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Lactose Induction of the lac operon in *Escherichia coli* B23 and its effect on the o-nitrophenyl ß-galactoside Assay

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This experiment expanded upon the results of a previous study involving the effect of carbohydrates on the induction of ß-galactosidase, in *Escherichia coli.* Previous results had indicated that lactose in the growth media caused an apparent decrease in enzyme induction and activity as compared to media containing glycerol. The intent of this experiment was to evaluate whether lactose had a biological effect on the induction of ß-galactosidase. In addition, we investigated whether different methods of lactose addition caused an interference effect with the o-nitrophenyl ß-galactoside (ONPG) enzyme assay through competitive inhibition.

The results showed that several factors potentially play a part in the apparent suppression of enzyme activity by lactose using an ONPG assay. The biological effect was seen when lactose was added to the growth media. Enzyme activity increased at a slower rate under this condition than in the glycerol control, but over the length of the experiment, increased to almost the same level. When a large concentration of lactose was added to assay samples, an immediate decrease in enzyme activity was observed, and this suppression continued throughout the experiment. Competition with ONPG limited the effectiveness of the enzyme assay; some of the ß-galactosidase produced by the bacteria probably cleaved lactose instead of the assay indicator. While the ONPG assay is generally a convenient enzyme assay, it was concluded from this study that an alternate type of enzyme assay might be more effective at measuring ß-galactosidase activity in the presence of lactose.

The lactose (*lac*) operon codes for the proteins involved with lactose transport into a cell and its subsequent cleavage into glucose and galactose. Transcription of the *lac* operon is induced by the intracellular presence of lactose and is regulated in two ways: through the processes of inducer exclusion and catabolite repression. In inducer exclusion, the intracellular presence of glucose inhibits the transport of lactose. The process of glucose repression is slightly more complex. When glucose levels are high, cyclic-AMP (cAMP) levels are low inside the cell. When glucose levels are low, cAMP levels are high and cAMP binds a cAMP receptor protein (CRP), which then binds a region of DNA upstream of the *lac* operon promoter and enhances its transcription (3).

In the following experiment, we have used the lactose analogue isopropyl thiogalactoside (IPTG) to induce *lac* operon expression in *E. coli* to high levels. IPTG is easily transported into normal *E. coli* cells where it binds and inhibits the action of the LacI repressor protein and effectively activates *lac* operon expression. Unlike lactose though, IPTG cannot be cleaved by ß-galactosidase and remains within the cell as a constant activator of *lac* operon expression. O-nitrophenyl ß-galactoside (ONPG) is another lactose analogue. It can be used to quantitatively measure the enzyme activity of ß-galactosidase. ONPG is a colourless substrate that can be cleaved by the enzyme ß-galactosidase to yield stoichiometric amounts of yellow o-nitrophenol and colourless galactose. As yellow o-nitrophenol is produced, its concentration can be measured with a spectrophotometer (2).

In a previous set of experiments (2), ONPG was used to assay ß-galactosidase activity in an *E. coli* cell whose *lac* operon expression had been activated by IPTG. The results seemed to indicate that lactose had a biological effect on induction of ß-galactosidase. Enzyme activity of ß-Galactosidase in cultures that were grown with lactose in the medium seemed to be lower than those grown in glucose, glycerol, or sucrose. These results appeared to be counter-intuitive. It is our belief that the presence of lactose in the media should have, in fact, increased the enzyme activity. It would therefore seem that the lactose, somehow, interfered with the ONPG assay to produce a misleading result.

One possible mechanism to explain the results is that the lactose competes with ONPG for the active site of the ß-galactosidase enzyme. O-nitrophenol concentration was used as a proxy for ß-galactosidase activity. One would predict that in the presence of another substrate (i.e. the lactose), ß-galactosidase would split its activity between cleaving ONPG and the other substrate thereby decreasing expected results.

We conducted an experiment to further investigate these observed results, and to see if this competition between ONPG and lactose actually occurs. Our hypotheses consisted of two separate branches. Either lactose interferes with the ß-galactosidase enzyme assay or it does not. If it does interfere, then it may have a biological affect on the culture or it may simply interfere with the assay (competition between substrates).

MATERIALS AND METHODS

E. coli strain B23 containing the *lac* operon was grown in M9 minimal media with 0.2% glycerol for the duration of the experiment. One culture was maintained, and prior to treatment with IPTG, an aliquot was removed to measure basal ß-galactosidase levels. The remaining culture was treated with IPTG and later aliquoted into three cultures that were maintained in separate environments. The first culture was maintained solely in M9-glycerol media, and served as the control. The second was enriched with lactose directly in the medium. The remaining aliquot was also maintained in only M9-glycerol media, but later had lactose added to it at the time of ONPG addition. Over set time intervals, these separate cultures were sampled and treated with toluene. These samples were later treated with an excess of ONPG and incubated until a suitable color formed. The samples were then measured for absorbance at 420 nm in a spectrophotometer (Spectronic 20) and used to calculate enzyme activities under the different conditions.

RESULTS

A comparison of the enzyme induction of all four conditions can be seen in Table 1. The curve of the condition with IPTG alone shows that the maximum enzyme activity was seen in media with no lactose present and IPTG as the sole inducer of ß-galactosidase (Fig. 1). The curve of the condition with IPTG and lactose in the media shows a consistently lower value of enzyme activity all throughout this experiment. It should be noted that the curve for this condition is not parallel to that of the IPTG alone over the entire range of data. The curve of the condition with IPTG and lactose in the assay is essentially parallel to that of the IPTG alone, though the enzyme activity is much lower. In the final condition, which contained no lactose or IPTG, no significant amounts of ß-galactosidase were detected.

TABLE 1. Induction of ß-galactosidase production in *E. coli* under different conditions

Condition	IPTG Present	Lactose Present	ß-galactosidase Activity
1	Yes	No	Increased
2	Yes	Yes (in media)	Increased (less than Condition 1)
3	Yes	Yes (in assay)	Decreased sharply, then increased
4	No	No	No change

DISCUSSION

Using ONPG to assay for ß-galactosidase contains inherent problems due to the fact that it is a lactose analogue. When assaying in the presence of lactose, the sugar interferes with the analysis, binding to the ß-galactosidase in lieu of the ONPG. This competition can therefore decrease the observed enzyme activity as reported by the ONPG. Our results indicated that a number of factors contribute to this competition, including the concentration of lactose and the ability of the cells to take up lactose.

When the control culture is compared to the lactose-treated culture, it can be seen that the lactose-treated culture has a lower trend line that generally diverges from that of the control culture (Fig.1). This divergence is contrary to expectations. One would expect that over time, the amount of lactose available would decrease due to consumption, and the assay would proceed at a rate closer to that of the lactose-free control. The cultures did not show a large difference in enzyme activity at the time of divergence (16 minutes). The assay samples with added lactose, however, showed an immediate decline in enzyme activity at 16 minutes. An immediate decrease is consistent with the hypothesis that a competition effect influenced the enzyme assay in whole or in part.

It is more difficult to explain why the lactose-treated culture did not show an immediate decrease in enzyme activity. One possible reason is that the amount of lactose in the culture was only 0.1%, thus it may not have had a significant impact on cellular expression. Some lactose was expected in the lactose-treated culture because the

sample was not washed before being permeabilized and therefore any free lactose in the media would have had the opportunity to interfere with the assay. This would have likely occurred with earlier samples instead of the later samples, as the amount of lactose in the environment should have decreased over time. However, with such a low concentration of lactose in the media (and thus in the assay), the competition effect with ONPG may have been negligible when the relative amount of ONPG was large.

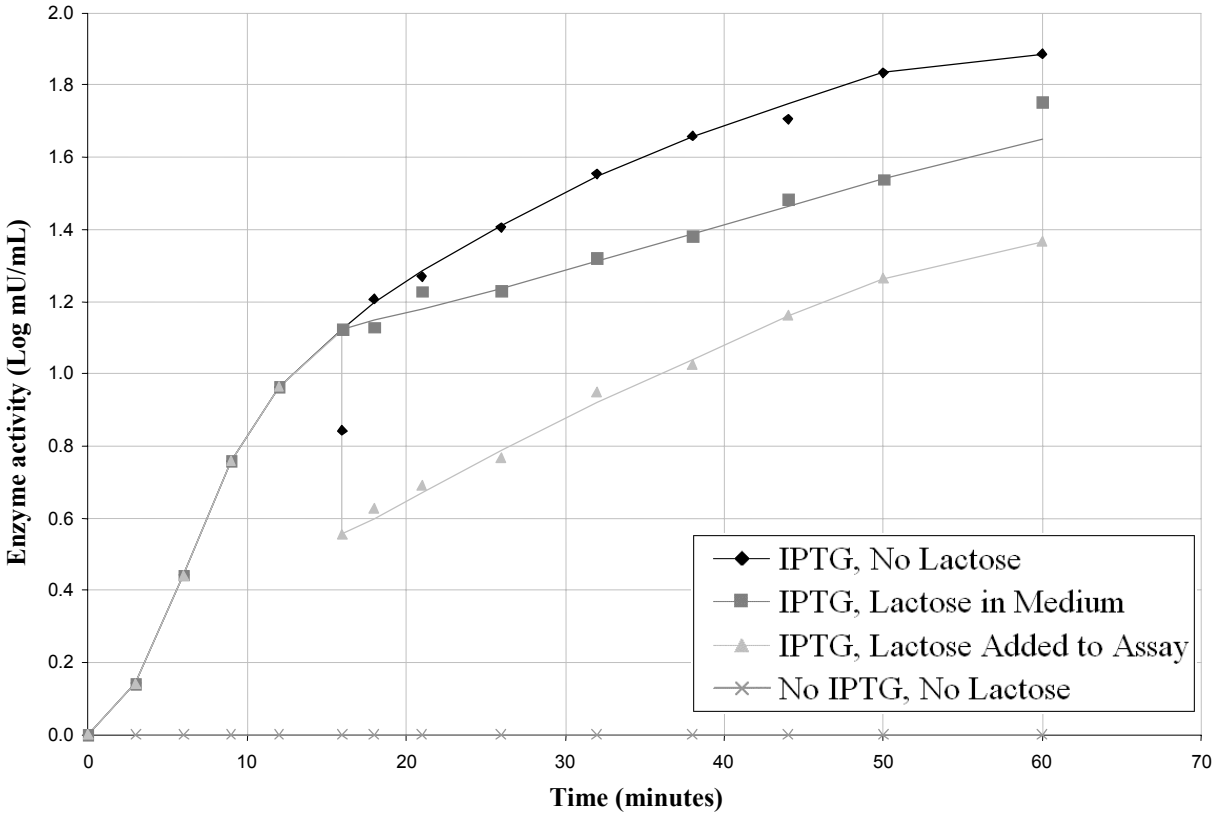

FIGURE 1. Induction of ß-galactosidase in *Escherichia coli* measured as enzyme activity vs. time.

Another possible reason is that in the lactose-treated culture, cells had to take up lactose in order for it to be cleaved by the enzyme. In the assay, however, the cells had already been permeabilized and were able to use ONPG readily. The "delay" seen therefore may have been the result of a slow competition effect, combined with the delayed uptake of lactose. In the lactose-treated culture, the trend line diverged from the control as the experiment progressed, suggesting that the negative effect on the assay increased over time. The effect on the assay may not have been entirely due to competition with ONPG. Intuitively, it would seem that lactose should be consumed as time progresses, causing the trend line to converge with the control. As time passed, the cells would have taken up more lactose so more β-galactosidase would have been competitively inhibited to cleave ONPG later in the experiment. This would explain why the lactose-treated culture appears to have diverged from the control.

The trend line for the assay samples treated with lactose is generally parallel to that of the control after the immediate decrease in enzyme activity. This decrease may have been due to several factors. First, the amount of lactose inadvertently added to each assay sample was approximately 45-fold larger than the relative amount present in the lactose-treated culture. Erroneously introducing this new variable means that the results from this assay could not be easily interpreted. However, the parallel lines indicate that the added lactose in the assay samples out-competed the ONPG in the assay, causing the apparent suppression of β-galactosidase activity. These parallel lines indicate that the extent of inhibition was constant at all times. It supports the argument that the lactose-treated culture did not have lactose in competition with ONPG because the enzyme activity did not vary over time (the slope of the curve remains constant). Also, the level of lactose in the media, when the cells were permeabilized was too low to cause significant inhibition in that sample. If the lactose present, when the cells were permeabilized, had a significant impact, it would have mimicked the other two curves.

To further develop this study, a comparison should be made of a lactose-treated culture and an assay sample using the same level of supplied lactose. Such an experiment would indicate whether lactose concentration was the main factor in the negative competition effect on the ONPG assay or whether it was the uptake of lactose that was the determining factor. In either case, our study has revealed that having lactose in the bacterial culture or in the assay samples directly causes interference with the ONPG assay. In experiments where the activity of β-galactosidase must be quantified, another enzyme assay or method would be more appropriate than the ONPG assay.

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Appendix:

A. Procedures

1. Media

E. coli inoculum

M9 minimal media with 0.2% glycerol	200mL
Lactose 10% solution	5mL
Toluene	5mL
ONPG (5mM)	8mL
IPTG (20mM)	2mL
Sodium carbonate	85mL
Tris buffer (20mM, pH 8.0)	55mL

2. Equipment

50 test tubes
10 - 5mL sterile pipettes
2 - 10mL sterile pipettes
15 - 1mL sterile pipettes
37°C shaking water bath
25°C non-shaking water bath
1 sterile 100mL graduated cylinder
4 sterile 250mL Erlenmeyer flasks
1 sterile 500mL Erlenmeyer flask
3 test tube racks
2 Spectronic 20 cuvettes
1 P-100 Pipetteman and 1 tip
1 Spectrophotometer

3. Method

Part I – Growth and Sampling

1) Aseptically add 200mL of M9 glycerol media with a sterile graduated cylinder to a sterile 500mL Erlenmeyer flask. Recap this cylinder and save it for later use.

2) Aseptically inoculate the media in the 500mL flask to about 0.15 OD_{460} by adding a calculated volume of inoculum. Incubate this culture for 25 minutes at 37°C.

3) Label four 250mL flasks.
   #1 – IPTG with No lactose
   #2 – Lactose in media
   #3 – Lactose in assay
   #4 – No IPTG, No Lactose
   Add 0.4mL of a 10% lactose solution to the flask #2.
   Place the four sterile 250mL flasks in a water bath shaker to warm up.

4) At 25 minutes, use a 5mL pipette to remove a 3mL sample from the inoculated culture to measure the basal enzyme activity levels.

5) At this point, remove 40mL's of inoculated culture from the flask and place it in Flask #4. This will be the control. You will add nothing more to this flask.

6) Begin the actual experiment by adding IPTG to the remaining culture in the 500mL flask to give a final concentration of 0.10 mM. (Should have 137mL of culture in the flask).
7) Place the culture flask back into the water bath and set the timer to zero.
8) At 3, 6, 9, 12 minutes, remove 3mL's from the culture flask to test for enzyme activity. At these points, you must also remove 3mL's from the control Flask #4 and test for enzyme activity as well.
9) At 15 minutes, use the graduated cylinder (from step 1) to rapidly place 40mL's of the culture into Flasks #1 and #3. Add 39.6mL's to Flask #2. Work rapidly to minimize the effects due to lack of aeration or chilling. Return all four 250mL flasks to the shaking water bath.
10) Continue to sample the four flasks at the following time points: 16, 18, 21, 26, 32, 38, 44, 50, 60 minutes. Ensure that the four flasks are all sampled in the same order at each time point.

Part II - Measuring Enzyme Activity Levels

1) After each sample has been removed from the flasks, immediately Toluenize the cells. This is done by adding 200 microlitres of toluene to each 3 mL sample. Ensure that the toluene actually enters the sample and does not hang-up on the test tube wall. Immediately vigorously vortex the sample for 20 seconds.
2) After vortexing, allow each sample to sit for at least 1 minute to allow the toluene to separate into a distinct upper phase. The transfer 0.4mL of the permeabilized cells from below the toluene layer into a clean test tube that will be used to do the assays. You may use the same pipette for all the samples from the same flask, as long as one begins at the first sample and works towards the last. Ensure that you make 2 sets of samples from Flask #1 and label each set (Set 1, Set 2). The second set will be used as blanks for reading the absorbance of all the other samples.
3) Now, add 1.2mL of Tris (20mM, pH 8.0) to each tube. Then place all of the samples into the 25°C non-shaking water bath. When they are warm, remove one sample at a time.
    a) Flask #1 samples (set 1) and Flask #2 samples – Add 0.2mL of ONPG (5 mM), immediately mix the sample and return it to the water bath. Record the time of addition and proceed to the next sample. Five tubes can be completed in a row by filling a 1mL pipette and measuring out 0.2mL of substrate (ONPG) to each tube before refilling the pipette.
    b) Flask #1 samples (set 2) – Add 0.2mL of distilled water instead of ONPG. Then add 2ml of 0.6 M sodium carbonate. These will be used as blanks for all the other samples. They should not develop colour and do not need to be incubated. Record the time of addition.
    c) Flask #3 samples – Add 0.2mL of ONPG (5mM) and 0.18mL of the 10% lactose solution and return it to the water bath. Record the time of addition.
    d) Flask #4 samples – Add 0.2mL of ONPG (5mM) and return it to the water bath. Record the time of addition.
4) During and after the substrate addition, keep an eye on the incubating assays and be prepared to stop any reaction that shows enough yellow colour to give a reading of about 0.4 absorbance units in the spectrophotometer. (To get a rough estimate of the time required, test your 50 minute sample first. Record the time taken for this sample to

develop enough yellow colour. You can now use this time as a rough estimate for all the other samples.)

5) To stop the reactions: Add 2mL of 0.6 M sodium carbonate to increase the reaction pH above the active pH of ß-galactosidase. Some will need to be stopped within a few minutes and others after much longer. Slow reactions that are taking longer than 40 minutes may be stopped and recorded.

6) Read the absorbance of the sodium carbonate treated samples at 420nm in a spectrophotometer zeroed with distilled water.

7) Plot four curves on semi-log graphing paper → Enzyme Activity (log units/mL) vs. Time (minutes), one for each experimental condition. Enzyme activity calculations may be found in the Microbiology 421, 2002 Lab Manual, Experiment A4, Part B, Step #8.

B. Sample Calculations

Enzyme Activity:

$$\frac{Enzyme\ Activity}{mL\ of\ Enzyme} = A \times \frac{1}{t} \times \frac{10^6}{15{,}000} \times Nv \times \frac{1}{Ev}$$

$$\frac{Enzyme\ Activity}{mL\ of\ Enzyme} = (0.334 - 0.000) \times \frac{1}{23} \times \frac{10^6}{15{,}000} \times 3.8 \times \frac{1}{0.4} = 9.197\ \text{mU/ml}$$