedly confirmed (24, 31). This does not necessarily imply that any other than the peptidoglycan layer grows throughout the cycle, since the inner and outer membranes are deformable and plastic. Kubitschek (16) has suggested that the envelope grows linearly throughout most of the cell cycle in steady-state systems but that transport machinery is formed just before cell division. Furthermore, Kubitschek's suggestion was that cell growth is limited by functional transport systems and that a new "cell's worth" of functional transport capacity is formed or becomes functional only over a brief period of the cell cycle near or at cell division. Kubitschek has subsequently supported his contention with transport measurements of cells in different phases of the cell cycle (17, 19) and by

measurements of the increase of volume of cells failing to divide because of an imposed thymine starvation (18) but continuing to elongate.

The present paper shows that galactoside permease is incorporated into the membrane and can become functional throughout the bulk of the cell cycle. This is inferred from studies of the lag in growth when inducible cells are transferred to low levels of lactose as their sole carbon source. Growth studies were done in an apparatus in which the turbidity data were continuously monitored and fed into a computer that calculated the lag and steady-state growth rate. The lags that occur with shift conditions in which galactosidase permease is necessary for growth indicate that most cells in the population can form functional permease. Two other independent types of experiments are discussed, also leading to the conclusion that galactoside permease can become functional at any time throughout the cell cycle.

MATERIALS AND METHODS

Culture conditions. *E. coli* strains ML 30 (*lac* $I^+Z^+Y^+A^+$) and ML 35 (*lac* $I^-Z^+Y^-A^+$) were grown in limiting glucose (0.02%) in M-9 medium with forced aeration at 37 C as described previously (14–16; A. L. Koch, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, G257, p. 69). Under these conditions, the cells first grow as rapidly as they do in 0.2% glucose. The growth decelerates over a short period of time (~10 min), corresponding to a very small increase (~10%) in biomass (see Fig. 1). After exhaustion, the cell mass remains constant. In other experiments, I have found it to remain constant for at least 24 h. Cells that have grown on the low level of glucose and then have been aerated at 37 C for at least 10 min after they have depleted the carbon and energy source are designated here as "glucose-depleted" cultures. Their ability to grow on added carbon source is immediate. This property does not change as the culture is further incubated with aeration at 37 C for many hours. For the studies reported explicitly in Table 1, the depleted culture had consumed the glucose at the times indicated. The inducible strain was also grown in a chemostat apparatus (14) in the same medium. Samples were taken directly from the chemostat and diluted into a cuvette. A third culture condition was batch growth in 0.2% glucose-0.2% potassium gluconate. The cells were then washed and used immediately. This combination of carbon sources enhances catabolite repression.

β-Galactosidase assays were run with the same spectrophotometer and computer system, with 1.83 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate at 28 C. The cells were lysed by the procedure of Putnam and Koch (29).

Growth measurements. A 1.0-ml sample of cell culture was added to 9.0 ml of M-9 previously placed inside a cuvette (2 by 2 cm) maintained at 37 C by water circulating through the cuvette block from a constant-temperature bath. The cuvette holder, adapted from a Zeiss model 50-54-88 to fit into the Cary model 16 spectrophotometer, allowed the sample to be magnetically stirred. In addition, the entire cuvette chamber was jacketted and also maintained at 37 C. The apparent-absorbancy measurements at 420 nm were sampled by a Wang 720 C computer through appropriate interfaces and clocks at 2-s intervals. One-minute averages of 29 values were stored in the computer. At the end of a run, these averages were converted to the corresponding dry weight concentration from the expression $[(1 - \sqrt{1 - 0.60881A}) \times 0.23091]$, where A is the apparent absorbancy in a 2-cm cuvette at 420 nm (13). These averages can be listed; however, usually only a data summary, including the initial absorbancy and time and the least-square regression of the natural logarithm of the dry weight concentration, was tabulated. The computer also calculated the precision of the slope and the least-square quadratic through the same data. The doubling time, once the culture's growth became exponential, was calculated from the relationship $T_2 = (\ln 2)/\lambda$, where λ is the slope of the linear regression of the logarithmically transformed data. Estimates of the lag time in all cases were calculated as the difference (Δ) of the intercept of the linear regression of the data collected in the exponential phase subtracted from the observed or extrapolated initial value divided by the slope of the linear regression. This is diagrammed in Fig. 1.

In almost all of the experiments, the cells could increase 10-fold in the spectrophotometer before they exhausted the carbon source (usually 0.02% lactose). Therefore, on logarithmic paper, the growth curves would look S-shaped (e.g., see lower curve in Fig. 1). Thus, there is an accelerating phase as the cells come out of lag and a decelerating phase as the carbon source is consumed. The present calculations require that an intermediate region, where growth is strictly exponential, be used in the extrapolation used to calculate the lag. This was usually done by asking the computer to calculate the statistics over limited regions of the data and picking a portion in which the error of the slope was less than 0.6%. The choice of the region was aided because a chart recording of the apparent-absorbancy measurements had been made on line through a Cahn automatic range expander. The 10-inch (ca. 25.4-cm) chart recorder effectively served as a 100-inch (ca. 254-cm) chart recorder because progressively the trace would be offset 10 inches as the signal increased.

RESULTS

Properties of the system. A high degree of photometric precision and long-term stability are afforded because of the double-beam mode of operation of the Cary model 16 spectrophotometer and because the cuvette is not moved once measurement starts. The 2-cm light path affords greater sensitivity than shorter ones. The magnetic mixing necessary for aeration causes fluctuations which are overcome by aver-

aging many successive measurements. To test the overall performance, several control experiments were run. In the first, a balanced growing culture in 0.02% glucose was diluted with equal portions of the same medium in the cuvette, and growth was allowed to ensue for 33 min. The computer output indicated a coefficient of variation of the slope of logarithm of dry weight concentration of 0.168% (this experiment is the first line of Table 1). A further indication of the accuracy is the size of terms of the quadratic regression. In this case, the coefficient of the square term is 1,760-fold less than the linear term. The 1-min lag (actually 0.94 min) was no doubt due to a small temperature fluctuation during pipetting of the culture from the water bath into the reaction cuvette. In the majority of experiments, the initial inoculum in the cuvette led to an absorbance of 0.11, corresponding to 7.8 μg (dry weight) per ml and about 5×10^6 cells per ml. This much growth represents the yield from 0.002% glucose. The additional growth resulting from the addition of 0.02% lactose gives an absorbance of 0.93, corresponding to 79 μg (dry weight) per ml.

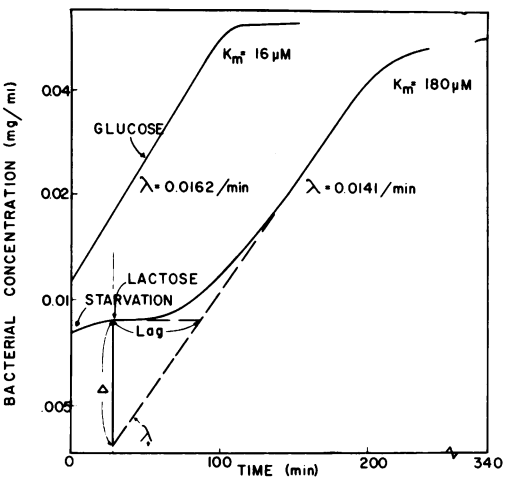

FIG. 1. *Example of automated measurement of growth.*

Another control experiment showed that in the presence of 0.2% glucose exponential growth at the same rate continues for at least a threefold increase above the level corresponding to the utilization of 0.02% carbohydrate. This was established by following the apparent absorbancy, which rises beyond the point where an accurate dry-weight determination can be inferred, but obtaining a reliable estimate by measurement of a diluted sample. Afterwards the growth rate diminished. It can be concluded, therefore, the aeration is not limiting in any of the experiments reported in the tables or figure.

Two experiments are given in Fig. 1. In the

TABLE 1. *Growth kinetics of cells from growing and glucose-depleted cultures of ML 30*

	Time since parental culture exhausted glucose (h)	Lag time (min)	Doubling time (min)	β-Galactosidase (μmol of ONPG/g per min)
Balanced growth:				
0.02% glucose		1	43	
		0	44	8
Depleted cells:				
Shifted to 0.02% glucose	2	−1	49	
	12	−2	54	
Shifted to 0.02% lactose	0.1	33	51	
Shifted to 5×10^{-4} M IPTG for 20 min, then 0.02% lactose added	3.5	~7	—[a]	
Shifted to 0.02% lactose + 5×10^{-4} M IPTG	3.0	23	50	
Shifted to 0.0002% glucose + 5×10^{-4} M IPTG for 8 min, then 0.02% lactose added	4	7	50	
Shifted to 0.0002% glucose + 10^{-3} M IPTG for 5 min, then centrifuged and into 0.02% lactose with no IPTG	1	11	45	341[b]
				5,582[c]

[a] —, Not accurately determined.
[b] After the 5-min induction period.
[c] After the exhaustion of the lactose.

upper line, the cells had been in balanced growth on 0.02% glucose and consumed the carbon sources during the run. In the lower line, the cells had been grown on 0.2% glucose-0.2% potassium gluconate. They were washed and immediately placed in 0.02% lactose medium in the cuvette. They exhibit a lag in adapting to the lactose and deceleration upon exhaustion of the carbon source. The growth data for the substrate exhaustion phases were fitted to Monod's relationship (25) by a computer program described previously (14), and the effective glucose concentration supporting half-maximal growth was found to be 16 μM, consistent with the value of 19.4 μM reported in that publication. The K_m for lactose consumption of cells shifted from glucose-gluconate cultures to 0.02% lactose that fits the data best is 180 μM, somewhat larger than the 120 μM reported previously (14) for cells grown indefinitely in lactose, where the levels of the *lac* operon products would be about twofold higher (see below).

The first line of Table 1 shows the growth kinetic parameter of cells of the inducible strain. Cells in balanced growth exhibited a doubling time of 43 min in 0.02% glucose until shortly before exhaustion. The third line shows that cells that had consumed the available glucose 2 h earlier exhibited an apparent lag of -1 min and then grew with a doubling time of 49 min. I had noted previously that no lag developed even if carbon-limited cells were aerated at 37 C in the absence of carbon for up to 24 h, and that after 7 h glucose-depleted cells recover their ability to form ribonucleic acid to the level exhibited by cells growing in rich medium as fast as do cells taken from slow, but balanced, growth (14). As part of the current experimental series, a -2 min lag after 2 h of incubation was found. This surprising situation was originally noted by Cohen and Arbogast in 1950 (4). I have discussed this phenomenon in relationship to the K_m of the uptake system for glucose (14) and concluded that the cell "sees" unlimited glucose until there is not sufficient energy and carbon to alter its physiology in response to the impending starvation. In the illustrated example shown in Fig. 1, the K_m of 16 μM, corresponding to 0.00029% glucose, implies that there is only a 13.5% increase in mass after the glucose concentration has been lowered to this value. So that even if cells in different phases of the cell cycle have different priorities for use of the residual glucose, they still will not be able to become synchronized. Here it is argued that cells from cultures that have exhausted their limiting supply of glucose do so, so rapidly that they represent faithfully all phases of the cell cycle with nearly the same incidence to be found in exponential culture.

The basic assumption of the experimental method is that cells in the presence of low lactose (0.02% or less) as the sole carbon source will not be able to grow until they have functional permease as well as β-galactosidase. A control to demonstrate this is given in Table 2, where it is shown that cells of strain ML 35, which constitutively form very high levels of β-galactosidase but no permease, do not grow in the low lactose medium. However, their capability for growth is not impaired since these cells, incubated for 2 h with no carbon source and then for 100 min with lactose (which they could not use), grew with only a 2-min lag in achieving balanced growth after the second carbon source, 0.02% glucose, was added. Strain ML 35 grows, albeit slowly, on 1% lactose; in part, this growth is on the contaminating glucose, but they must also slowly attack the lactose.

A reviewer of this paper pointed out that this is not an adequate control since these cells presumably have no permease at all but that the uninduced cells of strain ML 30 have a basal level of permease. This basal level might be important in initiating growth on lactose. I therfore carried out a different control experiment which will be only summarized here, because it will become part of another report. ML 30 was grown on 0.02% succinate and 5×10^{-4} M isopropyl-thio-β-D-galactopyranoside. As it approached carbon exhaustion, it was centrifuged, resuspended, and placed in a chemostat fed with the same growth medium but without inducer. Subsequently, as the culture became deinduced, samples were taken for both permease and β-galactosidase assays. Other samples were taken for growth measurements in

TABLE 2. *Growth kinetics of cells from glucose-depleted cultures of the permease-negative strain ML 35*

Cells	Lag time (min)	Doubling time (min)
Cell depleted for 1 h and then given 0.02% lactose		> 2,000
Above culture after 100 min of incubation and then 0.02% glucose added	2	51

the first 100 s after cells were taken from the chemostat to the growth cuvette and 0.02% lactose added. This is less time than it takes for synthesis of completed polypeptide chains of permease. In such a short time, growth measurements are only accurate to ±10 to 15% but are adequate to show that a net permease level capable of hydrolyzing 40 μmol of ONPG/g (dry weight) per min at 37 C is required to support half-maximal growth. The corresponding β-galactosidase level was 1,000 μmol/g per min. From the fully induced and basal levels of β-galactosidase under these growth conditions, it can be estimated that the basal permease level is about 1/50 of that needed to support half-maximal growth on 0.02% lactose. It is to be noted that the permease cannot be measured directly on uninduced cells because its effect is less than the uncertainty in measuring the background rates.

Shift of inducible cells from glucose-depleted cultures. When the cells were shifted from glucose-depleted culture into 0.02% lactose, a 33-min lag was observed (Table 1). This lag is, of course, a variant of the old diauxie phenomenon of Monod (25) in the sense that the cells need to form β-galactosidase and galactoside permease to consume the lactose and grow. In the idealized case in which the cells had neither any permease molecules nor β-galactosidase, no growth would ever be possible because the lactose would neither be able to enter nor be converted into allolactose (10), which serves as the actual inducer of the *lac* operon. Actually, such cells must have small no potential source of carbon to build protein and membrane needed for the function of the operon. Acturally, such cells must have small reserves of energy, they do have a few molecules of β-galactosidase, and they are permeable to some very slight degree to lactose either via other pathways or via basal level of galactoside permease. Although these reserves are evidently adequate to allow induction of the *lac* operon, that alone would not allow significant rates of mass growth until the permease so formed has become functional in any individual cell. The results of the control experiments presented above indicate that they would need to be induced to about 10% of the maximum level to support half-maximal growth. Consequently, we would expect a modest lag and slow growth while the permease gene product becomes functional to this level. On the other hand, the hypotheses to be tested supposes that all the permease is to be laid down in a small fraction of the cell cycle. Consequently, we would expect a very long lag if only 10%, for example, of the cells are in a phase in which membrane function is achievable and the remaining 90% never grow. Quantitatively, one would expect in this hypothetical situation a lag in excess of $\log_2$ (100/10) times the doubling time, or greater than (3.311 × 43 min) 143 min. Consequently, the observation of a 33-min lag is inconsistent with the hypothesis that functional cytoplasmic membrane synthesis takes place during only a small fraction of the cell cycle.

A stronger argument to support the conclusion that permease can become functional throughout the bulk of the cell cycle can also be drawn from the last experiment listed in Table 1. This has to do with the cells that were exposed for 5 min to 0.0002% glucose in the presence of 10^{-3} IPTG. This latter substance is a powerful inducer of the *lac* operon. The amount of glucose provided only let the cellular mass increase 10%, yet it reduced the lag by two-thirds from 33 to 11 min.

Controls were run (not shown) to show that this small amount of glucose does not shorten the lag if added first and the lactose added after it has been consumed, or if they are added together with the lactose. In the latter case, there is immediate growth to a degree corresponding to the glucose, and then the usual lag ensues as in Monod's classical experiments.

Since the lag was not completely abolished, we cannot conclude that functional permease can be laid down at all times of the cycle, but we can put limits on it. For example, the 7-min lag leading to a doubling time of 50 min (experiment on the next-to-last line of Table 1) implies that $2^{-7/50}$ = 97% of the dry weight of this culture is associated with cells capable of laying down functional permease at any instant. It can be shown mathematically that values calculated this way also apply to the percentage of the average cell cycle time during which synthesis is possible, if one assumes that all cells in balanced growth double in exactly the same time and that any membrane synthesis period is at the end of the cell cycle. This 91% is an underestimate because there must be at least a 4-min average lag after inducer is added under the best of conditions before the permease molecules are completed. But it is an over estimate since we have not taken into account the 8-min induction period. With these corrections the 91% becomes 83%. This type of calculation has been carried out for the experiments of Tables 3 and 4, and in all cases it can be concluded that throughout, at least, 75% of the cell cycle functional permease

TABLE 3. *Growth kinetics of glucose-limited chemostat ML 30 cells*[a]

Prior induction	Test conditions	Growth shift[b] to lactose (min)		β-Galactosidase[c] (μmol of ONPG per (g per min)	
		Lag	Doubling time	Before	After
None	0.02% Lactose added immediately	46	49		
	0.02% Lactose added after 8 min	64	53	32	3,591
	5×10^{-5} M IPTG; 0.02% lactose added after 8 min	25	49		6,047
5 min with 5×10^{-4} M IPTG, centrifuged	0.02% Lactose added after 8 min	17	49	345	6,747
183 min with 5×10^{-4} M IPTG, centrifuged	0.02% Lactose added after 8 min	−5 11	68[d] 52	9,150	6,422

[a] Doubling time, 109 min; glucose concentration in reservoir, 0.02%.
[b] 1.0 ml of cell suspension plus 9.0 ml of M-9, then 10 μl of 20% lactose.
[c] Before and after growth in the lactose in the cuvette assembly.
[d] First line calculated from data taken 30 to 60 min after lactose addition; second line calculated from data from 64 to 132 min after lactose addition.

TABLE 4. *Growth kinetics of ML 30 previously growing under highly catabolite-repressive conditions*

Culture	Lag time (min)	Doubling time (min)	β-Galactosidase (μmol of ONPG per g per min)
Balanced glucose-gluconate-growing culture	1	38	5, after
Washed, incubated 500 min in 5×10^{-4} M IPTG with no carbon source			483, after
Washed, shifted to 0.02% lactose	78	41	2,570, after
Washed, starved for 28 min, then shifted to 0.02% lactose	52	49	3, before 2,370, after
Washed, shifted to 0.02% lactose + 5×10^{-4} M IPTG	13	38	7,288, after
Growing culture washed, induced 5 min with 10^{-3} M IPTG, centrifuged	24	42	189, before 3,603, after
Growing culture centrifuged, induced 18 min with 5×10^{-4} M IPTG, centrifuged, shifted to 0.02% lactose	13	42	

can be formed. This is still an underestimate since the cells need to form about 10% of the maximal level of permease to grow rapidly, corresponding to a further increase of about 15% of the cycle time.

Shift of inducible cells from fast glucose-limited chemostat culture. Although I feel that the experiments presented above prove the point, objection has and can be made to the studies of cells starved of carbon source for several hours; therefore, two additional types of cultures of the inducible strain were studied in which the cells could be guaranteed to be in balanced growth at the time of the shift to low lactose. In the first type, cells were taken from relatively fast chemostat culture of ML 30 limited with glucose many generations after the turbidity stabilized. This type of culture must approach balanced growth very closely. The results of one chemostat culture are shown in Table 3. The uninduced cells from this 109-min doubling time chemostat have very little β-galactosidase, although more than cells from the glucose-depleted culture. They exhibit a 46-min lag if taken from the chemostat directly into the cuvette with lactose already present. There is a lag on shifting chemostat cells into an excess of the same medium in which they have been growing. For a 142-min chemostat culture there was a 14-min lag, and the doubling time had only shortened to 54 min after 100 min. The lag is considerably lengthened if an 8-min interval is interposed in the absence of any added carbon source. (Note: lags throughout this paper are computed from the time of lactose addition.) A considerable portion of the lag observed has to do with the slowness in the

induction process since the additional presence of even 5×10^{-6} M IPTG in conjunction with the lactose is enough to reduce the lag from 64 min to 25 min (see Table 3).

The critical experiment was to add inducer (5×10^{-4} M IPTG) to the chemostat (and to the reservoir bottle) and allow the chemostat to operate for 5 min. Some cells were immediately (<3 min) centrifuged and suspended in inducer-free medium in the growth cuvette in the spectrophotometer. IPTG rapidly goes in and out of uninduced cells (3) and because of the dilution involved, it could not possibly be present in the growth cuvette at a concentration higher than 5×10^{-7} M. Therefore, the reduction of the lag from 64 min to 17 min can only be accounted for on the assumption that most or all of the cells acquired functional permease resulting in the 5-min induction in the chemostat. As calculated above, these data correspond to $2^{-17/49} - 5/49 \times 4/49 = 77\%$ of the cell cycle, at least, available for functional permease formation.

The positive control was done by allowing the chemostat to continue under inducing conditions for 183 min. Cells sampled at this time exhibited a peculiar, complicated growth pattern on low lactose after inducer removal. The initial doubling time estimated from the data obtained in the first 10 min was 44 min. This fast rate then slows to a doubling time of 75 min before a return to the appropriate doubling time (49 min) for this low-lactose medium. Therefore, the lag can only be specified as being in the range −5 to +11 min when calculated by the routine used for the rest of the growth curves of this paper, depending on which portion of the data is analyzed.

β-Galactosidase assays are shown on the right-hand side of Table 3. These are consistent with the expectations that glucose-limited chemostat growth gives noncatabolite repression levels. Had the induction been continued indefinitely, the extrapolated enzyme level would be $9{,}150/(1-2^{-183/109}) = 13{,}305$, which is an appropriately high level for our strain of *E. coli* under these noncatabolite repressive conditions (15). A lower, partially catabolite-repressed, level is found after the 10-fold increment in mass in 0.02% lactose (with no IPTG). The final interesting point is that the previously uninduced cells shifted to lactose alone do grow a little slower (doubling time 53 min) even after the lag and do not have the definitive lactose-grown level of β-galactosidase even after 10 times as much new cellular mass is formed as inoculated initially.

Shift from catabolite-repressed culture. A third type of culture used in these studies was cells in balanced growth in 0.2% glucose-0.2% potassium gluconate. In such medium, the cells have a doubling time of 38 min and an extremely low level of β-galactosidase compared with the other two types of culture (9, 22; cf. Tables 3 and 4). Presumably they have an equally depressed basal level of galactoside permease. Consequently, these cells should be much slower in permitting lactose entry and converting it into the form actually binding to the repressor. On the other hand, they should have larger reserves of glycogen.

These energy reserves do not seem to be important since about the same lag is observed whether the cells are harvested, washed, and resuspended in 0.02% lactose (lag 78 min) or if the cells are centrifuged, resuspended, allowed to exhaust reserves for 28 min, and then given lactose (lag 54 min; total lag 82 min) (Table 4). The lag is decreased significantly (bottom two lines) if the cells are induced at higher (10^{-3} M) IPTG concentrations in the absence of an exogenous carbon source, harvested, washed, and placed in 0.02% lactose. The decrease was more evident with an 18-min induction in the absence of exogenous carbon source. During this time the cell dry weight increased by only 2%. The lag observed in this case is as short as that observed when uninduced, but washed, cells were exposed to 5×10^{-4} M IPTG and 0.02% lactose.

DISCUSSION

The growth experiments presented here bear directly on the formation of functional permease throughout the cell cycle and are fully independent of the other kinds of experiments that have been done (discussed below). The data show that inducing conditions (IPTG) are enough to decrease the lag after other carbon sources are removed and 0.02% lactose is introduced. The long lag phase (54 min) after highly catabolite-repressed cells are starved for 28 min and then given lactose points out the slowness with which those cells can adapt. This slowness is stressed further by the observation that the level of β-galactosidase after 11-fold growth is still only one-third of that characteristic of unlimited growth in the low-lactose medium. In considering growth of *E. coli* on lactose, it is necessary to understand that β-galactosidase inside the cell has an unfavorable K_m for lactose hydrolysis (10^{-2} M) (33). However, the permease has a very much lower K_m (7×10^{-5} M) (12) for lactose accumulation. In this case the active transport mediated by permease is clearly necessary to boost the internal concen-

tration high enough to be effectively attacked by the enzyme. This also may be the reason that very high levels of β-galactosidase can be produced in most isolates from nature. This circumstance is compounded by the peculiar fact that the natural substrate, lactose, only becomes an inducer after β-galactosidase action. They both conspire to make the control system more complicated with additional controls added on to the repressor-operator:promoter-catabolite repressor system so well studied in molecular biology. It gives the system the property of "remembering" how recently its ancestor had experienced lactose.

The effective level of induction depends on how fast lactose enters the cell, how fast it is converted to allolactose, and how fast it is hydrolyzed. In addition, galactose and glucose can effect permease and β-galactosidase function directly and they can also affect the cyclic adenosine monophosphate level. Consequently, it is not surprising that cells grown extensively in low lactose only have about one-half the fully derepressed level of β-galactosidase (and presumably all *lac* operon products as well). The data in Tables 3 and 4 clearly indicate that even one-third (2,000 to 3,000 units of β-galactosidase) of the *lac* steady-state level of induction allows growth at almost the low lactose steady-state rate, although in the steady-state cells growing on low lactose have 6,000 to 7,000 units.

Patently, it would do the starving cells little good to have their *lac* operons turned on by introduced lactose while unable to lay down functional permease. In such a case, the many cells would take many hours to double at all and many cells would be lost. Therefore, one might expect, from these a priori teleological considerations as well as from the observed decrease in lag after prior induction, that functional membrane can be laid down at any time in the cell cycle. A difficulty of the counter hypothesis is there is no way for the cell to progress through the cycle until it reaches the hypothetical phase in which membrane growth takes place. One can, of course, make the ad hoc assumption that there are two modes of membrane synthesis, one at a properly established phase of the cell cycle and one used in emergencies at other phases.

Even the assumption of an emergency mechanism can not save the periodic-transport synthesis models from the results of two other types of experiments. One type is synchrony experiments from this laboratory, which have been reported in brief (Koch, Abstr. Annu. Meet. Am. Soc. Microbiol.) and are the subject of a full paper in preparation. These experiments show that a pulse of inducer for one-tenth of a cell cycle gives rise to permease activity in cells of all sizes. These cells were undergoing balanced growth with adequate carbon sources at the time of induction, and carbon source was never rate limiting. The second type of experiment has been done by Cohn and Horibata (5) and by Novick and Weiner (26, 27; personal communication). Different media were devised in each of these two laboratories that had the property of permitting all cells to grow but that are only marginally inducing for the *lac* operon. These "maintenance" media allow growth of both previously induced and previously uninduced bacteria in such a way as to maintain their previous states of induction. Uninduced cells tend not to become induced because they cannot concentrate the inducer, whereas previously induced bacteria can pump the inducer from the outside to achieve an adequate inducer level to maintain the derepression of the *lac* operon. Both sets of workers report that 15 min of a high level of inducer was enough to convert the bulk of a previously homogeneous uninduced "off" culture to the "on" state. Although it was not explicitly pointed out, these findings also imply that cells in most phases of the cell cycle can lay down functional permease.

Although the present results and the work just cited argue strongly against Kubitschek's explicit hypothesis, since they all have been done with the inducible *lac* system, the argument cannot be judged conclusive. Perhaps the *lac* operon is special, or perhaps the incorporation of products of inducible operons are subject to different rules. It might also be suggested that the addition of inducer takes cells out of the state of balanced growth. This is probably not a valid objection because in all but one kind of experiment presented here the cultures were in balanced growth up until the experiment ended with the addition of inducer and the analysis of the experiment had begun.

The experiments with all three types of cell culture lead to the same conclusion regarding mostly continuous synthesis of functional galactoside permease throughout the cell cycle. The results with the three are complementary. The glucose-depleted cultures have the lowest energy reserves; the chemostat cells are closest to balanced growth with a minimum of other changes to be made in achieving balanced growth on low lactose; the glucose-gluconate cultures have the lowest β-galactosidase and presumably the lowest galactoside permease, but grow faster and are larger.

Of the three types of culture, the shortest lag is observed for the transfer of glucose-depleted culture to low lactose (33 min, see Table 1) ver-

sus chemostat culture (see Table 3) or glucose-gluconate culture (78 min, see Table 4). It might be argued that this is a result of a partial synchronization owing to glucose depletion. It was argued above that certainly there can be little mass increase after the external glucose concentration starts to fall precipitatiously and that there must be even a smaller differential in mass increase. However, some cells in the later phases of the cell cycle might proceed through cell division. This does take place when cells are treated with inhibitors of protein, ribonucleic acid, or deoxyribonucleic acid synthesis. Such cell division will tend to bunch the population at the early parts of the cell cycle and at a phase in which Kubitschek's hypothesis predicts no incorporation into the membrane. The tentative hypothesis we suggest is that glucose-depleted cells are even more catabolite derepressed than are the other two types of culture.

Very recently, Ryter et al. (30) have presented convincing evidence that the outer membrane is synthesized in a way dependent on the cell cycle. They find the receptor sites for phage lambda are only integrated into the outer membrane during the last quarter of each cell generation and that the integration process is initiated in the vicinity of forming septum. Shen and Boos (32) have claimed that the methyl galactosidase-binding protein is synthesized in a manner synchronized with cell division. This conclusion does not necessarily follow from their data, but is consistent with phenomena having to do with autogenous induction and catabolite repression. In any case, both of these systems are different in many ways from the galactoside permease, most notably in that the lambda receptor is attached to the outer membrane, the methyl galactoside-binding protein in the periplasmic space, and the galactoside permease tightly bound to the cytoplasmic membrane. Consequently, their synthesis and the processes involved in their becoming functional may be very different.

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