EXPERIMENT 3: GENE REGULATION AND ENZYME ACTIVITY ASSAY

DAY ONE: ENZYME INDUCTION

OBJECTIVES:

Today you will be setting up bacterial cultures to see how an organism's environment can affect gene expression. By the end of today's lab you should know

- **The difference between a regulatory and structural gene.**
- **The different kinds of regulatory genes that interact to control gene expression.**
- **How cells benefit from regulating their gene expression.**
- **The differences among enzymes, substrates, and products.**

INTRODUCTION:

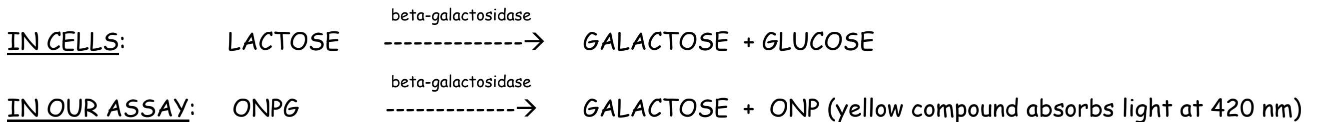
Although each single cell in a Moneran or Protistan population and each body cell in a multicellular organism carries DNA with all the information for the whole organism, not all of the information is constantly used. The processes of transcription and translation, respectively, result in an RNA copy of a particular gene and then the polypeptide specified by that information. Both processes require energy. Each cell needs different polypeptides, depending on its environment and stage in life and function (in a multicellular organism). Hence, gene expression is regulated so that energy is not wasted making unnecessary polypeptides and specific functions are not corrupted by the presence of competing or counteracting polypeptides.

In one of the early studies of gene regulation Jacob and Monod examined sugar metabolism of *E. coli*. Normally, *E. coli* is provided with the sugar glucose as a carbon and energy source in the mammalian gut; it makes the appropriate polypeptides for metabolizing glucose. If the sugar lactose is provided instead of glucose (as in the gut of a young mammal), a different set of genes, specifying lactose-metabolizing enzymes, is expressed. The cell expends energy to produce lactose-metabolizing polypeptides only when glucose is absent and lactose is present. Jacob and Monod's studies showed that there are segments of DNA, called **regulatory genes**, whose primary function is to bring about the appropriate expression of other genes. Some regulatory genes specify products (primarily proteins) that **interact with molecules from the environment** and then form a complex able to **bind to DNA (or RNA)**, to **block or facilitate gene expression**. Other regulatory genes are **base sequences in DNA recognized by the binding complexes**. The regulated genes specify proteins and RNA's for metabolism and development. They are called **structural genes**, because they **specify the amino acid sequences of the polypeptides**, rather than the time of the polypeptide's expression. Both structural and regulatory genes are found in prokaryotes and eukaryotes. Because the development of specialized cells and coordination of cell functions must occur in multicelled eukaryotes, they have more complex interactions of regulatory genes than do prokaryotes and unicelled eukaryotes.

One way to observe a simple example of gene regulation is to control the environment of *E. coli* cells and monitor the proteins made under different conditions. More specifically, we will follow the influence of sugar availability on the production of **beta-galactosidase** by *E. coli* grown on different sugars. This enzyme is used to break the disaccharide lactose into glucose and galactose and is

specified by the lac Z structural gene of *E. coli*. Lactose is a substrate for the enzyme beta-galactosidase. The expression of two other enzymes helping to make energy available from lactose is regulated along with beta-galactosidase. Although we will not be monitoring their activities, we would expect them to increase and decrease at the same time, because they are controlled by the same regulatory genes.

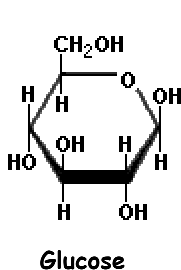
In the next laboratory period you will be assaying the enzyme beta-galactosidase, to see how its production is regulated by bacterial cells in response to available nutrients. In short, this enzyme is required to release glucose by cleaving lactose (milk sugar), and it is normally made only when lactose is present and glucose is unavailable. The production of beta-galactosidase is controlled by regulatory genes that allow specific transcription and translation to be coordinated with environmental conditions. Today you will be preparing media with different sugars and inoculating them with *E. coli*. Next lab period you will be measuring the enzyme activity produced by each of the cultures, growing in the different sugars.



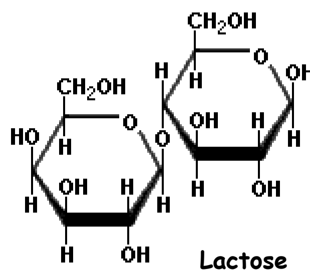
THINGS TO DO:

Form groups of 4 students and obtain: **4 sterile 6-inch tubes**; **bacterial medium lacking sugar (MS/YE); 1 tube each of 10% glucose, lactose, sucrose, and sterile water;** and **pipettes.**

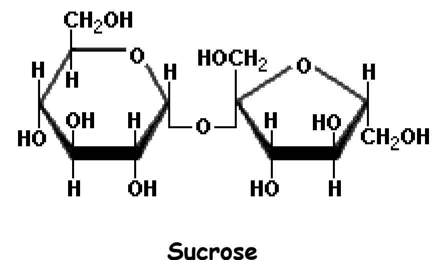
1. Label the tubes #1-#4.
2. To each tube add 7 ml bacterial medium lacking sugar.
3. Add 0.30 ml of the following to each tube:
   10% glucose to tube #1,
   10% lactose to tube #2,
   10% sucrose to tube #3,
   sterile water to tube #4.
4. Add 1 drop of well shaken wild-type bacterial suspension to each tube.
5. Place labeled tubes in a rack and incubate all tubes in the walk-in incubator on the shelf for your section, until the next lab period. They will be incubated at 37 degrees C to promote growth.
6. Review the structures of glucose, lactose, and sucrose diagrammed below. Explain why the enzyme beta-galactosidase can break the bond joining beta-galactose and beta-glucose (of lactose), but not the bond joining beta-glucose and beta-fructose (of sucrose).



Glucose



Lactose



Sucrose

DAY TWO: ENZYME ASSAY

OBJECTIVES:

Today you will be measuring enzyme activity per cell in the cultures you set up last time. By the end of today's lab, you should be able to

- **Properly adjust and use the Spec 20, including making appropriate blanking solutions.**
- **Explain how enzyme activities are measured.**
- **Know which of your samples acted as positive and negative controls.**
- **Know under what conditions *E. coli* produces the most beta-galactosidase, and why.**
- **Know on what carbon sources *E. coli* grows well, and why.**
- **Explain why a difference in enzyme activity may be due to a difference in transcription, translation, or enzyme activation.**

INTRODUCTION:

To see how cells have responded genetically to differences in their molecular environments, we will be looking at protein differences in the cultures we set up during the last lab period. Generally, to measure enzyme activities, cells are broken open and a cell extract is incubated with a known substrate. **Enzyme activity** is demonstrated by a **decrease in the substrate** and/or an **increase in the product** of the reaction. Differences in protein activity (gene expression) present could represent the cell's response of activating or inactivating the protein, translating a pre-existing mRNA, and/or transcribing a gene. Our enzyme assay will not shed light on which of these processes has occurred, but it will demonstrate whether or not the cells have responded to environmental differences by altering their expression of genes. Once a response is noted, other experiments can provide more information by looking at RNAs and/or seeing whether cells show the same response in the presence of drugs inhibiting transcription or translation.

In our exercise, cells grown in different sugars will first be treated with toluene to chemically create holes in the cells' walls and membranes. Among the enzymes leaking out should be beta-galactosidase. Recall that this enzyme speeds the cleavage of lactose into glucose and galactose. To assay how much of the enzyme is present from cells grown in different sugars, we take advantage of the fact that the enzyme will also cleave ortho-nitrophenyl- -d-galactoside (ONPG), because ONPG has a bond resembling the one between glucose and galactose.

If we add the cell extract to an excess of ONPG, the amount of ONPG cleaved will reflect the amount of enzyme in the cell extract. Conveniently, one product after cleavage of the galactoside is orthonitrophenol (ONP). ONP looks bright yellow and absorbs light of wavelength 420nm. So we can quantitate how much ONP is produced by measuring the absorbance of light of a wavelength of 420 nm (A420) of the enzyme reaction. The greater the A420, the more cleavage occurred, and the more beta-galactosidase must have been present in the cells.

Since we want to know how individual cells responded, we will also need to know the concentration of cells in each of the cultures. To estimate cell concentration, we will use the fact that bacterial cells deflect light moving through the cuvette, and thus appear to be absorbing light. Higher concentrations of cells produce higher absorbance readings of light at a wavelength of 600 nm. We will use an approximate equivalency value that 1 A600 corresponds to 8×10^8 cells/ml.

The instrument we will use to measure the light absorbed is a Spec 20 spectrophotometer. It sends light of a specified wavelength through a sample chamber into which is placed a cuvette containing at least 5 ml of sample solution. A photocell on the opposite side of the chamber measures how much light has gone through the sample and registers on a meter the % of incident light transmitted and the relative amount absorbed or deflected by the sample. The relationship between absorbance and concentration is linear up to a point (the cut-off is approximately absorbance = 1), beyond which your readings will underestimate your actual concentration. You may need to dilute your samples to work within the linear range of the instrument. For example if you make a 1:1 dilution (decrease concentration by 1/2) than you must multiply your estimated concentration by 2 to adjust for your dilution.

In order to obtain accurate readings, you must properly adjust the Spec 20. (Instructions are also posted next to each instrument.) First, the top dial must be set to the desired wavelength. Then, cuvettes must be free of smudges, contain sufficient sample, and be oriented with the logo facing the front of the sample chamber. Before the sample is placed in the chamber, the machine must be dark-zeroed and blanked. To **dark zero**, turn the machine on with the left front knob, leave the sample chamber empty, and make sure the chamber cover is shut. Under these conditions there should be no light reaching the photocell; turn that same left knob until the needle shows infinite absorbance (0% transmittance). To **blank the machine**, fill a cuvette with a solution containing everything but the component you are trying to measure. For example, if you are trying to measure the concentration of cells in culture medium, your blank will contain the medium in which the cells are growing. Place the cuvette holding the blanking solution in the sample chamber and then turn the right front knob until the needle shows 0 absorbance (100% transmittance). This eliminates any absorbance that is not due to your sample of interest. Once adjusted, the machine should be ready for your samples.

THINGS TO DO:

Work in groups of 4 to find the enzyme activity per cell for cultures you grew with different sugars as carbon sources. Assemble your required materials: **cultures growing in different sugars, 4 microcentrifuge tubes, pipettes, micropipettors and tips, cuvettes, 4 6-inch tubes, Tris buffer, ONPG.** Toluene is in the hood; vortexers, microcentrifuges, water baths, and Spec 20's are on side benches.

1. Transfer 1.2 ml of each culture into an appropriately labeled microcentrifuge tube. Add 3 drops of toluene to each tube (working in the hood), vortex the tubes, and incubate them for 10 min in the 37° C water bath. **CAUTION: TOLUENE HAS NOXIOUS FUMES AND CAN BURN YOUR SKIN; HANDLE IT WITH CARE AND KEEP IT IN THE HOOD.**
2. Take the remaining 5+ ml of each culture and use the Spec-20 to find the approximate cell concentration. Set the wavelength on 600 nm, dark zero, and blank the machine. Use medium with no cells as the blank. Record the A600 for each sample and **record the results in the table on the next page**. Return each sample from the cuvette back to the appropriate culture tube in case you have to repeat a reading.
3. Centrifuge the toluene-treated cells, at top speed, for 5 min, to pellet the leaking cells.
4. While centrifugation proceeds, prepare 4 6-inch tubes by labeling them and adding 4 ml of 100mM tris buffer, pH 8.0.

5. Transfer 1 ml of supernatant from each centrifuged tube into the appropriately labeled tube of buffer. The buffer is at the optimal pH for beta-galactosidase activity.

6. At the water bath, add 0.2 ml ONPG solution to each tube, noting the time of addition of this substrate to each tube. The time of addition marks the start of your reaction. Each reaction should be incubated in the water bath for exactly 15 minutes.

7. While the reactions are incubating, readjust the Spec-20 to read absorption by ONP (the product of the reaction) at 420 nm. Dark zero and blank the machine again. This time the blank should contain 1 ml medium + 4 ml tris buffer + 0.2 ml ONPG. Do you understand why?

8. Find the A420 of each sample and **record the results in the table below**.

9. When you are satisfied with all of your readings, rinse out the cuvette several times with water and leave it in the rack near the Spec 20 to drain. Leave the blanking solutions for other labs.

Sample	A600 reading	A420 reading
1 (glucose)		
2 (lactose)		
3 (sucrose)		
4 (water)		

10. Calculate the cell concentration for each culture, assuming that a reading of 1.0 at A600 corresponds to about 8×10^8 cells/ml. If your readings were taken in the non-linear range of the instrument's detection curve then you must dilute your sample and then multiply your final answer by the same proportion as the dilution. **Record your results in the table below**.
11. Calculate the units of enzyme activity per ml for each culture, based on the A420 readings. Assume that every 0.01 A420/15 min of reaction corresponds to 1 unit of enzyme activity. If your readings were taken in the non-linear range of the instrument's detection curve then you must dilute your sample and then multiply your final answer by the same proportion as the dilution. **Record your results in the table below.**
12. Calculate the enzyme activity (column 3, table below) for each culture. **Record your results in the table below.**
13. Calculate the enzyme activity per cell. **Record your results in the table below.**

Sample	# cells/ml (based on A600 reading)	Enzyme activity/ml (based on A420 reading)	Enzyme activity/cell
1 (glucose)			
2 (lactose)			
3 (sucrose)			
4 (water)			