Regulation of Gene Expression: Turning Genes On and Off

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Abstract: Regulation of Gene Expression

This experiment demonstrates that expression of lactose genes in *E. coli* is regulated by availability of sugars. Students predict the effect of sugars on β-galactosidase levels, and assess the validity of their hypothesis. Each group has one culture with lactose added and then glucose to determine whether providing the preferred sugar can turn off genes. For control culture, students choose glucose, lactose, or galactose sugar. Students analyze bacterial growth and quantitate the production of β-galactosidase. This workshop is for an undergraduate junior level lab course. Two other protocols follow for novice high school students and freshman students.

Introduction [For Instructor]

This Laboratory exercise is designed for students to study the regulation of the *lac* operon, specifically, the *lacZ* gene. Students are given two flasks of wild type bacteria strain B/r. In the first flask, they will add Lactose at 15 minutes, (which will cause de-repression of the *lacZ* gene, producing β-galactosidase), and add glucose at 30 minutes. The second flask serves as a control, where students may pick glucose, lactose, or galactose as their sugar. This experiment can be divided into two parts, the growth phase and the β-galactosidase enzyme assay. Students will monitor the growth of both cultures every 15 minutes by reading the turbidity in a spectrophotometer, taking an aliquot of the cell culture, and plotting the growth. After 90 minutes, the students will then measure the amount of enzyme produced by the cells using an ONPG assay. This laboratory exercise can be done in a 4-hour laboratory class. Prep time involves making of the lambda media, and starting an initial overnight culture of the bacteria strain (see APPENDIX B). The bacteria is kept growing in log phase until ready to provide to the students. Students learn sterile laboratory technique, *E. coli* growth phases, measurement of enzymes, and gene regulation. Students compare the growth of *E. coli* with the amount of β-galactosidase enzyme produced. This write-up is for an upper level Junior Laboratory class. It can also be adapted for high school students, by omitting the viable counts quantitation.

Student Outline

Overview

This experiment shows that expression of the lactose genes of *E. coli* are regulated by the availability of certain small molecules. You will monitor the growth of two cultures and remove samples at regular intervals to assay for the enzyme β-galactosidase, one of the three enzymes involved in the utilization of lactose. You will observe how the activity of this enzyme changes upon the addition of different sugars to the growth medium. You will also demonstrate that the genes can be turned off again by the addition of another sugar. Each group of students will have two cultures. To the first, you will add lactose and then glucose. You and your partner must choose whether to add glucose, lactose or galactose to the second culture. Based on what is known about the regulation of the genes of the *lac* operon, you should be able to predict the effect of each of these sugars on the intercellular level of β-galactosidase.

Introduction[1]

Over the years, the definition of the gene has changed; it was first defined in genetic terms—as a unit of inheritance. What a gene actually was chemically, and how such a unit brought about observable characteristics was not understood. It was not until biochemists began to unravel some of the complex series of chemical reactions carried out by cells that a new definition of the gene was possible. Using extracts of cell cytoplasm biochemists began to understand that the cell makes all the molecules it needs from simple components by a series of understandable steps. For example, all the 20 or more amino acids necessary for making proteins are each separately synthesized by a series of chemical reactions from other small molecules present in the cell.

At about the same time, enzymes had been discovered and their function as catalysts of chemical reactions was understood. It seemed obvious that enzymes must be participants in some or all of the steps in the complex series of chemical reactions going on in the cells.

These criteria led to the use of microorganisms such as bacteria, yeast, and fungi as the research organisms of choice. These organisms are exclusively haploid—or at least haploid during a portion of their life cycle. In addition, they are easy to culture in the laboratory and can grow and divide when supplied with only inorganic salts and a few other very simple molecules. That tells us that these organisms must be able to synthesize all the complex biological molecules from such very simple materials and, therefore, all the chemical reactions of those synthetic processes are going on inside each cell.

Out of a combination of genetic and biochemical studies of these organisms, first with the fungus *Neurospora crassa* and later with yeast and bacteria, arose the concept that the gene is the unit which specifies a single enzyme.

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[1] Adapted from: *Kauffman, Linda R. Experimental Genetics and Molecular Biology Lab Manual. Carnegie Mellon University, Department of Biological Sciences. 2005.*

It was already known that not all enzymes are produced all the time. For example, some bacteria use certain materials from their environment as a source of nitrogen atoms only when their preferred source – ammonia – is absent. In a similar manner, the bacteria *Escherichia coli* only uses the sugar lactose from its environment when its first choice – glucose sugar – is absent. To "use" lactose means that the bacteria takes up lactose from the environment and chemically alters it. Therefore, the cell needs an enzyme necessary for lactose digestion and we assume that a cell that cannot use lactose unless the cell has the necessary enzymes within the cell. If you found that cells could utilize lactose only after they were exposed to certain specific conditions, you would say that enzyme activity and the resulting lactose utilization were *induced* by something specific to the new conditions. This is the definition of gene regulation—genes can be turned on, and off. The first real experimental investigations of the process of gene regulation used the bacteria *Escherichia coli* and its response to the presence of the sugar lactose as a research tool.

A large number of mutants were isolated which, unlike the wild type strains, could not utilize lactose at all. Other mutants were isolated which were always ready to utilize lactose; that is, the cells were full of the enzymes involved in lactose utilization, even when no lactose was present. Lac^- mutants cannot utilize lactose at all. Lac constitutive mutants will always have β-galactosidase molecules present in the cell at all times. These two types of mutants are called Lac^- and Lac constitutive, respectively. The full genetic analysis of these mutants revealed, in the most elegant manner, the way in which the genes involved in lactose utilization are regulated and provided us with one model of how gene regulation works in general.

Measuring the amount of enzyme present

During this experiment we will quantitate the amount of the enzyme β-galactosidase that is present in the cells before and after the introduction of small molecule inducers – lactose or other sugars – to the culture medium. To directly determine the number of enzyme molecules in one cell or a small volume containing millions of cells is impossible or at least too difficult to be reasonable. We must therefore *indirectly* measure the amount of enzyme by measuring the amount of enzyme activity in a sample of cells. You must know the chemistry of the enzyme's catalytic function (activity) in order to do that.

An enzyme acts upon a specific molecule or class of molecules that is called its substrate. It chemically alters the substrate in a specific way, producing a new molecule or molecules that are called the product(s). By definition, as a catalyst, each enzyme molecule carries out these steps over and over, turning the substrate molecules into product molecules until the supply of substrate molecules is exhausted. We can take a sample containing enzyme molecules and add to it a supply of substrate molecules that is too large to be exhausted in a short time. Under the proper conditions of temperature, pH, etc., the enzymes will go about making product. We can stop this activity after a short time by changing the conditions (usually drastically). If we then determine how many product molecules are present in the sample we can say something about the number of enzyme molecules in the sample. We are assuming that every enzyme molecule present turns over the same number of substrate molecules in a given time. In this case, the action of the enzyme on its 'natural' substrate is illustrated in *Figure 1*.

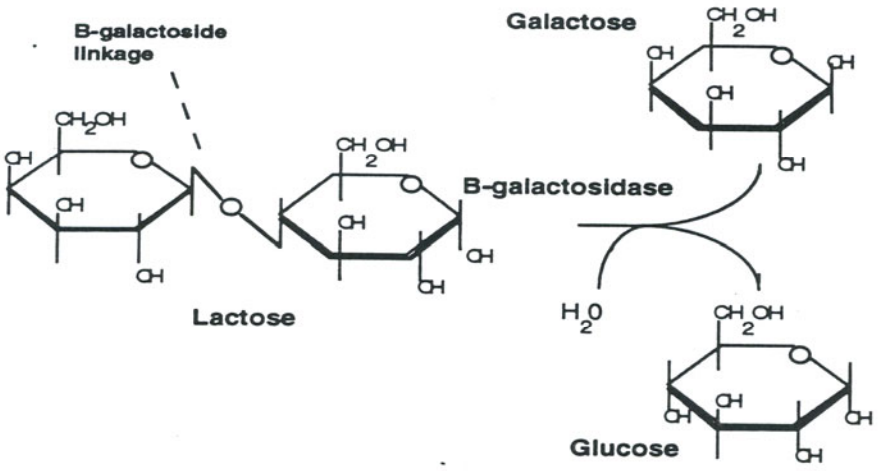


Figure 1: β-galactosidase cleavage of lactose to produce galactose and glucose.

In order to conveniently measure the number of product molecules generated in the test tube, we provide a substrate that is not the normal substrate of the enzyme, but one that the enzyme will still accept and act on. It contains a chemical group that will be part of the product and which is easy for us to quantitate. The action of the enzyme on ortho-nitrophenylgalactoside (ONPG), the artificial substrate is illustrated in *Figure 2*.

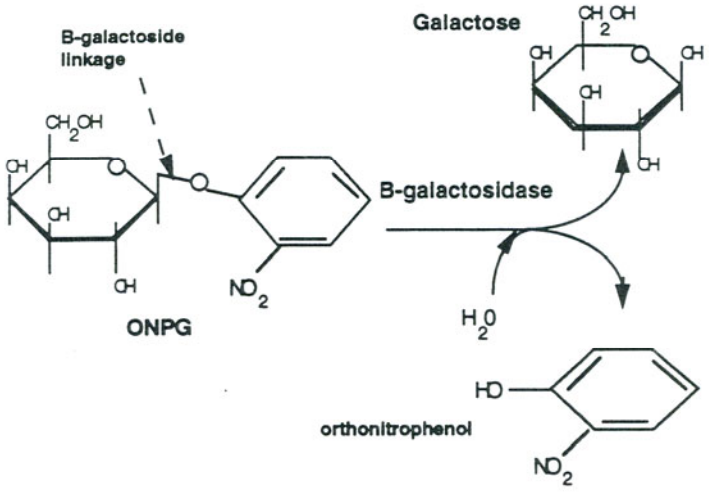


Figure 2: β-galactosidase cleavage of ONPG to produce galactose and orthonitrophenol.

This activity is similar to the activity of the enzyme on its natural substrate. It appears that the enzyme recognizes and is very specific for the galactose portion and the beta configuration between galactose

and the unit bonded to it. However, the enzyme does not seem to be very particular about the size and shape of the molecule bonded to galactose.

Ortho-nitrophenyl esters, such as ONPG, are much beloved by enzymologists because they are typically colorless until hydrolyzed by a specific enzyme; the o-nitrophenol liberated by hydrolysis has an intense yellow color that is easily measured spectrophotometrically. Transport of small molecules into the bacterium is normally under the cell's active control and molecules in the culture media are not available to the intercellular enzymes unless they are specifically transported. Therefore, to bring the substrate o-nitrophenylgalactoside (ONPG) into contact with the β-galactosidase enzyme you will add SDS to the samples of the culture that you remove at regular intervals. SDS kills the cells and disrupts their membranes sufficiently such that they become permeable to ONPG.

You can see that just a "simple" assay demonstrates that we know quite a lot about the enzyme and its tolerance for variation in its substrate, and we are able to synthesize a number of exotic substrates. This particular enzyme is especially well known and has some very distinct characteristics that will make us able to examine its activity in the *E. coli* cells.

Materials List

Two groups of students will share:

Na_2CO_3 (1 M), 40-mL bottle
Z buffer, 90-mL bottle
0.1% SDS (sodium dodecyl sulfate), in dropper bottle
chloroform, in dropper bottle
ONPG (o-nitrophenyl galactoside, 4 mg/mL), 25 mL in tube
vortex mixer
Klett spectrophotometer
Spectrophotometer
one extra side-arm flask (for blank)
culture medium (tryptone broth) for spectrophotometric blanks

Also needed by each group:

2 flasks of bacterial culture (approximately 15 mL), designated A, B
rack of 14 test tubes, 18 x 150 mm, for time points
one rack of 30 test tubes for enzyme assay
14 tubes with 9.0 mL sterile dilution fluid
test tube rack, coated for the water bath
pipets, 1.0 and 10.0-mL and Pi-pump pipettor
ice-water slurry in a tub
timer

Supplied by the instructor:

5% lactose, 5% glucose, 5% galactose
shaking water bath, 37 °C
bench top water bath, 28 °C

General Instructions

The Experimental Cultures:

Each group will have two flasks with 15 mL of *E. coli* growing in tryptone broth. These cultures will be labeled A and B, respectively. Lactose will be added to culture A at 15 minutes, followed by the addition of glucose at 30 minutes. Each group of students must choose whether to add glucose, lactose or galactose to culture B. Define what question you are asking that can be answered by this experimental control.

The Klett Spectrophotometer:

The cultures are growing in special side-arm flasks that can be used as simple cuvettes for reading the turbidity of the samples in the Klett-Summerson colorimeter. To read the turbidity of the culture simply tip the flask's contents into the side arm and insert the side arm into the colorimeter light-path, as shown in *Figure 3* below.

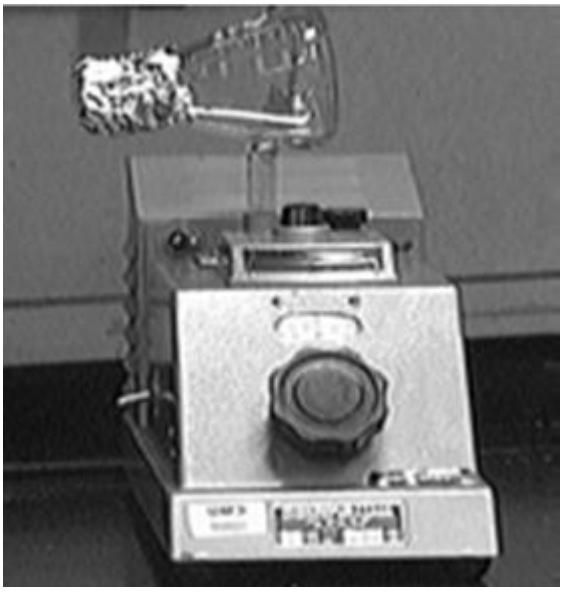


Figure 3: The Klett-Summerson Colorimeter with Side-Arm Flask inserted.

Protocol

Before You Begin

To be ready for sampling, put 14 test tubes (18 x 150mm) in an appropriate rack; you will need 14 tubes for samples of the cultures. Chill these in the tub of ice and water and keep them chilled even

after you have added the culture samples to them. Chill a rack of 30 more large tubes (18 x 150mm) and the bottle of Z buffer.

Obtaining Turbidity Readings and Samples

*Monitoring Growth**:***

To begin the experiment, read the turbidity of each of the cultures in the Klett and record the values observed. You will repeat this measurement every 15 minutes. The first reading established t = 0, the start of the experimental timing. Plot these turbidity measurements as their log (sometimes called O.D., optical density) against time on semi-log paper or on the computer-graphing program.

Samples for the Assay of β-galactosidase Activity:

Each time you make a turbidity reading, also remove 1.5 mL from each flask and place it in its own tube (ice cold). The first samples are the **t = 0** (time = zero) samples for the enzyme assay. Write the time and sample designation directly on each tube with indelible lab marker. Mix the contents of the tubes on the vortex and return them to the ice bath. **Be sure to return the flasks to the shaking bath as quickly as possible - bacteria do not grow well unless they are warm and have an ample supply of oxygen.**

Further Samples - Continuing the Experiment:

After the cultures have been growing for 15 minutes, remove them from the shaking water bath and again measure the turbidity of each using the Klett spectrophotometer. As before, remove samples from both flasks and place each in its own tube. These are designated **t=15**. **On your way back to the shaking bath, have the instructor add 0.2 mL of 2% lactose to culture flask A and 0.2 mL of either 5% galactose, glucose or lactose to flask B (your choice)**.

After an additional 15 minutes (a total of 30 minutes incubation), remove another sample from each culture (label them t=30), and read the turbidities of the cultures as you did before. Just before you return them to the water bath, **have the instructor add 0.2 mL of 2% glucose to culture A only.** Take similar assay samples from culture A and B at 15-minute intervals - at 45, 60, 75 and 90 minutes, total of 14 samples (7 time points). Transfer each sample to a separate tube in the ice bath. Continue to read the turbidities of the cultures at the same intervals.

Correlation of viable count to culture turbidity:

When your turbidity readings show that the cultures are growing steadily, remove a sample of culture B and carry out serial dilutions so that you can spread plate 0.1 mL of the 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions on nutrient plates. Repeat this at some later time during the experiment when the culture has

undergone several doublings. Incubate the plates at 37 °C. You must, of course, record the turbidity reading of the culture at the time the samples were removed.

Assay for Enzyme Activity

An enzyme assay measures the amount of enzyme activity present. Since this is a kinetic assay, not a physical measurement, there are a number of important parameters that must be met; these are outlined as follows:

Criteria for an Assay

- Provide an acceptable substrate for the enzyme.
- The enzyme and substrate must be freely accessible to each other.
- The amount of substrate must be sufficient to saturate the enzyme.
- Quantitate the product produced (or substrate consumed) in a given time under specific conditions.

When you have taken the first 3-4 samples from each flask you should begin to carry out the enzyme assay on those samples while you continue to take further samples and read the turbidities of the cultures.

Preparing the Samples for the Enzyme Assay

NOTE: For the enzyme assay, use the second rack of 30 tubes--14 time points, each one will be diluted 1/10 and 1/2 for a total of 28 tubes, plus two control tubes. Use the larger tubes (18 x 150mm) and label them appropriately.

Control Samples:

Label one tube 1:1 (1/2) control. Add 1.0 mL of tryptone broth and 1.0 mL of Z buffer to this tube. Label the other control tube 1:9 (1/10) control. Add .2 mL of tryptone broth and 1.8 mL of Z buffer to this tube. Carry out all the following steps on these tubes as well as those containing the culture samples. These control samples will be your blank for the spectrophotometer. DO NOT THROW THEM AWAY UNTIL ALL SAMPLES HAVE BEEN READ IN THE SPECTROPHOTOMETER.

Dilution:

The production of o-nitrophenol will be proportional to the amount of enzyme present only if ONPG is in excess. You will assay your samples at two different dilutions to meet these criteria - at a 1:1 (1/2) dilution and at a 1:9 (1/10) dilution. Transfer 1.0 mL of each chilled sample to a tube containing 1.0 mL of Z buffer and also transfer 0.2 mL to a tube containing 1.8 mL of Z buffer.

Rupturing the cells:

Add 1 drop of SDS to each tube of cells (or culture medium) and Z buffer that you are going to assay, and then add two drops of chloroform (in hood, if available). Mix thoroughly on the Vortex for 10 seconds. Place the samples that have been treated with SDS and chloroform in the coated rack in the bench top water bath (28 °C). Allow the samples to stand for 5 minutes and mix again. Let stand 5 minutes longer to equilibrate to bath temperature.

*Adding substrate**:***

Using a 1.0-mL pipet, add 0.4 mL of ONPG (4 mg/mL) to each tube in the water bath. Carefully note the time at which you add the ONPG substrate to the first tube in the series, mix the first tube and then continue down the line at a steady pace. After you mix each tube on the vortex, return it to the water bath. A yellow color will develop within minutes in some of the tubes; others will not develop yellow color.

Stopping the reaction:

Allow the enzyme-ONPG reaction to continue for just 15 minutes. Stop the reaction after 15 minutes by starting to add 1.0 mL of sodium carbonate (Na_2CO_3, 1M) to the first tube in the group in the water bath, mix and continue on down the line as before. Vortex the tubes carefully and don't splash the Na_2CO_3 on yourself or on the bench.

Measuring the amount of product formed:

Measure the absorbance of each assay tube using the spectrophotometer at **both 420 nm and 550 nm** (nanometers). You must use the plastic cuvettes; you need only two - one for your blank and one for each sample in turn. **Zero and blank** the instrument as the instruction sheet describes **at each** of the different wavelengths in turn. Use the control you have prepared as a blank and measure all other samples relative to this.

Pour each sample in turn into a cuvette and insert it into the spectrophotometer. When you have recorded the reading or obtained the printout, pour the sample back into its tube in the rack. Do not

discard any samples until you have all your data, and you are satisfied that you have taken your readings correctly!

Outline of β-galactosidase Assay

- Dilute the samples as appropriate with standard buffer.
- Add 1 drop 0.1% SDS and 2 drops chloroform to each tube. Vortex to mix well.
- Equilibrate at 28 °C.
- Add 0.4 mL 4 mg/mL ONPG to each tube. Mix. Incubate at 28 °C for 15 minutes.
- Add 1.0 mL 1M Na_2CO_3 to each tube and mix.
- Read O.D. at 420 nm and 550 nm.

The Report: Turning Raw Data into Results

The following outlines the tables, figures, calculations and discussion you should submit as your report of this experiment. The material requested comprises a concise presentation of the data, a complete description of the results and a rigorous discussion of its meaning. For this experiment, you do not need to submit an introduction or any other material other than that detailed below.

Data Tables:

Table 2 displays both primary data and values derived from them; it is appropriate to show them together because it allows you to see clearly how they are related and to see if you have made numerical errors. In addition, the whole of the experimental results and the biological events they (hoped to) reveal are fully and logically laid out in this table. Your own insight will come from the relationships that can be seen in this table.

Table 1: For Culture that is Assayed for Viable Count

Culture/Time (A_0, B_{15}, etc)	Turbidity Plate Count of Dilutions				Viable Count
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	

a. 0.1 mL of dilution was spread plated

b. Plate count used for determination is circled. Calculations shown in .....

Transfer your data for plate counts and related data to *Table.1*. Note that explanatory footnotes have been provided; expand these and add others as necessary. Provide an informative title.
Transfer your spectrophotometric readings from the colorimetric assay of ONPG to *Table 2* and include the information about sampling and turbidity as noted in columns 1–3. Decide, for each sampling time, whether the data from the more or less dilute sample is the "best". As noted in the foot, those data chosen should be circled and used in further calculations. Explain your criteria in the section on calculations. Note in the foot where the formulas and sample calculations can be found. When you have completed the calculations, put the calculated values into the table.

Table 2: _____

Culture Sampled	Elapsed Time (min)	Turbidity (Klett O.D.)	Est'd Cell Count[a] (cells/mL)	Assay of ONPG @ 1/10		Assay of ONPG @ 1/2		Enzyme Activity[b]	Specific Activity
				Ab_{420}	Ab_{550}	Ab_{420}	Ab_{550}		

a. Estimated from turbidity as shown in...

b. Data used for calculations is circled and corrected for scattering as shown in...

c. Formula and sample calculations:Calculations: Formulas and Examples

Determination of Viable Count from Plate Counts:

Show a formula that relates the number of colonies on a plate to the number of cells per unit volume (mL) in the culture plated. Provide one (only) sample of a calculation made using this formula and your own data. Explain as necessary and cross-reference to *Table 1*.

Estimation of Viable Count from Turbidity:

Use the data in *Table 2* to define a constant that can be used to estimate the number of cells per milliliter in a culture based on the measured turbidity. How should you proceed if you have only one data point? If you have more than one? Briefly outline the assumptions inherent in defining and using the constant. Show your work. This should be referenced in the footnote to *Table 2*. Use this constant to estimate the cell count for all samples assayed and put the values into the table (*Table 2*, column 4). Ask yourself if the values you obtain are reasonable ones.

Determination of Enzyme Activity:

Values of enzyme activity are determined from the absorbance of the ONP produced upon enzymatic cleavage of ONPG. However, since the cells in the assay scatter some of the incident light, the reading on the spectrophotometer at 420 nanometers includes the light lost by scattering as well as by absorbance. The effect of scattering alone can be determined at another wavelength (550 nm) from which the contribution at 420 nm can be estimated and a correction made. The corrected value for activity is expressed in terms of a constant denominator of one milliliter of original culture. The standard correction per unit volume for *E. coli* cells is:

$$\text{Enzyme activity/mL} = [Ab_{420nm} - 1.75\ (Ab_{550nm})]/\ v$$

v = volume (mL) of culture assayed

This formula is related to that used to express β-galactosidase activity in standard units (Miller, J.H., 1972). Provide one appropriate example of this calculation, using your data. Complete (but do not show) this calculation for each sample and add the values derived by calculation to *Table 2*.

You may find that you have negative values for enzyme activity. Does negative enzyme activity have any biological meaning? Include a brief discussion of what these negative values mean, what assumption has been made that has resulted in negative values. Discuss how, and whether, these negative values should be corrected or changed, and to what value they should be changed.

Determination of Specific Activity:

Describe your method for determining specific activity (activity/cell) from the corrected activity values. Show a formula and sample calculation. Briefly discuss why this value, rather than enzyme activity/mL, is used to define the increase in enzyme activity resulting from induction.
Values for specific activity will be difficult to compare to one another because they include negative exponents. Multiply all values by a single factor, which converts them to easy to understand decimals and integers (i.e. between 0.1 and 100). **Note the factor you have used in the column heading of *Table 2*.**

Figures:

Bacterial Growth:

Show the turbidities of both cultures A and B (*Table 1*) on a logarithmic scale (ordinate) versus time, the independent variable, on the abscissa. Provide labels, a key, and an informative title that is not simply a restatement of the labels of the two axes.

Enzyme Induction:

Plot the specific activities of culture A and B from *Table 2* versus time as *Figure 2*. On the same figure plot the turbidity of culture A versus time (as in *Figure 1*). This presents a problem; bacterial growth is a geometric function of time but specific activity is a linear function. Consider how to combine these data in the same figure and still represent them both properly and clearly. Remember that the curves should fill the figure, even if they are on different scales - a challenge!

Results

1.) Discuss the shape of the curves showing growth of the cultures. Is steady state growth apparent? What aspects of the curve define steady state? Why is it important that you know that growth is steady throughout the experiment even though you may not need values from the curves?

2.) Determine the generation time (minutes/generation) of cultures A and B from *Figure 1*. Describe briefly how this is determined and **indicate the method on the figure itself**. Discuss briefly any comparisons or relationships suggested by these data. Ask yourself if the values are reasonable.

3.) Describe the **general trends** in the growth data and the enzyme production shown in *Tables 1*, *2* and *Figure 1* and *2*. A few sentences should do. NOW NOTE any problems with the data—anything inconsistent? missing? Unexpected? Be specific about where to look - what column, or line in the table, which curve, at what time in the Figure. DONT JUST SAY THE growth increases or decreases, give specifics. Note any anomalies and explain them if you can.

4.) Explain the criteria you have used to choose the "best" of the colorimetric data in *Table 2*. YOU SHOULD NOT DO ALL CALCULATIONS WITH BOTH SETS OF DATA. PICK ONE DILUTION FOR EACH TIME POINT. Give specific examples from the data itself to illustrate your points.

Conclusions and Discussion

1.) In a few sentences describe and compare the changes in enzyme activity (per mL) and specific activity (activity/cell) observed for cultures A and B. Explain why specific activity is a better monitor of cellular events.

2.) The data shown in *Table 2* and *Figure 2* for culture A should demonstrate the strain's response to lactose; does your data shows that the strain is wild type (what <u>is</u> the wild type response)? constitutive? Lac^-? Indicate what data demonstrates this, and how it supports that conclusion. Describe what the data would look like if the strain were constitutive or Lac^-. Cite the data very specifically.

3.) Describe the **molecular** events at the *lac* operon that follow upon the addition of lactose to the culture media. Discuss how these events explain the changes in enzyme activity you observed. You will need review the structure and function of the *lac* operon. (Note: this question asks about molecular events, and does not ask about your data) NO DATA SHOULD BE CITED IN THIS QUESTION.

4.) What effect has the addition of glucose had on the specific activity of culture A? Did you expect activity to level off, drop or increase? What molecular events explain this response? Discuss.

5.) The data for culture B should demonstrate...what? In other words: what question was asked, and can be answered by the results for culture B? You must collect data for culture B from other groups in the class to have a full set of appropriate controls. Plan early. Discuss each one and make general conclusions.

Other Questions and Speculations

1.) Predict the change in specific activity of A after the addition of glucose if:
   a.) β-galactosidase molecules are degraded very slowly or
   b.) β-galactosidase molecules are degraded (turned over) very rapidly.

   How would the rate of degradation of the messenger RNA from the *lac* operon affect the data? Explain.

2.) Specific activity can be expressed in a variety of terms. For example:
   mmoles ONP/[min] x 1/[mg total cellular protein] = specific activity

What additional constants or data would we have to have in order to convert our data for Ab_{420nm} into this form of specific activity? Explain.

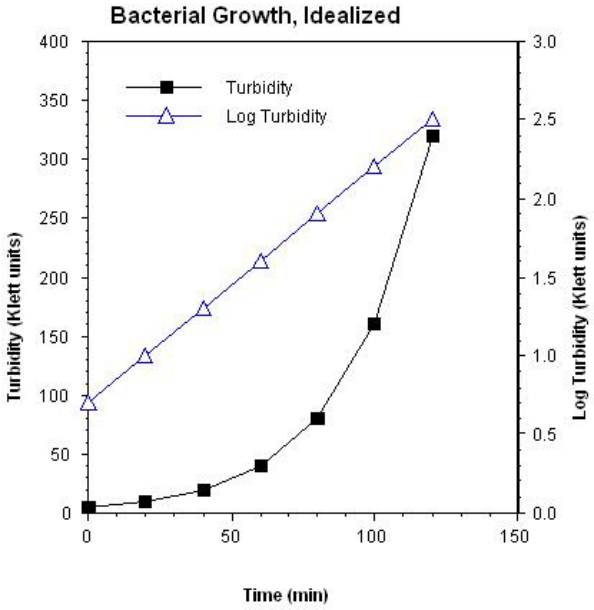
3.) Isoproylthiogalactoside (IPTG) is an artificial inducer of the *lac* operon, and is used in labs to induce the *lac* operon.
   a.) What is the structure of IPTG?
   b.) What would your specific activity data for culture B look like if you added IPTG at 15 minutes for your culture B? Indicate on *Figure 2.2* the specific activity for IPTG used in B.
   c.) Explain your reasoning for the graph in b).

4.) Are you really convinced that the results of your class exercise demonstrate that at least one gene is expressed *in vivo* when the cells are exposed to lactose? What data provides convincing evidence? What additional information would you like to have to demonstrate convincingly that lactose induces the expression of the *lac* operon?

5.) Do the results you have obtained clarify whether gene expression is regulated at the level of the transcription or at the level of translation? That is, could there be many β-galactosidase enzyme molecules in the cell all the time, but they are just not active unless lactose is present? Are additional experiments needed to clarify that point? What experiments should be carried out?

Hints and Help from the Instructors

Below is a graph showing the same data plotted in two different ways. Both are derived from the turbidimetric values for a culture growing steadily, and showing a constant growth rate. What change would you observe in each of these curves if the growth rate had abruptly increased at 40 minutes? at 90 minutes? Can you see why it is easier to evaluate the steady state of the culture from a plot of the log of turbidity? Can you determine the generation time of this culture? Which curve should you use? What simple assumption must you make to use the plot to determine the generation time?



Notes for the instructor: A 15.0 ml overnight starter culture of the B/r strain is set up the night before the experiment. On the morning of the experiment, inoculate 1.0 ml of the starter culture into 100 ml of lambda broth. When the Klett spectrophotometer reading is 20 Klett units, distribute 15 ml of culture into the flasks immediately before the students are about to begin. Cultures standing at the bench may not begin growing immediately, and exhibit lag phase growth. Add the sugars at the fifteen - minute time points only if the students have graphed the growth and have shown that the culture is in log phase growth. During the fifteen - minute intervals between sampling the cultures, students should be graphing the growth of the culture, to assess the growth phases. The control culture is designed by the student groups. It is a good idea to have many different controls so that the class can share the data. The control can also have two sugars, for example, two additions of lactose at fifteen and thirty minutes. After one hour, the student groups can separate the tasks. One partner can continue taking samples and culture readings, and the other can begin the enzyme assay. The chloroform should be added with a glass dropper, and done in the hood, one drop is all that is necessary to rupture cells. The final enzyme assay can be read in 4.0 ml disposable plastic cuvettes in any spectrophotometer at a wavelength of 420 nm.

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APPENDICES

APPENDIX A: Recipes for Solutions

1M Na_2CO_3				
Ingredients	**100ml**	**250ml**	**500ml**	**1000ml**
Na_2CO_3	10.6g	26.5g	53.0g	106g
H_2O	to 100ml	to 250ml	to 500ml	to 1000ml

Shelf life: ~2 weeks (if it precipitates, it can be heated to reconstitute)
Storage: RT, do not refrigerate

Z buffer				
Ingredients	**Final conc.**		**500ml**	**1000ml**
$Na_2HPO_4 \bullet 7H_2O$ **OR Na_2HPO_4 (anhy)**	0.06M		8.04g **4.26g**	16.08g **8.52g**
$NaH_2PO_4 \bullet H_2O$ **OR NaH_2PO_4 (anhy)**	0.04M		2.76g **2.71g**	5.52g **5.42g**
KCl	0.01M		0.37g	0.75g
$MgSO_4 \bullet 7H_2O$	0.001M		0.12g	0.25g
$MnSO_4 \bullet H_2O$	0.0001M		8.5mg	17mg
β-Mercaptoethanol	0.05M		1.75ml of 14.3M	3.5ml of 14.3M
H_2O			up to 500ml	up to 1000ml
Adjust pH to 7.0 before all of the water is added. This recipe is usually 7.0 without adjusting. No need to sterilize				

Shelf life: ~1 month
Storage: Refrigerate

0.1% SDS (Sodium Dodecyl Sulfate)				
Ingredients	**10ml**	**25ml**	**50ml**	**100ml**
SDS	0.1g	0.25g	0.5g	1.0g
H_2O	10ml	25ml	50ml	100ml

Shelf life: ~1 month
Storage: RT, do not refrigerate

ONPG, 4mg/ml (Sigma N-1127: 2-Nitrophenyl β-D-Galactopyranoside)				
Ingredients	**100ml**	**250ml**	**500ml**	
ONPG	400mg	1g	2g	
H_2O	100ml	250ml	500ml	

Shelf life: ~1-2 days (must be made fresh)
Storage: Refrigerate

5% Glucose, 2% Glucose, 5% Lactose , 2% Lactose, 5% Galactose
Dilute stock solution to corresponding %

Shelf life: ~1 month
Storage: RT

Lambda Broth				
Ingredients	**500ml**	**1000ml**	**2000ml**	**4000ml**
Tryptone	5g	10g	20g	30g
Sodium chloride	2.5g	**5g**	**10g**	**15g**
1N NaOH	0.575ml	1.15ml	2.3ml	3.45ml
H_2O	500ml	1000ml	2000ml	4000ml
Combine reagents in flask and solubilize. Dispense into bottles or other vessels and autoclave. Larger quantities (>1 liter) should be autoclaved 40-45 minutes. Small quantities should be autoclaved 20-25 minutes.				

Shelf life: ~1 month (assuming it will not get contaminated)
Storage: RT

Appendix B: Introduction Student Outline (High School)

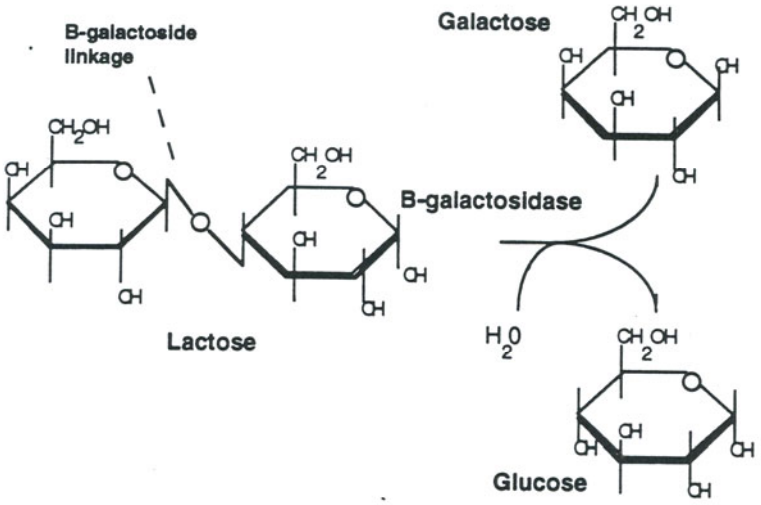
During the course of development of a fertilized egg (a single cell) into a mature animal (which can contain millions of cells) large numbers of genes are being turned on and off. The events, which occur during development—called differentiation—, result in an organism that has an amazing variety of different cell types. Consider for a moment the vastly different properties of the cells found in skin, the eyes, the lungs, the heart, or the digestive tract. These cells clearly differ both in their appearance (structure or morphology) and in the types of cell chemistry they perform (physiology). What is perhaps not so obvious is that the nucleus of each of these vastly different cell types contains exactly the same content of genetic information (DNA). In fact, each of these cell types contains all of the genetic information found in the original fertilized egg from which they were derived. Scientists have found that development, and the process of cell differentiation, depends not on the stepwise removal or loss of genes from calls as they become more and more specialized, but rather by selectively turning on and off various groups of genes, all of which are still resent in every cell of the mature organism

In order to study such gene action, scientists often try to make use of simpler (model) systems that are easier to manipulate in the laboratory. The common (and benign) bacterium *Escherichia coli*, isolated from the human gut, has proved to be especially useful in this regard. In fact, scientists today know more about *E. coli* than about any other living organism, and mainly because of the type of experiment we will be performing today.

E. coli is a versatile microorganism able to grow on a wide variety of carbon and energy sources. This is perhaps not too surprising since the bacterium lives as a scavenger of nutrients in the digestive tract of a number of mammals, including humans. There is however a disadvantage inherent in uncontrolled evolution toward metabolic versatility—it would be wasteful of resources for *E. coli* to manufacture (at a high energy expense) the enzymes needed to metabolize an energy source when that energy source is not presently available in the environment. *E. coli* has solved this dilemma by acquiring the genetic information (DNA) needed to utilize a wide variety of energy sources, while in most cases preventing the expression of that genetic information (in the form of enzymes needed to metabolize an energy source) unless that particular energy source is present in the environment

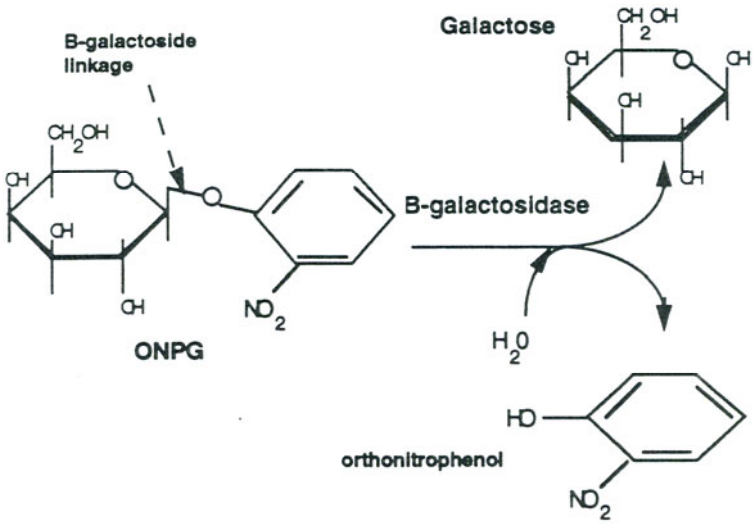
As our example of how genes can be turned on and off is bacteria (and presumably in other living cells) we will first determine that in the absence of milk sugar—lactose—*E. coli* does not manufacture the enzymes needed by the bacterium to utilize that sugar as an energy source (i.e. the *lac* genes are normally turned OFF). Secondly we will find that upon adding lactose to the growing culture of *E.coli,* the bacterium begins to manufacture the enzymes that are needed for lactose utilization (i.e. *lac* genes are turned ON).

The sugar lactose is a disaccharide—a dimer of two simpler sugars, glucose and galactose. In order to metabolize lactose, *E. coli* must hydrolytically cleave the lactose dimer into its two monomers, glucose and galactose (see figure, below).



The glucose can be directly used by the bacterium's metabolic machinery to generate energy. In order of the galactose monomer to be utilized however, the cell first rearranges the stereo-chemistry (3-D pattern) of one of its carbon atoms to convert galactose to glucose. (This step requires additional cell enzymes.) The galactose, now converted to glucose, is then also used for energy production

In order to follow the turning on and off of the genes needed for lactose utilization, we will assay cells for their content of B-galactosidase, the enzyme that catalyzes the cleavage of lactose into glucose plus galactose (see Figure, above). Since neither galactose nor glucose is quick and easy to assay, we will substitute for lactose in the assay a chromogenic substrate analog, orthonitrophenyl-b-galactoside (ONPG; See Figure, below).



After B-galactosidase claves the colorless compound ONPG, one of the products orthonitrophenol, is yellow in color. By measuring the amount of yellow color that is produced by extracts of cells grown under various environmental conditions, we will be able to quantitate the amount of b-galactosidase that they contain.

MEASURING BACTERIAL GROWTH

During this experiment we will be using cultures of bacteria that will be growing rapidly. We need to monitor growth quickly in a way that does not destroy the cells and which gives immediate results. One way to do that is to measure how much a beam of light is scattered by the bacterial cells. As the cell numbers increase, more and more of the light beam will be scattered by the increasing number of cells.

You will place the tube of the bacterial cells in culture fluid in the light beam in a Klett spectrophotometer. With this instrument you can quantitate how much light is dispersed or scattered by the cells. Within a reasonable range the amount of scattering is proportional to the number of cells present.

To make it even easier to make the measurements, our flasks have an appropriate tube fused to the flask. Simply tilt he flask to allow the culture fluid to flow into the tube and the tube may be inserted into the Klett spectrophotometer.

Follow the directions for the Klett that are in the lab bench next to the instrument. Have one of your partners or the lab instructors confirm your readings until you are sure that you understand how to use this instrument.

Student Protocol

Materials

Na_2NO_3 20ml bottle (1 Molar)
ONPG (o-nitro-phenyl galactoside) 10ml tube (4 mg/ml)
Z buffer 40 ml bottle
E. coli; two cultures labeled A and B
15 culture tubes 13 x 100 mm
Test tube rack, coated for use in water bath
Pipettes, 1.0ml and 10.0 ml
Pipettor
Timer
Ice bath
Desktop water bath, 20oC
0.1% SDS, in dropper bottle; chloroform; in dropper bottle
Spec 20 spectrophotometer, and 2 tubes (B & L)

Klett spectrophotometer
Shaking water bath with clamps, 37°C

Cultures

Your group will be supplied with a culture of *E. coli* growing in sterilized tryptone broth that is made of digested proteins, NaCl and water. As the experiment begins the culture will be divided into parts in separate flasks.

Flask A contains *E. coli* in tryptone broth – no supplements will be added.

Flask B contains *E. coli* in tryptone broth to which lactose sugar will be added (final concentration is 0.05%)

Protocol

1. Read the turbidity (scattering) of each culture (A, and B) on the Klett spectrophotometer. Record your results in the table provided.
2. Remove a 0.2ml sample from both of the flasks. These samples are for the assay for b-galactosidase activity. Add each of these samples to an appropriately labeled tube containing 2.0ml of ice-cold Z buffer. Mix the contents and return it to the ice bath. These samples are called A0 and B0.
3. Place the two flasks in the water bath in the clamps. Set the shaking speed for #5
4. After the first 15 minutes your instructor will add lactose to Flask B. Be sure to let the instructor know that your cultures are ready for these additions.
5. Read the turbidity of the two cultures approximately every 15 minutes. Be sure to record the turbidity in the appropriate place in the table. Record the time tat the measurements are taken.
6. Remove 0.2ml samples from the cultures as outlined below.
   Flasks A and B: samples at 15, 30, 45 and 60 minutes
7. These samples will be used for the assay of b-galactosidase activity. Each sample is placed in an appropriately labeled test tube containing 2.0ml of ice-cold Z buffer. Mix the contents and return the tube to the ice bath.

Preparation of Samples for Enzyme Assay

1. Each tube of Z buffer and cells must be treated with SDS (sodium dodecyl sulfate) and chloroform to break open the cells so that the enzyme from inside of the cells can act upon the substrate ONPG that we will be adding. Add 1 drop of SDS from the dropper bottle to each tube then add 2 drops of chloroform from the other dropper bottle in the hood. Mix thoroughly on the vortex for 10 seconds
2. When all the samples have been taken and treated with SDS and chloroform, place the rack of test tubes into the desktop water bath (20°C). Allow the samples to stand for 5 minutes.

Enzyme Assay

1. Using a 1.0 ml pipette at 0.4 ml of ONPG (4 mg/ml) to each tube in the desktop water bath. Note the time of addition carefully, since this part of the experiment is timed. Mix the tube on the vortex and return it to the water bath. A yellow color will develop within minutes in some tubes.
2. After 15 minutes the enzyme reaction is stopped by the addition of 1.0 ml of Na_2CO_3 (1 Molar).
3. In order to estimate the amount of the o-nitrophenol released by the enzyme we measure the amount of light absorbed by the yellow o-nitrophenol. We use a spectrophotometer that can read at a wavelength of 420 nm.

4. Measure the absorbance of each sample when the wavelength selector is set to 420nm. Record your result in the table. Your instructor will help you "zero" and Blank" the Spectrophotometer.

Determination of B-Galactosidase Activity

By straightforward inspection of your data you should be able to say which samples show that the B-Galactosidase enzyme was present in the cells. However, samples taken at later times have more cells than samples taken earlier and so they have more enzyme. In order to make valid comparisons, we need to have the data in the form of specific activity, that is, activity per standard amount of bacteria. We can do that by dividing the "crude" activity that you have measured in the spectrophotometer at 420, by the reading that you have obtained from the Klett spectrophotometer. The Klett reading, you will remember, is proportional to the number of bacterial cells present at that time.

$$\text{Specific Activity} = \frac{\text{Spectrophotometer reading at 420 for that sample}}{\text{Klett reading for that sample}}$$

Calculate the specific activity for each sample and enter your results in the table provided.

Data Table

Culture	Time (min)	Turbidity (Klett Units)	Activity (ΔAb420)	Specific Act x 10^{-3}
A				
B				

Notes for the instructor: A 15.0 ml overnight starter culture of the B/r strain is set up the night before the experiment. On the morning of the experiment, inoculate 1.0 ml of the starter culture into 100 ml of lambda broth. When the Klett spectrophotometer reading is 20 Klett units, distribute 15 ml of culture into the flasks immediately before the students are about to begin. Cultures standing at the bench may not begin growing immediately, and exhibit lag phase growth. Add the sugars at the fifteen - minute time points only if the students have graphed the growth and have shown that the culture is in log phase growth. During the fifteen - minute intervals between sampling the cultures, students should be graphing the growth of the culture, to assess the growth phases. The student groups design the control culture. It is a good idea to have many different controls so that the class can share the data. The control can also have two sugars, for example, two additions of lactose at fifteen and thirty minutes. After one hour, the student groups can separate the tasks. One partner can continue taking samples and culture readings, and the other can begin the enzyme assay. The chloroform should be added with a glass dropper, and done in the hood, one drop is all that is necessary to rupture cells. The final enzyme assay can be read in 4.0 ml disposable plastic cuvettes in any spectrophotometer at a wavelength of 420 nm.

Literature Cited

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Appendix C: Student Outline (Freshman)

Introduction

The definition of the gene has changed over the years. The gene was first defined in genetic terms - as a unit of inheritance. What a gene actually was chemically, and how such a unit brought about observable characteristics (phenotype) was not understood. It was not until biochemists began to unravel some of the complex series of chemical reactions carried out by cells that a new definition of the gene was possible. Using extracts of cell cytoplasm, biochemists began to understand that the cell makes all the molecules it needs from simple components by a series of understandable steps. For example, all the 20 or more amino acids necessary for making proteins are each separately synthesized by a series of chemical reactions from other small molecules present in the cell.

At about the same time, enzymes had been discovered and their function as catalysts of chemical reactions was understood. It seemed obvious that enzymes must be participants in some or all of the steps in these complex series of chemical reactions.

The notion that the separate concepts of genes and enzymes were related was first proposed by Garrod based on his studies of humans suffering from the heritable diseases alkaptonuria and phenylketonuria. He found that these diseases resulted from the absence of a necessary enzyme, and that this flaw, like other human characteristics, followed Mendelian inheritance. Other researchers worked to unravel the relationships between the genetics and biochemistry of insect eye pigments. It became obvious, however, that investigation of the relationship between mutations and individual chemical steps would be easier if one could work with an organism in which there was a simple direct way to observe the biochemical deficiencies resulting from mutations. An organism with only one set of chromosomes (haploid) would be a definite advantage; any new mutation, whether recessive or dominant (mutations are usually recessive) would be immediately evident because no second functional copy of the gene could complement the deficiency the resulted from mutation. It would also be desirable to have some simple means to look directly for biochemical or nutritional deficiencies rather than observing indirect effects like changes in the structure or color of body parts.

These criteria led to the use of single celled microorganisms such as bacteria, yeast and fungi as the research organisms of choice. These organisms are exclusively haploid, or at least haploid during a portion of their life cycle. In addition, they are easy to culture in the laboratory and can grow and divide when supplied with only inorganic salts and a few other very simple molecules. From this we know that these organisms must be able to synthesize all or most complex biological molecules from their constituent atoms. We must suppose therefore, that all the chemical reactions necessary to synthesize biological molecules are going on inside each cell - all the time.

Out of a combination of genetic and biochemical studies of these organisms, first with the fungus *Neurospora crassa* and later with yeast and bacteria, arose the concept that the gene is the unit which specifies a single enzyme.

It was already known that not all enzymes are produced all the time. For example, some bacteria use certain materials from their environment as a source of nitrogen atoms only when their preferred source - ammonia - is absent; the enzymes the would need to utilize alternative nitrogen sources are not present in the cell while ammonia is available. Sinilarly, the bacteria *Escherichia coli* does not use the sugar lactose, even though it is available in its immediate environment, unless it first choice - glucose sugar - is absent. Remember that "using" lactose means that the bacteria takes up lactose from the environment and chemically alters it, so we know that enzymes are involved in "using" lactose. If the cells were not using lactose, the cellular enzymes involved in the utilization of lactose were simply not present in the cell. If you switched the cells to a new set of conditions and you found that lactose was utilized, then you knew that the enzymes for lactose utilization were present. First the enzymes weren't in the cells, then they were; somehow the genes that code for these enzymes can be switched on - and off.

This bring us, and of course the scientists of the time, to the concept of gene regulation. The first real experimental investigations of the process of gene regulation used the bacteria *Escherichia coli* and its response to the presence of the sugar lactose as a research tool. A large number of mutants were isolated which, unlike the wild type strains, could not utilize lactose at all. Other mutants were isolated which were always ready to utilize lactose; that is, the cells were full of the enzymes involved in lactose utilization, even when no lactose was present. These two types of mutants are called Lac^- (minus) and Lac^c (constitutive), respectively. The full genetic analysis of these mutants revealed, in the most elegant manner, the way in which the genes involved in lactose utilization are regulated and provided us with one model of how gene regulation generally works.

Student Outline

We are going to carry out an experiment that shows that the lactose genes of *E. coli* are regulated. Each group of students will be given several cultures of *Escherichia coli*. We will observe the kinetics of their growth as well as their production of b-galactosidase, one of the three enzymes involved in the utilization of lactose. We can manipulate the environment of the bacterial cells to determine what sugars can induce the production of this enzyme. We can also demonstrate that genes which have been turned on can be turned off again by another change in the environment.

How do we know the cells are growing?

In the experiment, we will be trying to stimulate a change in the physiology of the cells and we know that only cells that are actively metabolizing and making all kinds of molecules can respond. The best

indication that our experimental population is in the appropriate condition is evidence that the cells are accumulating mass and increasing in number. Individual bacterial cells increase in size in a linear fashion, but the number of cells in the population as a whole increase not linearly (1 - 2 - 3 - 4 - etc.) but exponentially (l - 2 - 4 - 8 -) because the cells divide by binary fission.

To actually count the number of cells is not too difficult, but counting the number of cells every 10 or 20 minutes is very laborious. Instead we can simply monitor the mass increase associated with the growth of the population of cells, without concern for the absolute number of cells present. Liquid cultures of bacteria appear turbid because the cells scatter light that passes through the media. The more turbid a culture appears, the more cells are present per unit of volume. We can use a spectrophotometer to quantitate the amount of scattered light and, within certain limits, this is directly proportional to the number of cells (or cell mass) per unit volume. We get the necessary information immediately, and almost effortlessly.

Spectrophotometers - the fundamentals

There are many kinds of spectrophotometers; some can be adjusted so that they produce or respond to light of any wavelength from the ultra-violet to the far red. We will use two kinds of spectrophotometers in this experiment, a very simple Klett colorimeter to measure the turbidity of growing cultures, and a somewhat more elegant spectrophotometer to measure the amount of light absorbed by certain molecules. In principle these two, and all spectrophotometers, are the same. They have
a light source that produces a beam that passes through the tube of sample; the intensity of the transmitted light is measured by a phototube and translated into a numerical value by an electronic system.

The numerical value for the intensity of the transmitted light has meaning only when you first adjust the machine to an appropriate zero value. This operation is referred to as “blanking” and the appropriate sample used for this operation is called a “blank”. When, for example, you wish to obtain numerical values for the turbidity or light scattering of bacterial cells in culture media, you would use a sample of sterile culture media with no cells as a blank. With this sample in the light path the machine can be adjusted to “zero” and subsequent readings of the growing culture will be a comparison of media plus cells to media alone. In principle, the appropriate “blank” is a solution identical with the sample except that the particles or molecules which scatter or absorb light are absent.

One other adjustment must be made before using some spectrophotometers. The numerical scale must be adjusted so that when NO light is transmitted to the phototube, the numerical scale is set to read “zero light transmitted” or “null”. This process is called “zeroing” the spectrophotometer. This is not the same as “blanking” the spectrophotometer and is carried out before the blanking operation. No sample is used for the zeroing operation. Instead the phototube is turned off mechanically or the phototube and

the electronic read out system are disconnected by electronic intervention. Simple instructions for zeroing and blanking both the Klett and the Spec 20 spectrophotometers are provided with each instrument. At the appropriate time, practice these operations until you are comfortable with both the operations and the principles involved. Your partners or the instructors can help you.

Side-arm flask - the culture flask with integral spectrophotometer tube

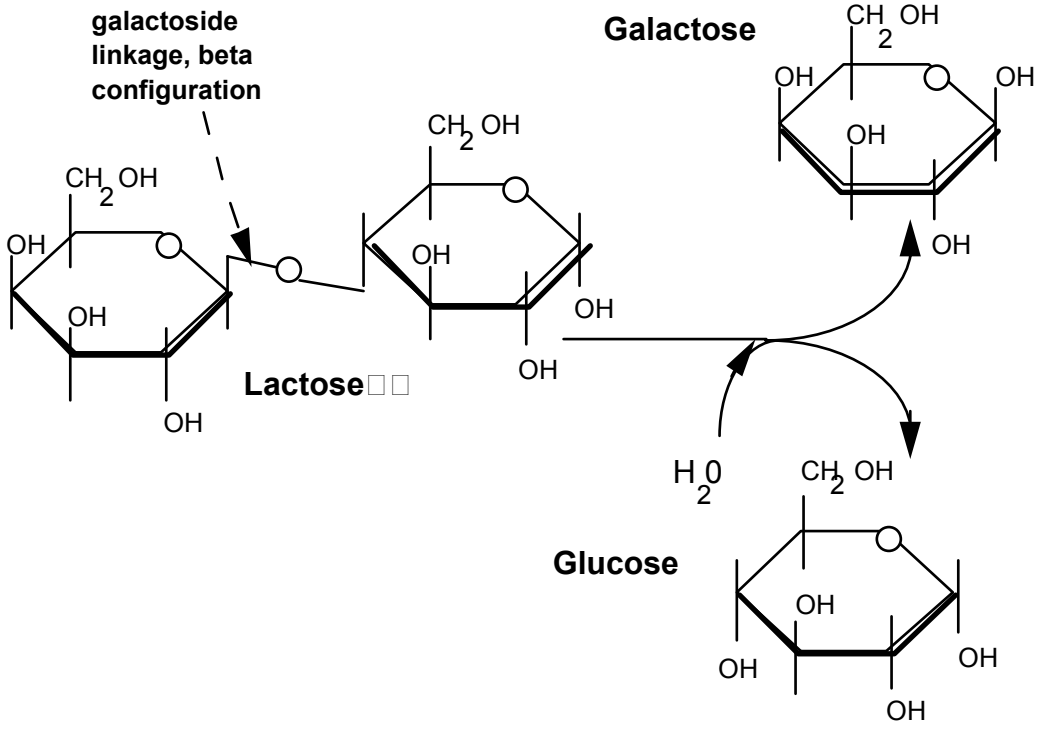
If we must remove a portion of the culture each time we measure its turbidity, we will use up most of the culture and probably contaminate it as well. We can avoid this by using culture flasks to which a spectrophotometric tube has been fused. These side-arm flasks allow you to measure the culture's turbidity by simply tilting the flask to let the culture media and suspended cells fill the tube on the side. The tube can be inserted into the spectrophotometer for a reading. The flask can then be returned to the temperature controlled shaker bath until another reading is required. These readings will indicate the increase in the numbers of cells as the culture grows.

Measuring the amount of enzyme present

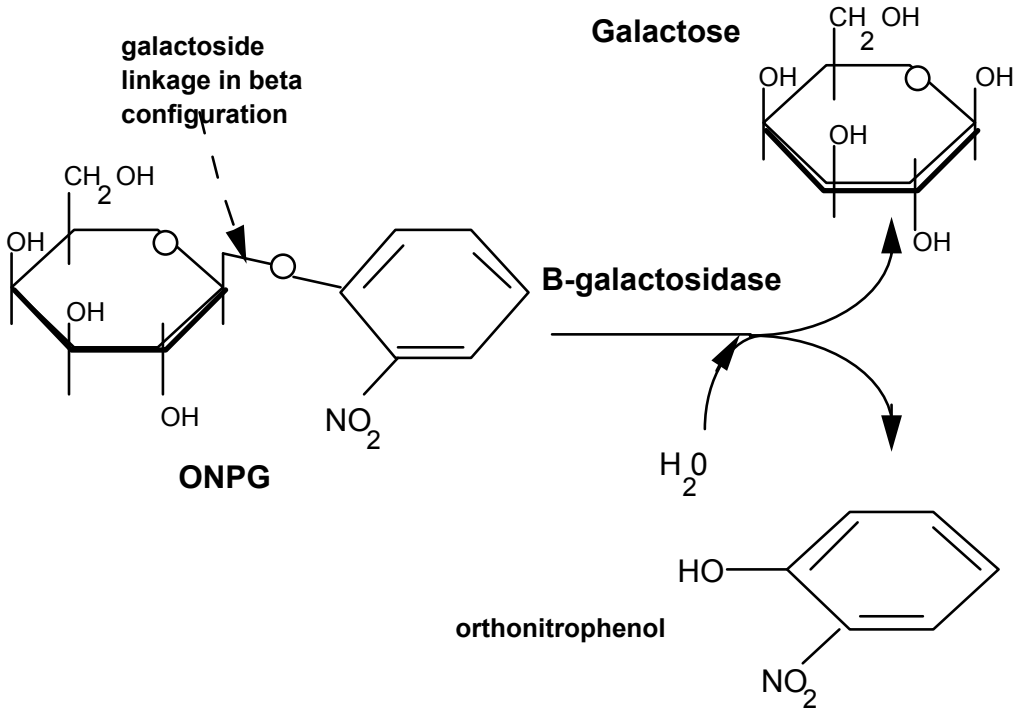
During this experiment we will also quantitate the amount of the enzyme b-galactosidase that is present in the cells before and after the introduction of lactose or other possible inducers to the cultures. To directly determine the number of enzyme molecules in one cell or a small volume containing millions of cells is impossible or at least too difficult to be reasonable. We must therefore **indirectly** measure the amount of enzyme by measuring the amount of enzyme activity in a sample of cells. Obviously, you must know the chemistry of the enzyme's catalytic function (activity) in order to do that.

An enzyme acts upon a specific molecule or class of molecules that is called its substrate. It chemically alters the substrate in a specific way, producing a new molecule or molecules that are called the product(s). By definition as a catalyst, each enzyme molecule carries out these steps over and over, turning the substrate molecules into product molecules until the supply of substrate molecules is exhausted. We can take a sample containing enzyme molecules and add to it a supply of substrate molecules that is too large to be exhausted in a short time. Under the proper conditions of temperature, pH, etc., the enzymes will go about making product. We can stop this activity after a short time by changing the conditions (usually drastically). If we then determine how many product molecules are present in the sample we can say something about the number of enzyme molecules in the sample. We are assuming that every enzyme molecule present turns over the same number of substrate molecules in a given time.

In this case, the action of the enzyme on its 'natural' substrate is:



In order to conveniently measure the number of product molecules generated in the test tube, we provide a substrate that is not the normal substrate of the enzyme, but one that the enzyme will nevertheless accept and act on. It contains a chemical group that will be part of the product and which is easy for us to quantitate. The action of the enzyme on ONPG, the artificial substrate is:



This activity is analogous to the activity of this enzyme on its natural substrate. Note that the enzyme recognizes and is very specific for the galactose portion, and the beta configuration between galactose and the unit bonded to it. However, the enzyme does seem to be very particular about the size and shape of the molecule bonded to galactose.

Ortho-nitrophenyl esters such as ONPG are much beloved by enzymologists because they are typically colorless until hydrolyzed by a specific enzyme; the o-nitrophenol liberated by hydrolysis has an intense yellow color that is easily measured spectrophotometrically.

Transport of small molecules into the bacterium is normally under the cell's active control and molecules in the culture media are not available to the intercellular enzymes unless they are specifically transported. Therefore, to bring the substrate o-nitrophenylgalactoside (ONPG) into contact with the b-galactosidase enzyme you will add toluene to the samples of the culture that you remove at regular intervals. Toluene kills the cells and disrupts their membranes sufficiently that they become permeable to ONPG.

Science builds on prior work

You can see that just a "simple" assay depends on extensive knowledge of bacterial physiology and the specificity of this enzyme. Experiments many years ago examined in detail the enzyme's tolerance for variation in the structure of its substrate. To do that basic research, scientist synthesize a number of exotic galactosides as test substrates, some of which are commonly used today. This particular enzyme has been thoroughly studied and our knowedge allows us to use the enzyme as a tool for molecular and developmental research. It is an unusual enzyme in some respects - it is very large (>150,000 daltons molecular weight) and unusually stable. It functions even when another protein is fused to its tail end, and it can be transported into membrane bound compartments of the cell if properly 'tagged' for transport. Geneticists and molecular biologists are very fond it.

Materials

2 groups will share:

Na_2CO_3 (1 M) 40 ml. bottle
Z buffer 75 ml. bottle
0.1% SDS (sodium dodecyl sulfate) in a dropper bottle
chloroform, in dropper bottle
ONPG (o-nitrophenyl galactoside, 4 mg/ml),15 ml in tube
vortex mixer
Klett spectrophotometer
Spec 20 spectrophotometer and several BL tubes
one extra side-arm flask (for blank)
culture mediuam (tryptone broth) for spectrophotometric blanks

Also needed by each group:
2 flasks of of bacterial culture (approximately 15ml), designated A, B or unknowns X or Y
rack of 25 tubes, 18 x 150 mm.
test tube rack, coated for the water bath
pipets, 1.0 and 10.0 ml and Pi-pump pipetor
ice-water slurry in a tub
timer

Supplied by the instructor:
5% lactose, 5% glucose, 5% galactose
shaking water bath, 37^{o} C.
bench top water bath, 28^{o} C.

Protocol

Starting the Experiment:

To begin the experiment, place your cultures in the shaking water bath and set the shaking speed at #5. Allow 5-10 minutes for thermal equilibration. During that time, carry out the preparations outlined below.

Preparations:

To be ready for the enzyme assay, get 11 test tubes (the larger ones, 18mm x 150mm) and start adding 2.0 ml of Z buffer to each tube. You need not be concerned about sterile technique. You will need 7 tubes for each of the cultures and several to spare. Chill these in the tub of ice and water and keep them chilled even after you have added the culture samples to them.

Learn to use the Klett spectrophotometer as described in the instruction sheet provided. Use sterile tryptone broth as a blank and practice making readings of your cultures. When you have time, read the material in the Introduction about spectrophotometers.

Monitoring Growth and Collecting Samples:

Read the turbidity of each culture and record your results in the Table. Remove an 0.2 ml sample from each flask and place each in its own tube of 2.0 ml Z buffer (ice cold). These are the t = 0 (time = zero) samples for the enzyme assay. Write the time and sample designation directly on each tube with indelible lab marker. I suggest you designate these samples A-0, B-0,X-0 as appropriate, and later samples are A-15, etc. Mix the contents of the tubes on the vortex and return them to the ice bath. Be

sure to return the flasks to the shaking bath as quickly as possible. Bacteria do not grow well unless they are warm and have an ample supply of oxygen.

Designing an Experiment:

You will continue to monitor turbidity and remove samples for the enzyme assay in order to see the effect of these sugars on the growth rate and enzyme activity of **both cultures**. Shortly you will add lactose to culture A and a little later, you will also add glucose. However, if your group has culture B, you will have to decide what additions you want to make to this culture. Think in terms of a question. For example, if you are asking "Will galactose also induce synthesis of b-galactosidase?" Then you should add galactose to culture B at t=15 minutes. If you are asking what effect the addition of glucose has on culture B, then you will design your experiment accordingly.

All groups will have to define whether their bacterial strain's lac phenotype is constitutive or Lac^-. What response would such strains have to the addition of lactose? When should you add lactose? Should you also add glucose? Discuss this with your partner or the instructor and design an appropriate procedure for this culture.

Further Samples:

After the cultures have been growing for 15 minutes, remove a second set of samples from each flask and place each in its own tube of Z buffer. These are designated **t = 15**. **Have the instructor add 0.2 ml of 2% lactose** to culture flasks A and specify what addition should be made to culture B; we have 5% glucose, 5% lactose, and 5% galactose to choose from. Return both flasks to the shaking water bath. (Do not add any sugars to the samples that have been removed and placed in the tubes!!). Request additions to X and Y as per your experimental design.

After a total of 30 minutes have elapsed, remove another sample from each culture, and also read the turbidities of the cultures as you did before. Just before you return them to the water bath, **have the instructor add 0.2 ml of 2% glucose to culture A.** Depending on your own experiment, some additions may also be made to B, X or Y.

Continue to remove samples from both flasks at 15 minute intervals - at 45,and 60 minutes; add each sample to a separate labelled tube containing the ice cold Z buffer.

Make a paper graph of the turbidity readings as you go along or use a graphing program on the lab computers. Use the semilog plot or graph paper so that the values for the cells growth are on the vertical geometric (log) scale and time is on the linear horizontal scale. Make bold

symbols centered on the values of each reading and, when you have all the data points, draw in a smooth curve.

When you have taken the first 3-4 samples from each flask you should begin to carry out the enzyme assay on those samples. Don't forget to continue to remove the additional samples. When these last samples have been collected you can carry out the assay on them as well.

Preparing the Samples for the Enzyme Assay:

Add 0.2 ml of sterile tryptone broth to one of your extra tubes of Z buffer. Mark this C for control. Carry out all the following steps on this tube as well as those containing the culture samples. This control sample will be your blank for the spectrophotometer.

Each tube of cells in Z buffer must be treated with SDS and chloroform to disrupt the cell wall and membrane so that the substrate ONPG can reach the enzyme inside the cells. Treat the control sample the same way.

Add 1 drop of SDS to each tube of Z buffer and cells that you are going to assay, then add two drops of chloroform. Mix thoroughly on the Vortex for 10 seconds. Place the treated samples in the bench top water bath ($28^{O}C$) for 5 minutes.

Timed Enzyme Assay:

After 5 minutes, add 0.4 ml of ONPG (4 mg/ml) to each tube in the water bath (use a 1.0ml pipet). Note the time of each addition carefully, or you may simply note the time at which you add the ONPG substrate to the first tube in the series, then continue down the line in a regular fashion. Mix each tube on the vortex and return it to the water bath. A yellow color will develop within minutes in some of the tubes; others will not develop yellow color.

Allow the enzyme-ONPG reaction that is going on in the tubes in the bath to continue for just 15 minutes. When 15 minutes have elapsed since you added ONPG to the first tube, start adding 1.0ml of sodium carbonate (Na_2CO_3 , 1M) to the first tube in the group in the water bath, and continue on down the line as before. This should ensure that all the tubes have had the same time for reaction.

Caution: Na_2CO_3, IS CAUSTIC.

Vortex each tube carefully, don't splash the Na_2CO_3 on yourself or on the bench.

Measuring the Amount of Product Formed:

> *The enzyme b-galactosidase normally hydrolyzes (cleaves, with the addition of a water molecule) the disaccharide lactose to the two monosaccharides galactose and glucose (see diagrams in the Introduction). It will, however, hydrolyze a bond between galactose and any number of groups other than glucose. We have provided it with galactose bonded to o-nitrophenol (ONPG). When the enzyme cleaves this bond, o-nitrophenol is released. This molecule appears yellow. By measuring the amount of light absorbed by the o-nitrophenol we can estimate the amount of product released by the enzyme. We will not determine the absolute number of o-nitrophenol molecules, since relative values for the o-nitrophenol and, indirectly, the amount of enzyme are sufficient in this experiment.*

Measure the absorbance of each assay tube from the water bath using one of spectrophotometers. For the Milton Roy spectrophotometer, use square plastic cuvettes and read absorbance. Set the wavelength selector to 420 nm (nanometers). Zero and blank the instrument as the specific instruction sheet describes. Use the "blank" you have prepared and measure all other samples relative to this blank. Pour each sample in turn into the tube or cuvette. When you have taken the reading from the meter or digital read-out screen and recorded the value in the data table, pour the sample back into its tube in the rack. Do not discard any samples until you have all you data, and you are satisfied that you have taken your readings correctly! These values are a measure of "crude" activity.

Literature Cited

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