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The Effect of Isopropyl-beta-D-thiogalactopyranoside (IPTG) and
Methyl-alpha-D-glucopyranoside (MgG) on Escherichia coli
B-Galactosidase Production and Growth on Lactose

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Abstract

It has been shown that methyl-alpha-D-glucopyranoside (mNG) retards the growth of E. coli on lactose. Inhibition of growth on lactose has been explained by the competing theories of inducer exclusion and catabolite repression. The purpose of this research was to further test these theories by determining if induction of the lac operon overcomes the mNG retardation of E. coli growth on lactose. Growth of E. coli in medium containing lactose and mNG with and without isopropyl-B-D-thiogalactoside (IPTG), a lac operon inducer, was measured with a spectrophotometer interfaced with a computer. Beta galactosidase activity in these cultures was also measured. Results have shown there is no difference in B-galactosidase activity or growth of E. coli in medium containing lactose and mNG with or without IPTG, nor does IPTG induce the lac operon before the onset of growth on lactose. It is concluded that IPTG gets into the cells and derepresses the lac operon, but transcription and translation does not occur in the absence of a usable energy source.

Introduction

Previous studies (Diehl and Wirth, 1990) had shown that Methyl-α-D-Glucopyranoside (mNG) severely retards the onset of growth of E. coli on lactose. Lactose, a disaccharide composed of galactose bonded beta 1-4 to glucose, is a
carbohydrate that can support the growth of *E. coli*, but mG
will not support the growth of *E. coli* (Diehl and Wirth, 1990).
The bacterium has separate and different transport systems for
transferring glucose and lactose across the membrane from the
environment to the cytoplasm (Scarborough, 1985). The glucose
transport system, known as the phosphoenolpyruvate carbohydrate
phosphotransferase system (PTS) consists of at least four enzymes
that catalyze group translocation of glucose across the membrane.
This transport system was recently reviewed by Saire
(Microbiological Reviews, Mar. 1989). Briefly, the PTS functions
as follows: the energy for glucose transport and activation
is derived from phosphoenolpyruvate (PEP) in the form of a high
energy phosphate bond which is transferred to PTS enzyme I\textsuperscript{Glc},
then to a heat resistant protein HPR, then to PTS N\textsubscript{II} III\textsuperscript{Glc},
and finally to the sugar, glucose, as it enters the cell by
way of PTS N\textsubscript{II} II\textsuperscript{Glc}.

Lactose is transported by a single integral membrane protein
called B-galactoside permease. This protein is coded by the
structural gene of the lac operon, which is an inducible operon
(Jacob and Monod 1961). The lactose permease is a symport that
catalyzes the simultaneous movement of H\textsuperscript{+} and lactose through
the membrane from environment to cytoplasm (Prescott et. al.,
1990). The energy for transport is provided by the
electrochemical gradient that exists when the H\textsuperscript{+} concentration
is greater on the outside of the cell than it is on the inside
of the cell. PTS enzyme III\textsuperscript{Glc} in the dephosphorylated state
inhibits lactose permease such that it can not efficiently
this new lactose permease will also be inhibited by the
dephosphorylated Nz III. However, when 75 of these lactose
permeases are present in the cell membrane, all of the cells
Nz III in the dephosphorylated state are being utilized to
inhibit each of the lactose permeases. This could occur
approximately every 15 minutes, which is the approximate time
required for a new permease to be formed. Once the lactose
permease level reaches 76 or more, the additional lactose
permeases are not inhibited by Nz III in the dephosphorylated
state because there are no more Nz III's to inhibit. With the
additional lactose permease, lactose can be transported into
the cell, free from inhibition, resulting in the total induction
of the lac operon. B-galactosidase then initiates lactose
catabolism to provide the cell with the energy it needs to
transcribe and translate the lac operon, for the secondary active
transport of lactose, and for other endergonic metabolic
processes required for bacterial growth and cell division.

Isopropyl-β-D-thiogalactopyranoside (IPTG) is a gratuitous
inducer of the lac operon, meaning it enters the cell, induces
the operon permitting transcription of the structural genes
occur, but IPTG is not catabolized by the resulting
B-galactosidase. In other words, IPTG is a noncatabolizable
inducer of the lac operon. Theoretically, IPTG added to E. coli
culture media containing lactose and mιG should induce
the lac operon. This would increase the cellular content of
B-galactosidase and permease and thus overcome the mιG
retardation of B-galactosidase production and growth of E. coli
transport lactose into the cell (Saier, Mar. 1989).

Theoretically mG sufficiently mimics glucose so it can be recognized as substrate by PTS II_Glc of the PTS system. mG may either bind to II_Glc or be transported by II_Glc, both of which could dephosphorylate Nz III. When mG theoretically is bound or transported by II_Glc, III_Glc would be in the dephosphorylated state and causes the lactose permease to be transiently inhibited, retarding the entry of lactose and hence the growth of E. coli on lactose. However, the bacteria eventually overcome the mG inhibition of growth on lactose. There are at least two possible mechanisms whereby this is achieved. First, a lactose molecule occasionally may be able to get into the cell when permease is transiently deinhibited by Nz III. Once lactose is in the cell, it is converted to allolactose (1-6-o-beta-D-galactopyranosyl-D-glucopyranoside) by B-galactosidase (B-gal), which derepresses the operator and allows RNA polymerase to attach to the promoter region and transcription of the genes, lac z, lac y, and lac a can occur to make a polycistronic mRNA (Freifelder, 1987). Translation of mRNA by ribosomes results in synthesis of B-gal, permease, and transacetylase. B-gal is now present in the cell and the permease for lactose is inserted in the membrane. Secondly, the repressor on the lac operator transiently derepresses the lac operon, thus allowing RNA polymerase to transcribe the genes (Freifelder, 1987). This also allows for the gradual accumulation of B-gal in the cytoplasm and permease in the membrane. Now, let us say there are 75 II_Glc in the membrane,
on lactose.

The purpose of this research was to culture *E. coli* in medium containing lactose and mβG in the presence and absence of IPTG, a gratuitous inducer of the lac operon, to determine if IPTG overcomes the retardation of growth and 8-gal production caused by mβG.

**Methods and Materials**

**Bacterium**

The genetically well defined *E. coli* used for this research was strain MG 1555. For a history of this strain see Guyer et. al. (1980). Every three to four weeks the stock *E. coli* was recultured in Leuria Broth (LB medium) to keep the culture fresh and viable.

**Culture Media**

Most culture media used in this research was formulated by adding selected ingredients to a minimal salt solution (MSS). The minimal salts solution consisted of 1.0 g of NH₄Cl, 11.33 g Na₂HPO₄·7H₂O, 3.0 g KH₂PO₄, 0.266 g MgSO₄·7H₂O, and diluted to a volume of 500 ml with distilled H₂O at pH of 7.0. Adjustments were made in pH, if needed, using 10% NaOH or 10% HCl. Portions of 100 ml volumes of MSS were autoclaved to sterilize the solution.
Three types of media used in this research were stock culture medium (LB medium), starter culture medium, and a computer culture medium. The stock culture was composed of 1.0 g of tryptone, 0.5 g yeast extract, 1.0 g NaCl, and 100 ml distilled H₂O (pH 7.2). The starter culture was composed of 1.0 ml of (MSS), 0.3 ml of distilled H₂O, and 33.3 microliters of 0.5 M glucose. The computer culture medium always consisted of a total volume of 4.0 ml with carbohydrates at a concentration of 3.5 mM, prepared by combining 3.0 ml of MSS, 25 or 200 microliters of starter culture, 25 microliters of carbohydrates and/or nonutilizable carbon sources, and the necessary amount of distilled H₂O to bring the total volume to 4.0 ml.

ATP & EDTA Concentration

To achieve a concentration of 10⁻³ M in spectronic 20 cultures, ATP and EDTA concentrations were calculated as follows: ATP in the amount of 44.64 mg was dissolved into 0.5 ml of aqueous solution to give a .08 M solution. Fifty microliters of .08 M ATP in 4.0 ml of culture will produce 10⁻³ M final concentration of ATP. EDTA in the amount of 0.2047 g in 10 ml of distilled H₂O produces a concentration of .05 M. Eighty microliters of .05 M EDTA in 4.0 ml of culture produces a concentration of 10⁻³ M.
Computer Setup

Growth curve data was spectrophotometrically measured and recorded by an Apple IIe computer equipped with an analog digital board (Bioanalytical Systems, State College, Pa.) as previously described by Diehl and Angstadt (1989). Briefly, this methodology allows values from the spectrophotometer in the form of light energy to be transduced into electrical energy for the computer to detect and process. The computer recorded the change in transmittance of light through the spectronic 20 tubes as *E. coli* grew in the culture medium. Data was harvested at intervals of six minutes for experiments set up for either 24 hrs or 48 hrs with the Spectest program by Diehl and Angstadt. Raw data were saved and transmittance values were converted into absorbance values using a program entitled Convert AB. A graph of these absorbance values was produced using a program entitled Scientific Plotter. Hard copies of these graphs were printed using a program by Appleworks entitled Image Writer. Data disk file management was done using a Data DOS ver. 3.3 program by Appleworks.

The method to raise the starter culture baseline value from an absorbance of 0.00 to an absorbance value of 0.05, involved preparing a computer culture medium and adding 24 hr starter culture of *E. coli* to it in 25 microliter intervals. Twenty five microliter increments of *E. coli* starter was added by a micro Finnpipet until a transmittance value of about 90% resulted in order to provide the necessary absorbance of 0.05.
The total volume of starter required to give an absorbance of 0.05 in the computer culture medium was recorded.

Sample Collection for B-galactosidase Assays

Samples were taken from the culture tubes in the spectronic 20 whenever experimental protocols required assays for B-gal. To collect samples, the door on the spectrophotometer was opened and the cotton filter plug was removed from the culture tube and 50 microliters of culture was collected using a Finnpipet. The sample was placed in the Z-buffer at pH 7.0 (described below) and the cotton was replaced and the door was shut. To record the time/position sample was taken, the spacebar was manually depressed while the door of the spec 20 was open. Samples were paraflimed and placed in the refrigerator unless immediately assayed.

Experiments were done in order to determine whether or not prolonged storage on refrigeration had any effect on the amount of B-galactosidase activity present in medium containing lactose and mWG in the presence and absence of IPTG. A total of twelve samples of 50 microliters of culture in .45 ml of Z-buffer were assayed in groups of three at 0 hrs, 12 hrs, 24 hrs, and 48 hrs in the presence of IPTG and also in the absence of IPTG. B-galactosidase assays were done in the same manner as described above. Using an Apple IIc computer, graphs showing the B-gal activity with respect to storage time were generated from data obtained from the above procedures.
Sample Collection for Glucose and Lactose Assays

Fifty microliters was removed from the spectronic 20 culture tube by the same manner as for B-galactosidase assays except the 50 microliters was placed in 0.5 ml microcentrifuge tube. The centrifuge tube was spun for 10 min at 9,000 rpm in Eppendorf model no. 5415 centrifuge to sediment the E. coli cells. Thirty five microliters of cell free supernatant was drawn off by Finnpipet and placed in a new centrifuge tube and stored under refrigeration until assayed.

Assays

B-galactosidase

Units of B-gal activity were determined according to the method of Jobe et. al. (1974). Fifty microliter samples of culture from the spectronic 20 were placed in .45 ml of pH 7.0 \( L \)-buffer containing 1.61 g Na\(_2\)HPO\(_4\)*7 H\(_2\)O, 0.55 g NaH\(_2\)PO\(_4\)*H\(_2\)O, 0.075 g KCl, 0.0246 g MgSO\(_4\)*7 H\(_2\)O, .27 ml Beta mercaptoethanol diluted to 100 ml with distilled H\(_2\)O. PH adjustments, if needed were done with 10% NaOH or 10% HCl before the addition of Beta mercaptoethanol. Early sampling during growth were refrigerated before assays were initiated. To the samples, 50 microliters of chloroform and 1% aqueous sodium dodecylsulfate were added by pipet to kill and lyse the cells. A control without any culture sample was run with 4X the amount of ingredients because
twice the volume was needed to establish 0 and 100% transmittance on the double beam spectrophotometer. Fifteen one hundredths of a ml of ortho-nitrophenylgalactoside (ONPG) was added to the reaction mixture with the exact time of addition recorded. Samples were incubated at 28°C for a period of 2-3 hrs for samples taken before the onset of growth and 5-10 minutes for samples taken with growth on lactose. When a pale yellow color had developed in the mixture, the reaction was stopped by the addition of .25 ml of Na₂CO₃, mixed thoroughly and the exact time recorded. The solutions of each sample were transferred by a micropipet into 1.5 ml micro centrifuge tubes to be centrifuged for 10 min at 9,000 rpm in the Eppendorf. The transmittance of each sample was read at 420 nm against the control. The spectrophotometer used was a Beckman DBGT. B-gal activity was calculated by the equation:

\[
\text{Absorbance at 420 nm} = \frac{\text{Units of B-gal}}{\text{(Absorbance at 490 nm)} \times \text{(Change in Time in min.)}}
\]

Glucose Assay

Twenty five microliters of centrifuged (as previously described) culture medium is added to 1.5 ml of Trinders (1969) glucose reagent (Sigma, St. Louis, Mo) and incubated at 37°C for 10 min. Controls consisted of 1.5 ml Trinders without
carbohydrate and 1.5 ml Trinders with 25 microliters of 3.5 mM glucose. Absorbances of the rose color that developed, which is proportional to glucose concentration, were read spectrophotometrically at 505 nm.

**MwG Assay**

The MwG assay was performed by the method of Diehl (1992). Twenty five microliters of centrifuged culture medium sample were mixed with 25 microliters of 12 M HCl, then boiled for 20 min, cooled and 25 microliters of hydrolysate were neutralized with 25 microliters of 6.0 M NaOH in a separate tube. Twenty five microliters of neutralized hydrolysate was added to 1.5 ml of Trinders solution, incubated at 37°C for 18 min and the resulting red color was measured spectrophotometrically at 505 nm. A control consisted of Trinders without carbohydrate and a positive control containing Trinders and 25 microliters of glucose at 3.5 mM.

**EDTA Inhibition of Bacterial Growth**

Glucose and lactose starter cultures with and without $10^{-3}$ M EDTA were inoculated with stock culture and incubated for 24 hrs. at 37°C. The starter cultures were: glucose alone, glucose and EDTA, lactose alone, and lactose and EDTA in sterilized 13 X 100 mm test tubes.
Sterilization

Solutions that could be decomposed by elevated heat or pressure were filter sterilized with a syringe and 0.2 micron Acrodisc filter (Gelman Science, Ann Arbor, Mich). Solutions or substances that are not affected by elevated heat or pressure were autoclaved at 121º for 15 min. All laboratory techniques were aseptically performed unless sterility had no effect upon the environment of the E. coli and on the assay being performed.

Results

Previous studies in our lab had shown that mmG severely retards the onset of growth of E. coli on lactose and retards the induction of B-gal production (Diehl and Wirth, '990). As indicated in figure 1, 25 microliters of starter added to lactose and MSS starts growth on lactose at 4 hrs and finishes growing at 18 hrs at a maximum of 0.37 A. B-galactosidase activity of 0.06 units is detected at 4 hrs and reaches a maximum of 0.22 units. As indicated in figure 2, 25 microliters of starter added to media containing lactose and mmG begins growth on lactose at 18 hrs and finishes growing at 38 hrs at 0.35 A. B-galactosidase production began at 18 hrs at 0.01 units and reached a maximum of 0.24 units.

Results of E. coli grown in media with lactose and mmG in the presence and absence of IPTG are presented in figures 3 and 4. Twenty five microliters of starter added to media
containing lactose, mXG, and IPTG begins growth on lactose at 18 hrs and finishes growing at 36 hrs at a 0.36 A while in control cultures not containing IPTG, the delay in onset of growth is also 18 hrs. B-gal is not produced before the onset of growth, but B-gal was produced at 18 hrs at 0.05 units and reaches a maximum of 0.55 units. Results from the mXG assay done with samples from these cultures indicated that mXG is not utilized. The initial concentration of mXG at the beginning of the experiment was equal to the final concentration at the completion of the experiment.

The results of experiments to determine if B-gal activity changed with sample storage time are presented in figures 5 and 6. As indicated in these graphs, samples taken from cultures formulated with and without IPTG and stored for 50 hrs in Z-buffer on refrigeration did not lose substantial B-gal activity.

Results of the experiments designed to determine what volume of starter culture must be added to computer medium to obtain a baseline absorption value of 0.05 showed that 200 microliters of starter added directly to computer culture medium produced the desired value.

As seen in figures 7 and 8, the onset of growth dropped from 18 hrs to 7.25 hrs in cultures of lactose and mXG in the presence and absence of IPTG, inoculated with 200 microliters of starter. However, maximum growth and B-gal activity did not substantially change from the cultures inoculated with 25 microliters of starter. Also, B-gal activity was not detected
before the onset of growth on lactose in either culture.

The results of cultures in lactose, mwG, IPTG, 200 microliters of starter with and without ATP are presented in figures 9 and 10. ATP caused a further delay in the onset of growth from 7.25 hrs to 15 hrs, but there was no B-gal production before the onset of growth.

Computer cultures with 200 microliters of \textit{E. coli} in media containing lactose, mwg, IPTG, ATP with and without EDTA are shown in figures 11 and 12. EDTA was found to totally inhibit growth of \textit{E. coli} and B-gal activity for cultures containing lactose, mwg, IPTG, and ATP, whereas, without EDTA, the growth curve and B-gal activity was normal for cultures in lactose, mwg, IPTG, ATP, and 200 microliters of starter.

EDTA tested in starter cultures of \textit{E. coli} with either glucose or lactose alone proved to grow as turbid as starter cultures without EDTA at 37°C for 24 hrs.

All experiments done to find an energy source that is not diauxic with glucose yielded negative results. The sugars, xylose, ribose, and rhamnose were tested and all were diauxic with glucose.

Cystine, an amino acid, was considered as a potential energy source, but due to its limited solubility, the desired concentration of 3.5 mM could not be achieved in pH 7.0 aqueous culture medium at 37°C.
Discussion

Initial experiments were designed to determine if IPTG overcomes the retardation in B-galactosidase production and the onset of growth on lactose caused by mwg. There was no difference in results of experiments with E. coli cultured in lactose and mwg in the presence and absence of IPTG. Since samples collected for B-gal assays harvested early in the experiment were stored on refrigeration for up to 48 hrs before analysis, there existed the possibility that B-gal activity could be lost during refrigeration. However, the results of experiments designed to determine if B-gal activity is lost on storage indicated this was not the case. When duplicate culture samples were harvested with one being assayed immediately and the other being assayed after 50 hours of refrigerated storage, B-gal activity was not substantially different. One interpretation of the results of the initial experiment is that IPTG is not inducing the lac operon in the presence of mwg; or alternatively, IPTG may be inducing the lac operon, but 25 microliters of starter does not provide sufficient cells to detect B-galactosidase even if it is being produced before the onset of growth. Previous experiments (Diehl, 1989) revealed that cells in culture to give .05 absorbance is more than sufficient to detect B-galactosidase if it is being produced. Thus, it was experimentally determined that 200 microliters of starter in 4.0 ml of medium gives 0.05 absorbance. Results showed that increased starter reduced the time of growth onset
from 18 hrs to 7.25 hrs. The probable reason for the shorter time in onset of growth is due to an 8 fold increase in starter with no increase in any of the nutrient or nonnutrient ingredients. B-galactosidase was not detected in either culture before the onset of growth on lactose. These results show that IPTG is not inducing the lac operon before the onset of growth or an alternative hypothesis is that IPTG is inducing the lac operon before onset of growth, but B-galactosidase is not being synthesized because the cells do not have adequate energy (ATP) to drive transcription and translation.

To test this alternative hypothesis, ATP was added directly to the medium. When ATP was added to the culture, a lag period in the onset of growth went from 7.25 hrs to 15 hrs. Possibly, ATP was binding to the lactose permease in the cell membrane causing lactose to delay entry or it binds Mg$^{2+}$ altering the integrity of the outer membrane which slows growth. ATP did not provide energy for the cell to produce B-galactosidase, and since there is substantial evidence to support the concept that nucleotides are not normally transported into the cell, it was reasoned that ATP probably wasn't getting in the cell. A literature search led to the discovery by Perlman and Pastan (1968) that $10^{-3}$ M EDTA would open the cell to cAMP, the structure which is similar to ATP. In an effort to render cells permeable to ATP, EDTA was added to the medium. When EDTA was added to lactose, MgG, IPTG, and ATP, growth and B-galactosidase production was totally inhibited. Possibly, this mixture was unique in a way that something reacted with
EDTA to cause total inhibition of growth and β-galactosidase production. Starter cultures with glucose and lactose with EDTA were grown for 24 hrs at 37°C. EDTA caused no inhibition of growth in the starter cultures which leads to the conclusion that some reaction among the ingredients in the mixture of lactose, mαG, IPTG, ATP, and EDTA is causing total inhibition of growth on lactose. Instead of studying this phenomenon, a search for an energy source was continued.

Some of the carbohydrates which were tested to determine if they could serve as an energy source were xylose, ribose, and rhamnose, but growth curve experiments showed that each of these carbohydrates is diauxic with glucose. If these sugars are diauxic with glucose then they could also be diauxic in the presence of mαG and their transport into the cell inhibited by the dephosphorylated III or some other mechanism. The idea is to find a sugar that is not diauxic with glucose to provide the cell with the energy needed for transcription and translation of the lac operon in the presence of IPTG and mαG.

Cystine, an amino acid, was considered for use as an energy source, but it would not dissolve into solution at a concentration of 3.5 mM in 4.0 ml of aqueous solution. With the addition of NaOH, more cystine would go into solution at a pH of about 10.0, however, once added to the culture containing the buffer at pH of 7.0, some of the cystine would most likely precipitate out.

After searching the literature for possible non PTS energy sources that are not diauxic with glucose, none were discovered.
However, previous studies (Diehl and Wirth, 1990) of E. coli growth and B-gal production in medium containing glucose, lactose, mW, and IPTG show that B-galactosidase and presumably permease are synthesized before lactose was utilized as seen in Figure 1). IPTG is turning on the production of B-galactosidase in the presence of a usable energy source long before the growth on lactose and in the presence of mW. In the presence of IPTG, no diauxy is present as it is in cultures containing glucose and lactose alone and in cultures containing glucose, lactose, and mW (Figures 14 and 15). With IPTG, no diauxy is present because the lac y structural gene is turned on by IPTG with glucose as the energy source and permease is produced and inserted into the membrane, so when glucose is completely utilized, lactose can flood into the cell without a lag period for permease production. However, in the absence of IPTG, the lac operon is repressed and a lag period results after glucose is utilized because as lactose is utilized, it takes time for the lac operon to produce enough B-galactosidase and permease to allow lactose into the cell and to be broken down.

From our studies on mW inhibition of onset of growth and B-gal production and the studies of cultures containing glucose, lactose, mW, and IPTG, it appears that IPTG gets into the cells and induces the lac operon, but transcription and translation does not occur in the absence of a usable energy source.
Literature Cited


Absorbance And
Beta Galactosidase Activity

Figure 1

Absorbance And
Beta Galactosidase Activity

Figure 2
Asay Time After Refrigeration

B-gal Activity of SGC 713

\[ y = 0.556 + 0.0032143x \quad R = 0.2793 \]

Assay Control with IPTG Present
Figure 6: Assay Activity of SGC 7.13

\[ y = 0.3694 + 0.0005074x \]

B-gal Activity
Growth curve of E. coli cultured in MSS containing glucose, lactose, MgG and IPTG all at 3.5 mM. Units of β-galactosidase activity plotted as solid circles. Concentrations of glucose plotted as squares, lactose as open circles and MgG as triangles.
Growth curves (solid line) of E. coli cultured in MSS containing glucose, lactose and methyl-alpha-glucoside (all at 3.5 mM). Units of absorbance and beta-galactosidase activity are plotted as solid circles, while glucose and lactose are open circles.

Growth curve (solid line) of E. coli cultured in MSS containing 2.5 mM glucose and lactose both at 3.5 mM. Units of absorbance and beta-galactosidase activity are plotted as solid circles, while glucose absorbance and lactose as open circles.

Figure 14

Figure 15