Laboratory 5
Measurement of β-Galactosidase Activity in Lactaid™ Tablets

I. Introduction

Enzymes are an important class of proteins which catalyze chemical reactions. Each individual cell contains hundreds of enzymes. Some enzymes are found in the cytoplasm but others are found in membrane-bound organelles such as the nucleus, mitochondria, lysosomes and peroxisomes. Many proteins that participate in specialized cellular functions such as signal transduction and cellular movement also have enzymatic activity. The purpose of this experiment is to study the basic properties of enzymes, using the β-galactosidase activity found in Lactaid™ tablets as an example. This enzyme is of practical importance to humans because many adults are lactose intolerant. By consuming a tablet containing β-galactosidase, the symptoms of lactose intolerance such as diarrhea, gas, bloating, cramping, and flatulence can be avoided. The activity of the enzyme β-galactosidase will be measured quantitatively using a synthetic substrate called ONPG (o-nitrophenyl-β-D-galactopyranoside). Hydrolysis of this substrate results in the formation of o-nitrophenol, a yellow compound whose absorbance can be measured with a spectrophotometer.

As part of this lab, you will:

- prepare an extract of one of the commercially-available forms of Lactaid™
- construct an o-nitrophenol standard curve
- measure the β-galactosidase activity in an extract of Lactaid™ tablet quantitatively
- compare β-galactosidase activities is different forms of Lactaid™
- learn how to write an introduction in the style of a scientific journal article
- learn how to use PubMed to find scientific journal articles

As you carry out the experiment, make notes, describe your observations, and record your data. This lab session is adapted from an experiment described in a paper by Deutch, C. E. 2007. Degradative Enzymes from the Pharmacy or Health Food Store: interesting examples for introductory biology laboratories. **The American Biology Teacher** 69: available online at

http://www.nabt.org/websites/institution/File/pdfs/publications/abt/2007/069-06-0038.pdf

II. Pre-Lab Preparation

Read the Introduction, Background Information, and Experimental Procedures for this laboratory session. Also read the sections of your Cell Biology textbook which deal with protein structure and with enzymes. It will be helpful to review the Background Information of **Laboratory 3 (Spectrophotometric Analysis of Membrane Stability in Beet Root Cells)** and **Laboratory 4 (Determination of Protein Concentrations by Spectrophotometry).** After preparing for the lab, you should be able to answer the following questions.

A. What is the purpose of taking a Lactaid™ pill?

B. What is the reaction catalyzed by β-galactosidase?

C. How are amino acids linked together to form a protein?

D. What are the four levels of protein structure?

E. What is an enzyme?

F. How does an enzyme increase the rate of a chemical reaction?

G. What is the difference between a fixed-time enzyme assay and a kinetic enzyme assay?

H. What is the difference between V_o and V_{max}?

I. Why do enzyme-catalyzed reactions show substrate saturation?

J. Why is ONPG used as a substrate for measuring β-galactosidase activity?

K. What is the absorption maximum for o-nitrophenol?

L. What is the purpose of constructing an o-nitrophenol standard curve?

M. What are the units of enzyme activity?

III. Background Information

A. Lactose Intolerance

Lactose is a disaccharide composed of glucose and galactose, which is normally found in milk and other dairy products. It can be degraded into its component sugars in the small intestine by an enzyme called β-galactosidase or lactase. Most persons of Northern European origin have adequate levels of this enzyme as adults and so have no trouble digesting milk products. However, people from other parts of the world often have a condition called lactose intolerance. These individuals have high levels of this enzyme at birth and so can digest the lactose in breast milk. However, they gradually produce less of the enzyme as they grow older and so cannot digest lactose as adults. If they do consume milk, yogurt,

ice cream, or other dairy products, the lactose accumulates in their digestive system. This results in diarrhea and gas formation, with the associated symptoms of bloating, cramping, and flatulence.

There are several over-the-counter products on the market such as Lactaid™ which contain the enzyme β-galactosidase and can be used to treat lactose intolerance. In these preparations, the enzyme has been combined with several other ingredients and pressed into tablets or caplets. These additional ingredients include microcrystalline cellulose, calcium carboxymethylcellulose, dextrose, sodium citrate, silicon dioxide, and magnesium stearate. The exact amounts and functions of these ingredients are not stated, but they are included to protect the enzyme protein as it moves through the digestive system.

B. Conformation of Proteins

While a protein can be described in terms of its sequence of amino acids, proteins in a living cell do not exist as simple linear chains. Rather, each polypeptide chain is folded through weak chemical bonds between the peptide bonds and R groups into a complex three-dimensional conformation. This conformation may be relatively extended (fibrous) or compact (globular). The weak chemical bonds that contribute to a protein's conformation include hydrogen bonds, ionic bonds, van der Waal's interactions, and hydrophobic associations.

The structure of a protein is often described in term of four levels (Figure 5.1). The amino acid sequence of a protein is called primary structure. The regular arrangement of the amino acids in a polypeptide chain as coiled α-helices or more extended β-strands is called secondary structure. The further folding of the polypeptide chain in three dimensions as a result of interactions between the sidechains of the amino acids is called tertiary structure. The stable association of several identical or non-identical polypeptide chains or subunits in a protein is called quaternary structure.

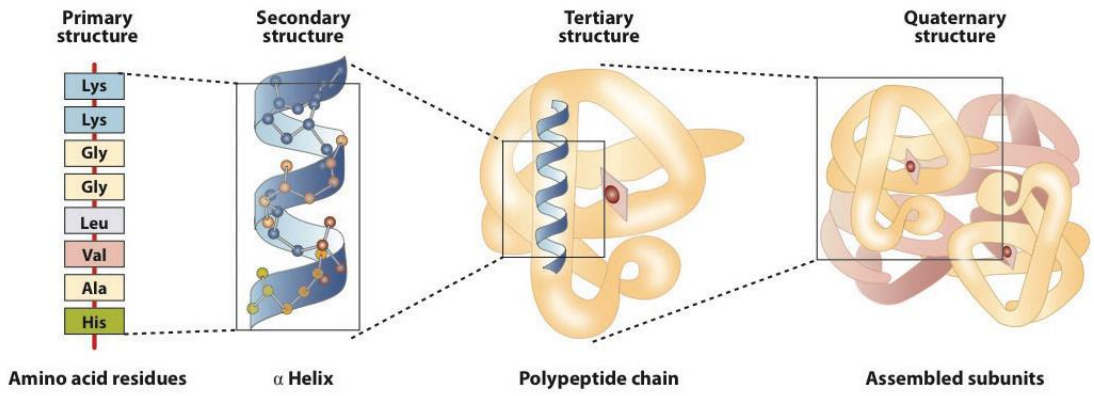

Figure 5.1. Protein Structure; Primary, Secondary, Tertiary, Quaternary

The folding of a polypeptide chain into a conformation creates a unique three-dimensional surface with which only certain other molecules can interact. A molecule that can bind more or less stably to a protein through weak chemical bonds is called a ligand. Most of the functions of proteins in a cell can be explained in terms of protein-ligand interactions. For example, enzymes bind specific ligands (substrates), which then undergo a chemical reaction. Membrane receptors bind specific ligands (hormones), which then leads to a signal transduction process in which cellular functions change. Transport proteins bind specific ligands (solutes) and facilitate their transport across a membrane. Antibodies bind specific ligands (antigens), which then can lead to an immunological response.

C. Enzymes

Enzymes are biological catalysts that increase the rates of chemical reactions, and while a few RNAs have catalytic activity, most enzymes are proteins. An enzyme binds a particular compound (a substrate) at a particular site, which leads to a chemical change and the formation of a different compound (a product)(Figure 5.2). The site in the three dimensional structure of a protein where the substrate binds to the enzyme is called the active site. Because binding of the substrate and the product to the enzyme occurs through weak chemical bonds, binding is reversible. An enzyme is not permanently changed by its participation in the chemical reaction and so can be used again and again.

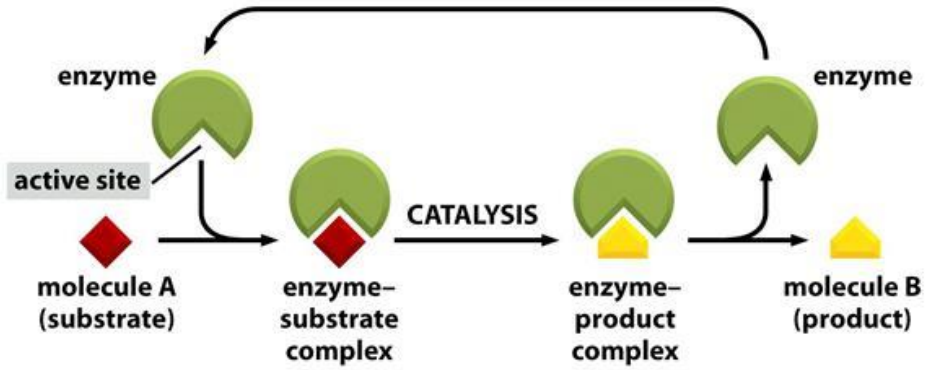

Figure 5.2. How enzymes work. Each enzyme has an active site to which one or two substrate molecules bind, forming an enzyme-substrate complex. A reaction occurs at the active site, producing an enzyme-product complex. The product is then released, allowing the enzyme to bind additional substrate molecules.

The active site of an enzyme is often small compared to the structure of the protein as a whole. In some cases, (Figure 5.3), substrate binding leads to a change in the conformation of the protein.

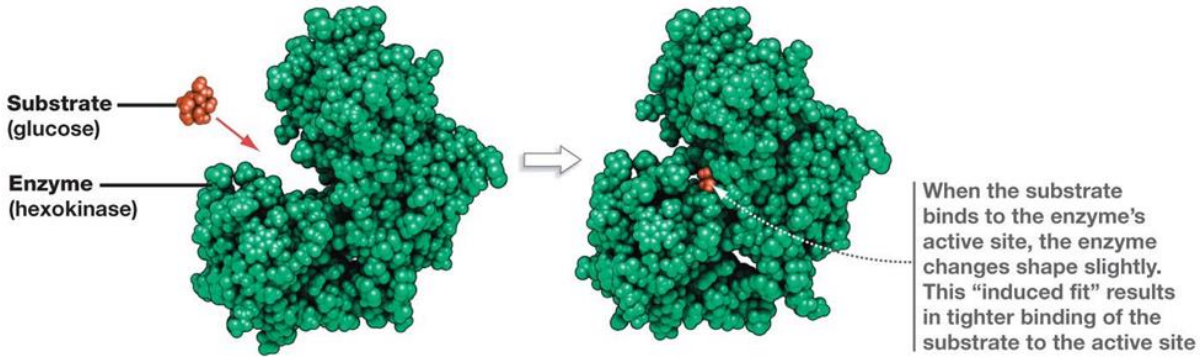

Figure 5.3. Induced fit in hexokinase. The ends of the U-shaped enzyme hexokinase pinch towards each other in a conformational change induced by binding to glucose (red). (rhodopsinreader.wordpress.com)

1. How Enzymes Work

Enzymes are catalysts that do not change the fundamental energetics of a chemical process. That is, whether a reaction will tend to go in one direction or another depends entirely on the chemical structures and concentrations of the substrate and the product. The directionality of a reaction can be expressed in terms of an equilibrium constant (K_{eq}) or a change in free energy (ΔG). However, an enzyme does increase the rate at which equilibrium is reached by reducing the activation energy (E_a) for the reaction (Figure 5.4).

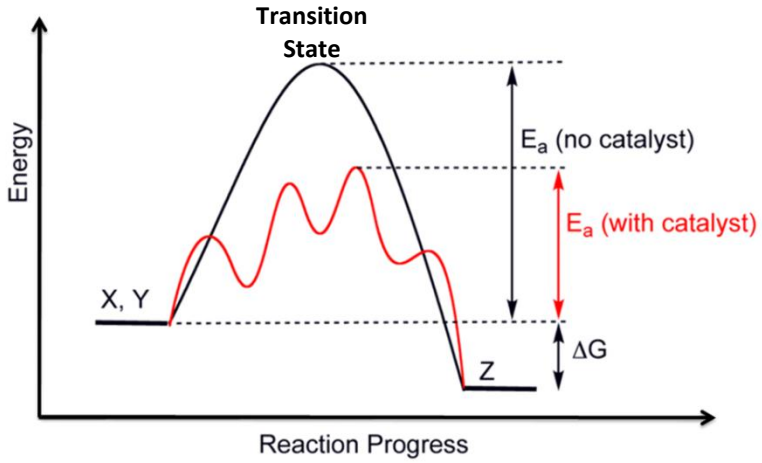

Figure 5.4. Reaction Coordinate Diagram comparing enzyme catalyzed and uncatalyzed reactions. The catalyst offers an alternate reaction pathway (shown in red) where the activation energy (E_a) for the overall process is lower when the enzyme catalyzes the reaction. (By Smokefoot - Wikimedia Commons, CC0, https://commons.wikimedia.org/w/index.php?curid=29514035

In order for a substrate to be converted to a product, the compound must go through a transition state. In an uncatalyzed chemical reaction, the amount of energy needed to reach this transition state (the activation energy) is relatively high. This means that the reaction is not likely to occur, particularly at the temperatures compatible with life and protein function. In a catalyzed chemical reaction, the amount of energy needed to reach the transition state is much lower. This means that the reaction is more likely to occur within a living cell. Enzymes decrease the energy needed to reach the transition state in several ways. They bind one or more substrates to a reactive surface and they remove water molecules that can interfere with a reaction. They also may participate directly in a reaction by using amino acids as proton donors or proton acceptors or as nucleophiles. In some cases, an enzyme may contain cofactors such as divalent metal ions and organic coenzymes which also contribute to the catalytic process. These cofactors bind functional groups or electrons in ways that amino acids cannot.

2. Types of Enzymes

Enzymes are usually classified according to the type of reaction they catalyze. Most reactions are relatively simple:

SUBSTRATE(S)		PRODUCT(S)
Molecule A	→	Molecule B
Molecule C	→	Molecule D + Molecule E
Molecule F + Molecule G	→	Molecule H

Table 5.1 summarizes the major types of enzymes. **The enzyme to be used in these experiments - β-galactosidase or lactase - is an example of a hydrolase** because it uses water to break covalent bonds.

Table 5.1. International Classification of Enzymes

No.	*Class*	*Type of reaction catalyzed*
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer reactions
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to water)
4	Lyases	Addition of groups to double bonds, or formation of double bonds by removal of groups
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C—C, C—S, C—O, and C—N bonds by condensation reactions coupled to ATP cleavage

Note: Most enzymes catalyze the transfer of electrons, atoms, or functional groups. They are therefore classified, given code numbers, and assigned names according to the type of transfer reaction, the group donor, and the group acceptor.

3. Enzyme Assays

Enzymes are usually detected by measuring the rate of the chemical reaction they catalyze. That is, an assay or procedure is developed for detecting a particular substrate or product. The amount of that compound then is measured as a function of time. From the rate at which the substrate is removed or the product is formed, the amount of enzyme which is present is inferred. The rate of substrate utilization or product formation is affected by the amount of enzyme protein added to the reaction and by the substrate and product concentrations. It is also affected by the pH or temperature of the solution and by the presence of other compounds that may activate or inhibit the protein.

Two types of enzyme assays are commonly used: Kinetic (continuous) and Fixed-time.

- **Kinetic or continuous assay:** The enzyme and substrate(s) are combined and the amount of substrate remaining or product formed is monitored continuously. This is most easily done with spectrophotometric assays where the substrate or product absorbs or fluoresces light at a particular wavelength. The reaction is usually set up in a cuvette which is placed in a

colorimeter, spectrophotometer, or spectrofluorometer. The instrument is connected to a recorder or computer so that absorption or fluorescence can be measured directly as a function of time. Kinetic assays are very convenient because the rate of the reaction is immediately apparent. Unfortunately, however, they are not applicable to most enzymes.

- **Fixed-time assay:** The enzyme and substrate(s) are combined and incubated for a defined period of time. The reaction is terminated by an abrupt change in temperature or pH and the amount of the substrate remaining or product formed is then determined. Fixed-time assays are usually done if some sort of processing of the sample is required to detect the substrate or product. Such processing is often necessary when the substrate or product does not intrinsically absorb light or fluoresce, when the substrate is radioactive, or when antibodies are employed as secondary reagents.

D. Biochemical Characteristics of Enzymes

Enzymes catalyze chemical reactions by binding specific substrates and decreasing the energy needed to reach a transition state at which a chemical transformation can occur. Even though each enzyme has a different protein structure and catalyzes a different chemical reaction, all enzymes have some common characteristics.

1. Kinetics of Enzyme-Catalyzed Reactions

The rate of an enzyme-catalyzed reaction (its kinetics) is usually determined by measuring the amount of product formed or the amount of substrate consumed as a function of time. This does not require a completely purified enzyme but it does require a sensitive assay for either the product or substrate. The rate of the reaction depends on several factors: 1) the amount or concentration of the enzyme; b) the structure of the substrate; c) the amount or concentration of the substrate; d) the physical environment, including the temperature, pH, and ionic strength (salt concentration) of the solution.; and e) the presence of other molecules that might act as activators or inhibitors of the reaction.

For the simple reaction in which A ---> B, the rate of the reaction might be assessed by measuring the amount of product (B) present in a solution containing a certain amount of enzyme and a certain amount of substrate. The amount of product is initially 0, but it then increases with time as the reaction occurs (Figure 5.5). The amount of product increases linearly for some time, but then begins to approach a plateau. The initial rate of the reaction is called the initial velocity or V_o.

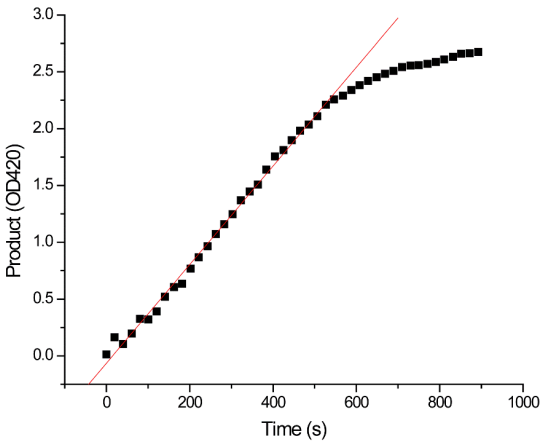

Figure 5.5. Rate of an Enzyme-Catalyzed Reaction. The amount of product increased with time (s). The amount of product formation increases linearly at first (red line), but then approaches a plateau.

One of the characteristic features of all enzyme-catalyzed reactions is that they show substrate saturation. If the rate of product formation is measured using a fixed amount of enzyme and increasing amounts of substrate, data like those shown Figure 5.6 are usually obtained. The initial velocity gradually increases as the substrate concentration increases. However, at high substrate concentrations, no further increase in velocity occurs.

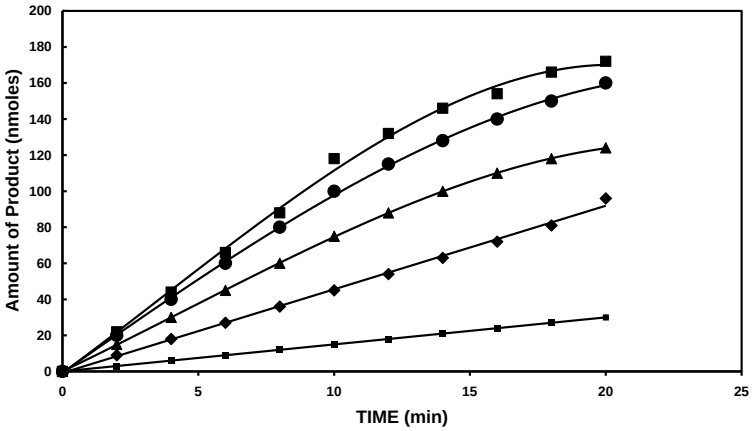

Figure 5.6. Rate of an enzyme-catalyzed reaction with increasing amounts of substrate.

This can be seen more easily by plotting initial velocity (V_o) for each reaction as a function of substrate concentration. This is shown in the next figure. The rate of the reaction gradually increases with the substrate concentration but then reaches a plateau. The maximum rate of reaction is referred to as the maximal velocity or V_{max}. Note that in Figure 5.7, the velocity is expressed in μM/min rather than nmoles/min. Some scientists prefer to express product formation as a concentration, which takes into account the actual volume of the enzyme reaction.

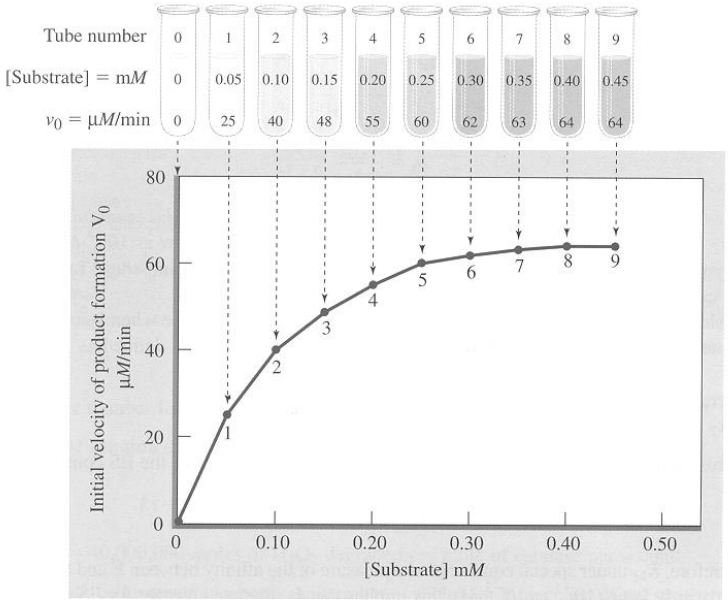

Figure 5.7. Experimental procedure to study the kinetics of an enzyme-catalyzed reaction. An identical amount of enzyme is added to a set of tubes containing increasing amounts of a substrate. The reaction rate or initial velocity is measured for each reaction mixture by determining the rate of product formation. The velocity is plotted against substrate concentration. Most enzymes yield a hyperbolic curve as shown.

In addition to being dependent on the substrate concentration, the rate of an enzyme-catalyzed reaction is related to the amount of enzyme that is present. In the presence of an excess of substrate, the initial rate of the reaction increases proportionately with the amount of enzyme (Figure 5.8).

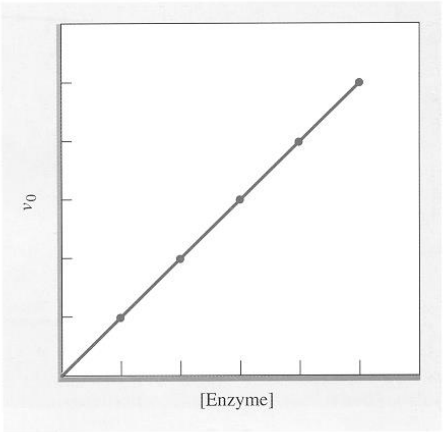

Figure 5.8. A plot of initial reaction velocity versus the concentration of enzyme. Note that the velocity increases in a linear fashion with an increase in enzyme concentration.

2. Substrate Specificity

The specificity of any enzyme depends on its interactions with ligands (substrates) that can bind to the active site. The degree of this specificity is quite variable. Some enzymes show very high degree of specificity and will only act on one or two structurally related substrates. Others show a lower degree of specificity. For example, alcohol dehydrogenase (an enzyme involved in the formation or detoxification of compounds containing a hydroxyl group) tends to be relatively nonspecific. It will act on ethanol as a substrate, but also on propanol, butanol, and pentanol. Likewise, D-amino acid dehydrogenase (an enzyme involved in the oxidation of amino acids) will work on most amino acids that have a D configuration even though their side chains are quite different.

E. Detection of β-Galactosidase Activity

The enzyme β-galactosidase, which is also known as lactase in animals, catalyzes the hydrolysis of the disaccharide lactose into its constituent monosaccharides, D-galactose and D-glucose (Figure 5.9)

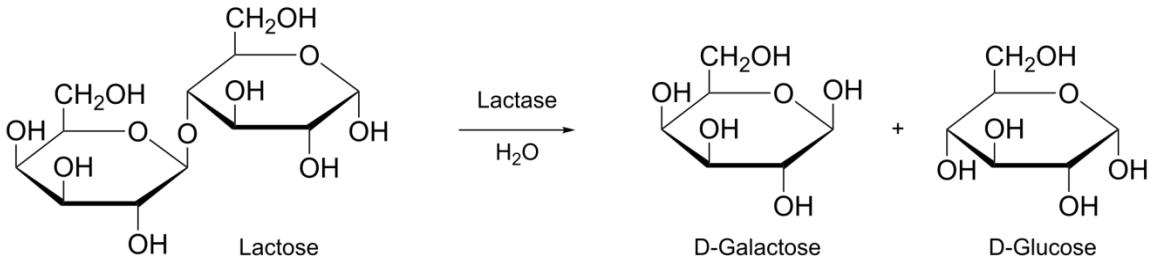

Figure 5.9. Reaction in which lactose is broken down by β-galactosidase to glucose and galactose

1. Colorimetric Assay for β-Galactosidase

The enzyme β-galactosidase does not itself exhibit any unique light absorption or fluorescence; neither do the natural substrate (lactose) and products (galactose and glucose). Moreover, the natural substrate and products are very similar in structure, which makes it difficult both to separate them and to assay specifically for one compound or another. However, β-galactosidase can hydrolyze several artificial substrates which do give products that are easy to detect. The most commonly-used of these compounds is o-nitrophenyl-β-D-galactopyranoside (ONPG) (Figure 5.10).

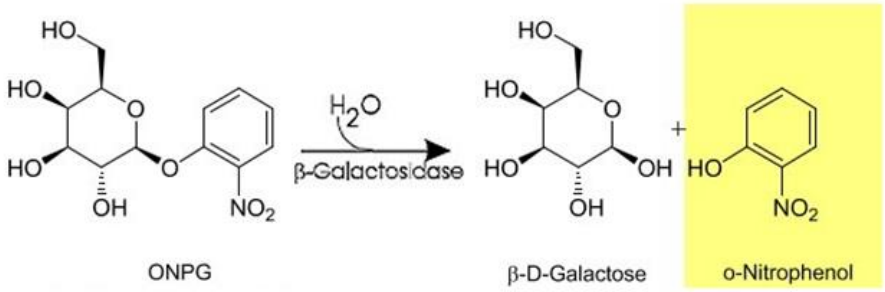

Figure 5.10. Reaction used to quantify the activity of β-galactosidase.

ONPG consists of D-galactose and o-nitrophenol (also called 2-nitrophenol), joined through a β-linkage similar to that found in lactose. Hydrolysis of this bond by β-galactosidase results in the formation of free D-galactose and o-nitrophenol. D-galactose is again difficult to detect specifically, but o-nitrophenol has a distinct yellow color. At pH 7, which is normally optimal for β-galactosidase activity, o-nitrophenol has a pale yellow color. However, at more alkaline pH (>8), the hydroxyl group in o-nitrophenol loses its hydrogen (deprotonates) to form the o-nitrophenolate anion. This ion has a more intense yellow color. ONPG is commercially available at relatively low cost and so is very convenient to use.

2. Measurement of β-Galactosidase Activity

The use of ONPG as a substrate makes the measurement of β-galactosidase or lactase particularly simple. A solution containing the enzyme is usually combined with a buffer of salts designed to keep the pH initially constant at pH 7.0. The substrate (ONPG) is then added and the solution incubated at a certain temperature. As ONPG is broken down and o-nitrophenol is formed, a pale yellow color develops. When this color is readily apparent, the reaction is terminated by adding a basic solution such as sodium carbonate (Na_2CO_3). This raises the pH to about pH 10, where the enzyme no longer functions and where the color intensifies. The exact interval between the time the substrate was added and the time the reaction was stopped is noted. The yellow color is then measured quantitatively in a colorimeter or spectrophotometer at about 420 nm. Using a standard curve that relates the amount of color to the amount of o-nitrophenol, the amount of product formed within a particular time interval can be calculated. This is a measure of the amount of enzyme activity.

Figure 5.11 gives the absorption spectra of o-nitrophenol, which was constructed in the same way as you used in **Laboratory 3 (Spectrophotometric Analysis of Membrane Stability in Beet Root Cells)** to determine the absorption maximum for betacyanin. Note that the wavelength of maximal absorbance (λ_{max}) is about 420 nm.

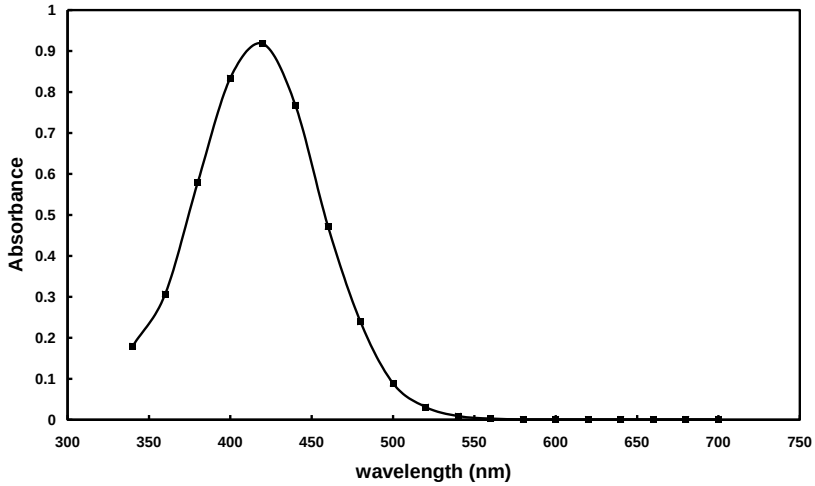

Figure 5.11. The absorption spectrum for o-nitrophenol

3. Standard Curves

The relationship between the amount of a compound such as o-nitrophenol in a solution and its absorbance can be summarized in a standard curve. Recall from **Laboratory 4 (Determination of Protein Concentrations by Spectrophotometry)** that a standard curve is constructed by setting up a series of solutions that contain varying amounts of the compound of interest in exactly the same volume. The amount may be expressed in molar units (for example, nmoles or μmoles) or in weight units (μg or mg). The absorbance of each solution is then measured against a reference solution or blank that lacks the compound of interest. The absorbance values of the solutions are then plotted as a function of the amount of compound (Figure 5.12)

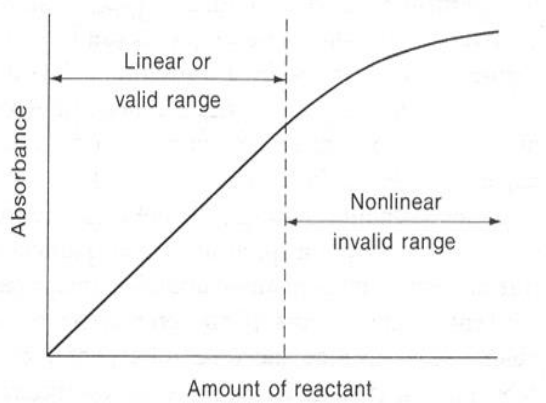

Figure 5.12. Typical standard curve for a color-forming quantitative reaction.

Once a standard curve has been created, it can be used to determine the amount of the compound of interest in an unknown solution. The absorbance of this unknown solution is first measured using the same instrument at the same wavelength as the standards. As noted in **Laboratory 4**, there are

then two ways to estimate the actual amount of the compound of interest in the unknown solution. One is graphically and the other is by using the slope of the standard curve as a conversion factor. The graphical method is easiest if only a few values need to be determined. The mathematical method is easiest if there are many values that need to be determined.

4. **Outline of Basic Protocol for β-Galactosidase Assays** *(This is not the procedure it just give the outline of the process you will use.)*

The basic strategy for measuring the activity of β-galactosidase will be to add a series of reagents to a 13 x 100 mm test tube and to look for the development of a yellow color. The following steps should be done in the order given:

Basic protocol for a β-Galactosidase Assay

To a 13 x 100 mm test tube:

a. add 3.5 mL of Z buffer
b. add 0 to 0.5 mL of water
c. add 0 to 0.5 mL of an enzyme solution (from the lactaide tablet)
d. mix by inversion
e. incubate at room temperature for 5 minutes
f. add 0.5 mL (500 μL) of ONPG substrate solution and start timer
g. mix by inversion
h. watch for yellow color formation
i. add 0.5 mL of 1.0 M Na_2CO_3 to stop reaction and note time when pale yellow color formation is observed.
j. mix by inversion
k. read absorbance at 420 nm

IV. Experimental Procedures

This experiment has several parts which must be done sequentially. In order to compare the efficacy of different forms of Lactaid, several types of tablets will be available: 1) the name-brand form; 2) several generic forms: and 3) a more concentrated form. Each group will use one type of tablet. We will then compile the class results and compare the data at the end of the lab. Because it takes some time to prepare the extract from the Lactaid Tablets, you should start on the construction of the o-nitrophenol standard curve while the extraction is occurring. As before, divide up the work among the members of your group.

The following is a flow chart for this laboratory session:

Extraction of β-Galactosidase (Lactase) from Lactaid™ Tablets (Section IVA)

↓

Construction of an o-Nitrophenol Standard Curve (Section IVB)

↓

Preliminary Measurement of β-Galactosidase (Lactase) Activity in Lactaid™ Tablets (Section IVC)

↓

Precise Measurement of β-Galactosidase (Lactase) Activity in Lactaid™ Tablets (Section IVD)

A. Extraction of β-Galactosidase (Lactase) Activity from Lactaid™ Tablets

The purpose of this part of the experiment is to prepare an extract of a tablet of Lactaid™ which will contain β-galactosidase (lactase) activity. Each group will use one type of tablet. Note the label on the box which indicates the dosage and contents of the tablet. Most tablets or caplets constitutes a "serving size" and are reported to contain 9000 FCC units (but these units are not defined).

1. Obtain a tablet of Lactaid™ and place it in a clean mortar. With a pestle, grind the caplet into a fine powder. Add 5.0 ml of Z buffer to the powder and mix the suspension by drawing the liquid up and down in a Pasteur pipet at least 10 times.

2. Transfer the suspension to a clean 15 ml plastic centrifuge tube and allow it to sit at room temperature for about 15 minutes. Invert the solution periodically so that the contents dissolve as much as possible.

3. Centrifuge the solution for 3 minutes at 3,000 rpm in a clinical centrifuge and pour off the supernatant liquid to a clean 13 x 100 mm tube. **Keep the tube in an ice bucket throughout the lab period** as your stock enzyme solution.

B. Construction of an o-Nitrophenol Standard Curve

The purpose of this part of the experiment is to construct a standard curve for the ionic (deprotonated) form of o-nitrophenol. This standard curve will be used throughout this lab in assays for β-galactosidase. By using this standard curve, you will be able to convert absorbance values in your assays to actual amounts of reaction product.

1. Turn on the Spectronic 20 Genesys spectrophotometer and allow it to warm up for about 15 minutes. Set the wavelength to 420 nm.

2. Set up a series of eleven (11) 13 x 100 mm test tubes as shown in Table 5.2. Use a 5.0 ml pipet and a green Pipet Aid to dispense the Z-buffer and micropipetters with large tips to dispense the o-nitrophenol, water, and sodium carbonate. All volumes are given in milliliters (ml), but remember that 1.0 ml = 1000 μl. The total volume is 5.0 ml in each case. The stock solution of 1.0 mM o-nitrophenol contains 1.0 millimole of o-nitrophenol per liter, which is the same as 1.0 μmole per ml, or 1.0 nmole per μl.

Table 5.2. Construction of an o-Nitrophenol Standard Curve

Tube	Z-Buffer (mL)	1.0 mM o-nitrophenol (mL)	Water (mL)	1.0 M Na_2CO_3 (mL)
1	3.5	0	1.0	0.5
2	3.5	0.1	0.9	0.5
3	3.5	0.2	0.8	0.5
4	3.5	0.3	0.7	0.5
5	3.5	0.4	0.6	0.5
6	3.5	0.5	0.5	0.5
7	3.5	0.6	0.4	0.5
8	3.5	0.7	0.3	0.5
9	3.5	0.8	0.2	0.5
10	3.5	0.9	0.1	0.5
11	3.5	1.0	0	0.5

3. Mix the solutions carefully by covering the tubes with a piece of Parafilm and inverting them.

4. Wipe off the outside of tube # 1 (the reference solution or blank) with a Kim-Wipe and place it in the instrument. Set the instrument to zero absorbance.

5. Now read the absorbance values of each of the other tubes. **Record your results in Table 5.3 or in your lab notebook.**

Do not discard any of the solutions until all of the absorbance values have been determined and your instructor has checked your standard curve.

Table 5.3. Data Collection for o-Nitrophenol Standard Curve

Tube	A(420 nm)	1.0 mM o-nitrophenol (mL)	o-nitrophenol (nmoles)
1		0	
2		0.1	
3		0.2	
4		0.3	
5		0.4	
6		0.5	
7		0.6	
8		0.7	
9		0.8	
10		0.9	
11		1.0	

6. Calculate the number of nanomoles (nmol) of o-nitrophenol in each of the 11 tubes in the following way. The stock solution has a concentration of 1.0 mM, so there are 1.0 millimoles per liter of solution. Each milliliter of solution would contain 1.0 micromole (μmole) or 1000 nanomoles (nmoles). A volume of 0.1 ml thus would contain 100 nmoles. Using this procedure, calculate the nmoles of o-nitrophenol in each of the 11 tubes for your standard and record them at the right above.

Step 1: Determine how many mmoles per mL of the 1.0 mM o-nitrophenol stock solution.

$$\textbf{1.0 mM} = \frac{\textbf{1.0 mmoles}}{\textbf{L}} \times \frac{\textbf{1 L}}{\textbf{1000 mL}} = \frac{\textbf{0.0001 mmol}}{\textbf{mL}}$$

Step 2: Convert mmoles to nmoles

$$\frac{\textbf{0.0001 mmol}}{\textbf{mL}} \times \frac{\textbf{1000 µmol}}{\textbf{1 mmol}} \times \frac{\textbf{1000 nmol}}{\textbf{1 µmol}} = \frac{\textbf{100 nmoles}}{\textbf{mL}}$$

Step 3: Multiply the concentration of your stock solution (in nmoles/mL) by the volume of stock added to each tube.

$$\textbf{0.1 mL} \times \frac{\textbf{100 nmoles}}{\textbf{mL}} = \textbf{10 nmoles}$$

7. Now, using a sheet of graph paper, plot the observed absorbance of each solution as a function of the nmoles of o-nitrophenol in the tube. Draw the best straight line through as many of the data points as you can, using 0,0 as a defined value. Note any deviation from linearity at high amounts of o-nitrophenol. (**Include this graph in your lab manual**)

8. Using the standard curve, define a conversion factor (slope of best fit line) for converting absorbance values to amount of o-nitrophenol. **The conversion factor indicates that 1 nmol =**

_____**A.** This conversion factor will make it easier to do the calculations needed for a large number of assays. **Write the equation for the line from your graph here or in your lab manual:**

Equation for the line from your standard curve: _____

C. Preliminary Measurement of β-Galactosidase (Lactase) Activity in Lactaid™ Tablets

The purpose of this part of the experiment is to learn how to measure β-galactosidase activity and to identify an appropriate dilution of the stock enzyme solution to use for the other assays.

1. Since you do not know how much β-galactosidase activity is present in your extract of the Lactaid tablet, make four serial 1/10 dilutions of the stock enzyme solution using Z buffer. Obtain four (4) clean 16 x 125 mm tubes and add 9.0 ml of Z buffer to each tube with a 10 ml pipet and a green Pipet-Aid. Mix your stock enzyme solution (your Lactaid extract) by inverting the tube several times and then add 1.0 ml of it to the first tube. Mix the solution carefully by covering it with Parafilm and inverting it several times or by holding it firmly at the top and "thumping" the bottom of the tube with your finger. **DO NOT USE A VORTEX MIXER**. Then add 1.0 ml of this first dilution to the second tube. Mix this solution and continue this process (adding 1.0 mL to the next tube and so on) until you have made all four dilutions. **Keep the dilutions in an ice bucket** along with your stock extract from the Lactaid tablet.

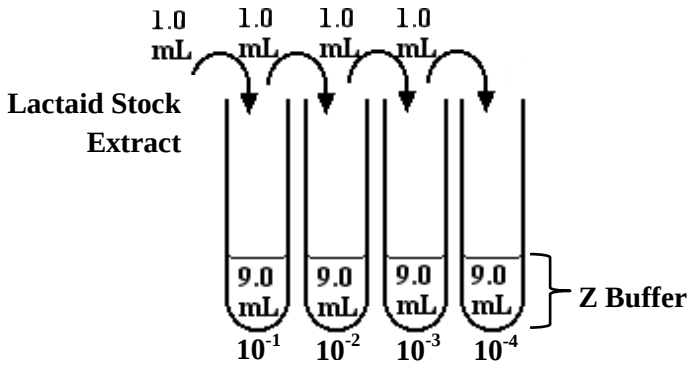

Figure 5.13. Serial dilutions of Lactaid Stock Extract

2. To estimate the amount of activity in each of these dilutions, set up a series of six (6) 13 x 100 tubes as shown in the following table. All volumes are given in ml. Notice that tube 1 is a "reaction blank" and has no enzyme, and that tubes 2, 3, 4, 5, and 6 contain 0.1 ml (100 μl) of one of the lactase solutions. You can use a 5.0 ml pipet to dispense the Z buffer but use a micropipetter to add the water and enzyme solutions.

Table 5.3. Reaction mixtures for preliminary measurement of Lactaid™ extract activity

Tube	Z-Buffer (mL)	Water (mL)	Lactase Solution to Use	Volume of Lactase Solution (mL)
1	3.5	0.5	None	0.1
2	3.5	0.4	Undiluted	0.1
3	3.5	0.4	10^{-1} Dilution	0.1
4	3.5	0.4	10^{-2} Dilution	0.1
5	3.5	0.4	10^{-3} Dilution	0.1
6	3.5	0.4	10^{-4} Dilution	0.1

Note that the total volume in each tube at this point is 4.0 mL. Mix each of the solutions by covering the tubes with Parafilm and inverting them several times or by "thumping" the tube on the bottom. Allow the tubes to sit at room temperature for about 5 minutes to equilibrate the solutions to this temperature.

3. To start the reactions, add 0.5 mL of 10 mM ONPG to each tube using a micropipetter and a large tip. Since you need to know the exact time interval of the reaction, it is best to do this by adding the substrate to the tubes at **30 second or 1 minute intervals**. Mix each tube **immediately** after adding the substrate by inversion or by thumping it a few times on the bottom. **DO NOT VORTEX** these solutions.

   Fill in the chart shown below as you begin the assays. You will need to know the exact time each reaction was started, the time at which it was stopped, and the total elapsed time. You will eventually measure the absorbance of each solution and convert the value to nmoles.

Table 5.4. Data Collection: Preliminary Lactaid™ extract activity measurement

Tube	Lactase Solution	Time Reaction Started	Time Reaction Stopped	Total Time	A(420 nm)	nmoles o-nitrophenol
1	None					
2	Undiluted					
3	10^{-1} Dilution					
4	10^{-2} Dilution					
5	10^{-3} Dilution					
6	10^{-4} Dilution					

4. Watch the tubes carefully and note the appearance of a yellow color. As each of the tubes develops a **readily detectable but not too dark yellow color**, stop the reaction by adding 0.5 ml of 1.0 M Na_2CO_3 with a micropipetter and a clean tip. **Do not stop the reaction until the yellow color in the tube is darker than the blank (tube 1).** Mix the tube **immediately** after adding the sodium carbonate and note the exact time the reaction was terminated. You should find that the reactions with the concentrated enzyme solution develop color very quickly and that the reactions with the more dilute enzyme solutions develop color more slowly**. If there is no visible color after 15 minutes in a particular tube, stop the reaction and proceed.**

5. After the reactions in tubes # 2-6 have been terminated, stop the reaction in tube # 1 by adding 0.5 mL of 1.0 M Na_2CO_3 to it.

6. Use the solution in tube # 1 as the blank. Then read the absorbance in each solution in the spectrophotometer at a wavelength of 420 nm

7. Using the either the o-nitrophenol standard curve constructed in Part B or the conversion factor derived from it, calculate the number of nmoles of product formed in each reaction.

8. You might find that some of the reactions are too dark and outside of the range of the standard curve. In that case, make a 1/10 dilution of the sample in water (0.5 ml of the sample combined with 4.5 ml of water) and read the absorbance again. Then calculate the number of nmoles of product from the standard curve. Multiply this value by 10 to estimate the amount of product in the original solution.

9. Calculate the activity of each of the enzyme reactions in the following way:

$$\text{Relative activity} = \frac{\text{nmoles of o-nitrophenol formed (from standard curve)}}{\text{(total time of reaction in } \textbf{minutes}\text{) (volume of lactase in mL)}}$$

The units of relative activity are nmoles/min mL.

For example, if you find that 34.2 nmoles of o-nitrophenol was formed in 4.6 minutes with 0.1 ml of the 10^{-3} dilution, the activity is:

$$\text{activity} = \frac{34.2 \text{ nmoles}}{(4.6 \text{ min})\ (0.1 \text{ mL})} = \frac{74.3 \text{ nmoles}}{\text{min mL}}$$

Note: **this calculation does <u>NOT</u> include an adjustment for dilution** that was used, that will be addressed later. This is just a preliminary calculation.

Include those calculations here or in your lab notebook:

10. Fill in the following chart to summarize these data:

Table 5.5. Summary of Preliminary Lactaid™ Activity Measurements

Lactase Solution	Activity (nmoles/min mL)
Undiluted	
10^{-1} Dilution	
10^{-2} Dilution	
10^{-3} Dilution	
10^{-4} Dilution	

Does the activity fall off with each dilution as you might have expected?

11. Based on these results, decide on which dilution of the enzyme solution to use for the remaining experiments today. The ideal dilution would be one that results in 0.1 ml of the enzyme solution giving a reasonable absorbance in a reasonable period of time (for example, an absorbance of 0.2 to 0.5 in 5 to 10 minutes). You will be running several assays and you need to be able to do them efficiently but very accurately.

D. Precise Measurement of β-Galactosidase (Lactase) Activity in Lactaid™ Tablets

1. Once you have selected a particular dilution to use, repeat the basic assay in triplicate so that you can get an accurate measure of the enzyme activity in the stock solution. Set up a new series of four (4) 13 x 100 mm tubes containing 3.5 ml of Z buffer. The control tube should contain 0.5 ml of water and the three other tubes 0.4 ml of water and 0.1 ml of your diluted lactase solution. **Be sure to use the diluted solutions of the enzyme in the ice bucket, not the reactions from Part C. Fill in a table as shown in Table 5.5:**

Table 5.5. Data Collection: Precise Measurement of Lactaid™ Extract Activity

Tube	Z Buffer (mL)	Water (mL)	Diluted Lactase	Time Started	Time Stopped	Total Time Elapsed	A (420nm)	nmoles
1	3.5	0.5	0					
2	3.5	0.4	0.1					
3	3.5	0.4	0.1					
4	3.5	0.4	0.1					

Mix the solutions and allow the tubes to sit at room temperature for about 5 minutes to equilibrate to this temperature. To start the reactions, add 0.5 ml of 10 mM ONPG to each tube. Again, add the substrate to the tubes at **30 second** intervals. Mix each tube **immediately** after adding the substrate by inverting it or thumping it a few times on the bottom. As before, record the time each reaction was started.

2. Watch the tubes carefully and note the appearance of a yellow color. As each of the tubes develops a **detectable but not too dark color**, stop the reaction by adding 0.5 ml of 1.0 M Na_2CO_3. Mix the tube **immediately** after adding the sodium carbonate and note the exact time the reaction was terminated. Stop the reaction in tube # 1 after tubes # 2-4 have been terminated.

3. Read the absorbance of the solutions in tubes 2, 3, and 4 in the spectrophotometer using the solution in tube # 1 to set the instrument to zero. Using the o-nitrophenol standard curve constructed in Part B, calculate the number of nmoles of product formed in each reaction. **Include these calculations here or in your lab notebook:**

4. Then calculate the activity of each of the reactions as before.

$$\text{Relative activity} = \frac{\text{nmoles of o-nitrophenol formed (from standard curve)}}{\text{(total time of reaction in \textbf{minutes}) (volume of lactase in mL)}}$$

Again, the units of relative activity are nmoles/min mL

Note: Since we used 5 mL of solution in each of the tubes that we used to create our standard curve and 5 mL in each of the tubes used to measure the activity of the enzyme in solution we do not need to correct for the volume of the reaction, as we will do in later experiments.

Include the calculations for relative activity here or in your lab notebook:

5. Calculate the average relative activity for the three samples. Then calculate the activity in the stock enzyme solution by multiplying by the inverse of the dilution factor. For example, if you used a 1/100 (10^{-2}) dilution and found an activity of 345 nmole/min mL, the stock solution would have 100 times this activity or 34,500 nmole/min mL. This can be expressed more simply as 34.5 μmol/min mL. **Show those calculations here or in your lab notebook.**

6. Finally, calculate the amount of total lactase activity in the Lactaid™ tablet. Remember the caplet was initially dissolved in a total of 5.0 mL of Z buffer. In addition you must convert from nanomoles to micromoles.

   The total activity is thus:

$\frac{X\ \mu mol}{min\ mL\ extract}$	x	$\frac{5\ mL\ extract}{tablet}$	=	_____ μmol/min tablet

   Include your final value here:

   _____

7. **Add your data to the chart** in the front of the room with the activities of the different forms of Lactaid™. Alternatively your teacher may have you email the data to them or have you post it on Blackboard.

   Include the class data here or in your lab notebook:

Table 5.6. Class Data for Lactaid™ Tablets

Group	Type of Lactaid™	Total Activity (μ mol/min tablet)

V. Post Lab Analysis

In addition to a data sheet for Lab 5, you will **write up the Introduction for Lab 5**.

A. Writing an Introduction Section

The **Introduction** section describes the context for the question being asked in this paper – i.e., what is the larger question that this study is a part of? There is often a useful summary of the current state of knowledge of the field and some comment on previous research. Then it focuses on a particular aspect of the field, describing a result or set of results from which the current work flows. There will be some statement separating the research conducted in this paper from previous research, i.e., describing why this research represents an advance in the field. Often there is a statement of one or more of the major conclusions from the current research presented at the end of the introduction. This is done so that the reader knows the answers to the major questions just posed. Conceptually, the Introduction section is a funnel – it focuses your attention from the broad field to the specific research conducted in the current paper.

Introduction Section Checklist

- Make a general statement likely to interest the reader in your topic
- Provide background information that is relevant to your experiment
- Do not reference information that is common knowledge (i.e., familiar to your audience)
- Review current literature (primary journal articles) on the topic – must be relevant!
- Paraphrase information from the literature and cite the source; do not use direct quotations
- Final paragraph should contain the objectives of your experiment
- Use proper citation format (Name–Year format)
- Include the full citation information for each reference in a **Literature Cited Section.**

One goal of the introduction is to review current literature related to the topic of your experiment. Current literature comes in the form of scientific journal articles. These journal articles must be cited within the text but also in a **Literature Cited** section that appears at the end of your paper.

The **Literature Cited (or references) Section** is a list of the references cited in the various sections of the journal article. There are two general styles of citing references. In the (author, year) style, which is to be used in this course, the references are cited in the text using the authors' last names and the year of publication. Give the names of one or two authors, but for articles with three or more authors, use the name of the first author followed by *et al.* (*et alia*, which is Latin for "and others"). The articles should be listed in the Literature Cited section in alphabetical order. In the numerical style, the references are cited in the text using numbers in brackets, beginning with [1] and continuing to the last citation. The articles then should be listed in the Literature Cited section in numerical order.

B. Organization of the Scientific Literature

The scientific literature is organized in a hierarchical fashion.

- **Primary journal articles** (first level): Scientists who do research on cell biology or another area of science normally publish their results in scientific journals. These journals are organized, edited, and printed either by scientific societies or by commercial publishers. A typical journal article is 6-8 pages in length and describes the results of single focused investigation. The article usually contains an abstract, an introduction, a summary of the materials and methods, a section of results, a discussion, and a list of references. The results are commonly presented in the form of graphs or tables, although in the area of cell biology, photographs may be of particular importance. In the area of cell biology, the major primary journals include **Cell**, **Eukaryotic Cell**, **Experimental Cell Research**, **Journal of Biological Chemistry**, **Journal of Cell Biology**, and **Molecular Cell Biology**. However, many journals are quite broad in scope, and so an article related to a topic in cell biology may also be found in a genetics journal, a biochemistry journal, or a physiology journal. There are literally hundreds of journals in which thousands of articles are published each year.

Most of the references cited in BIO354 Lab Reports should be Primary Journal Articles!

- **Short review articles** (second level): Short reviews are summaries of the results related to a particular topic and are based on articles published in primary journals. Short reviews are usually written by active investigators in a field, and often reflect results from both their laboratory and other labs. These reviews usually focus on the work published in the last 2-3 years, and they often are designed to both indicate what is known and what is not known about that topic. Most short reviews are 4-8 pages in length, although some are shorter and some are longer. Short reviews often contain summary diagrams or cartoons that are designed to illustrate current concepts in that field. In the area of cell biology, short reviews commonly appear in some primary journals such as **Cell**. They are also are found in journals such as **Current Opinion in Cell Biology** and **Trends in Cell Biology** that consist entirely of short reviews, letters, and commentaries.

- **Major Review Articles** (third level): Like short reviews, major reviews are based on the results published in the primary literature and are usually written by key scientists in a particular field. However, major reviews tend to be much longer 20-50 pages and often tend to survey a field over a longer time frame (5-10 years). Major reviews often contain hundreds of references. In the area of cell biology, major reviews are commonly published in the **Annual Review of Cell Biology** and **Current Topics in Cellular Regulation**. Again, because they are often quite broad, review related to cell biology may also be found in review journals in Biochemistry, Plant or Animal Physiology, and Genetics.

- **Monographs** (fourth level): A monograph is usually a hard-bound book that is 100-300 pages in length. Some monographs are published as standalone volumes, but others are part of a continuing series such as the **Annals of the New York Academy of Sciences**. As with major reviews, a monograph tends to take a broad view of a topic, and while primary articles may be cited, it is also possible that many of the references reflect short or major reviews. In the area of cell biology, there are monographs on cell membranes, the cytoskeleton, mitochondria, chloroplasts, centrioles, cell division, and so on.

- **Textbooks** (fifth level): Most textbooks are surveys of a whole field such as cell biology, genetics, biochemistry, or animal physiology, and are often 800-1000 pages in length. Textbooks tend to focus on major, well-accepted concepts and are often lavishly illustrated. Most textbooks contain little experimental data and so are far removed from primary journal articles. The references in textbooks are often either to short or major reviews or to older but key papers from primary journals. Textbooks differ somewhat in complexity and level of detail. The cell biology book we are using is an intermediate-level book. There are some other very good texts in this field such as **Molecular Cell Biology** and **Molecular Biology of the Cell,** which are larger and more advanced.

C. Searching the Scientific Literature

The primary database used for search the scientific literature in cell biology is PubMed. PubMed is a very large data base that is run by the National Center for Biotechnology Information (NCBI) at the National Library of Medicine. The data base includes titles and abstracts of thousands of articles, and now has electronic links to many complete articles. Most of the journals used in the data base are available either in a hard copy form or electronically through the ASU library system.

Go to the following link to learn how to use PubMed to access journal articles:

https://sites.google.com/a/asu.edu/bio354/

The link contains a tutorial along with a short PubMed Quiz. Ask your instructor if this quiz is required for your laboratory section.

The following sections are from the following article:

Otomo, T., Schweizer, M, Kollmann, K. et al. 2015. Mannose 6 phosphorylation of lysosomal enzymes controls B cell functions. Journal of Cell Biology. 208(2): 171-180.

Mannose 6 phosphorylation of lysosomal enzymes controls B cell functions

Takanobu Otomo,[1,2] Michaela Schweizer,[3] Katrin Kollmann,[1] Valéa Schumacher,[4] Nicole Muschol,[1] Eva Tolosa,[4] Hans-Willi Mittrücker,[4] and Thomas Braulke[1]

[1]Department of Biochemistry, Children's Hospital; [3]Department of Electron Microscopy, Center for Molecular Neurobiology; and [4]Department of Immunology, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany
[2]Department of Pediatrics, Osaka University Graduate School of Medicine, 5650871 Suita, Osaka, Japan

Antigen processing and presentation and cytotoxic targeting depend on the activities of several lysosomal enzymes that require mannose 6-phosphate (M6P) sorting signals for efficient intracellular transport and localization. In this paper, we show that mice deficient in the formation of M6P residues exhibit significant loss of cathepsin proteases in B cells, leading to lysosomal dysfunction with accumulation of storage material, impaired antigen processing and presentation, and subsequent defects in B cell maturation and antibody production. The targeting of lysosomal and granular enzymes lacking M6P residues is less affected in dendritic cells and T cells and sufficient for maintenance of degradative and lytic functions. M6P deficiency also impairs serum immunoglobulin levels and antibody responses to vaccination in patients. Our data demonstrate the critical role of M6P-dependent transport routes for B cell functions in vivo and humoral immunity in mice and human.

First sentence introduces the reader to the topic.

Introductions will often present medical relevance (why should your reader care?)

Introduction

Lysosomes function in the degradation of macromolecules delivered by the biosynthetic, endocytic, or autophagic pathway and depend on the concerted action of ~60 lysosomal enzymes at low pH (Saftig and Klumperman, 2009; Schröder et al., 2010). Newly synthesized lysosomal hydrolases are modified on their N-linked oligosaccharides with mannose 6-phosphate (M6P) residues, which can be recognized by M6P-specific receptors in late Golgi compartments mediating their segregation from the secretory pathway and delivery to endosomal/lysosomal structures (Braulke and Bonifacino, 2009). The key enzyme in the formation of M6P residues is the *N*-acetylglucosamine-1-phosphotransferase complex consisting of three subunits that are encoded by two genes, *GNPTAB* and *GNPTG* (Reitman et al., 1981; Waheed et al., 1981; Bao et al., 1996; Raas-Rothschild et al., 2000; Kudo et al., 2005; Tiede et al., 2005). The loss of phosphotransferase activity in individuals with mucolipidosis II (MLII or I-cell disease), a rare lysosomal storage disease with an incidence of 1:650,000, prevents the formation of the M6P recognition marker, which subsequently leads to missorting and hypersecretion of multiple lysosomal enzymes associated with lysosomal dysfunction and accumulation of nondegraded material (Braulke et al., 2013). However, in certain cell types in MLII patients such as hepatocytes, Kupffer cells, or cytolytic lymphocytes, the absence of lysosomal storage material and nearly normal level of selected lysosomal enzymes were observed, suggesting the existence of alternate M6P-independent targeting pathways (Owada and Neufeld, 1982; Waheed et al., 1982; Griffiths and Isaaz, 1993; Glickman and Kornfeld, 1993). Data on the direct consequences of variable targeting efficiency of nonphosphorylated lysosomal enzymes on cell functions in vivo are lacking.

Previous mouse studies have demonstrated that in antigen-presenting cells several lysosomal enzymes, in particular cathepsin proteases, are implicated in the limited degradation of proteins destined for the major histocompatibility complex (MHC) class II processing pathway. Furthermore, cathepsins have been shown to be involved in the stepwise proteolytic removal of CD74 (invariant chain), which regulates the assembly,

First paragraph presents general information.

Describes other relevant studies that have been done.

ed.med.osaka-u.ac.jp; or

enetics, Osaka University
ka, Japan.

se; αMan, α-mannosidase; βGal, β-galactosidase; βHex, β-hexosaminidase; CFSE, carboxyfluorescein succinimidyl ester; Cts, cathepsin; DC, dendritic cell; Lamp1, lysosome-associated membrane protein 1; LPS, lipopolysaccharide; M6P, mannose 6-phosphate; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MLII, mucolipidosis II; OT-II, Ova-specific T cell receptor transgenic mice; Ova, ovalbumin; WT, wild-type.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 208 No. 2 171–180
www.jcb.org/cgi/doi/10.1083/jcb.201407077

Supplemental material can be found at:
http://jcb.rupress.org/content/suppl/2015/01/15/jcb.201407077.DC1.html
Original image data can be found at:
http://jcb-dataviewer.rupress.org/jcb/browse/9152

peptide loading, and export of MHC II molecules for recognition by CD4 T cells (Riese et al., 1998; Driessen et al., 1999; Nakagawa et al., 1998, 1999; Honey and Rudensky, 2003).

To examine the significance of variable targeting efficiencies of lysosomal enzymes in the absence of phosphotransferase activity on cells of the immune system in vivo, *Gnptab* knock-in mice (MLII mice) were analyzed. These mice mimic the clinical symptoms of MLII patients (Kollmann et al., 2012, 2013) and we find that the levels of lysosomal proteases are severely decreased in MLII B cells and impair the proliferation, differentiation, and antigen presentation as well as their interaction with T helper cells, resulting in reduced immunoglobulin production. Compared with MLII B cells, MLII T and dendritic cells (DCs) maintained higher lysosomal protease activities, and their cell functions were only moderately affected. Importantly, defective humoral immunity was also observed in MLII patients.

References are cited within text using the Author-Year format

Last paragraph states the objective of the study

Last paragraph summarizes findings of the study

References

Full citations are listed in the References section.

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Citations are in alphabetical order based on first author's last name.

Note that all of the references are scientific journal articles (Not Textbooks or Websites)