MASSACHUSETTS INSTITUTE OF TECHNOLOGY
Department of Chemistry
5.310 Laboratory Chemistry

EXPERIMENT #5[1]

A Study of the Kinetics of the Enzyme Catalase and its Reaction With H_2O_2.[2] A Further Study on Protein Assay Quantitation of Catalase[3]

I. OVERVIEW OF THE EXPERIMENT

In this experiment, the student will investigate the enzyme activity of catalase by studying the decomposition of H_2O_2 to form water and oxygen. Using an oxygen based pressure sensor the student measures the amount of oxygen produced and then calculates the rate of the enzyme catalyzed reaction under various conditions. The student also completes a Protein Assay on an unknown sample of catalase using the Coomassie® Plus Protein Assay from Pierce to determine the protein concentration of the sample. The correlation between the actual and calculated concentration gives an indication of the experimental skills used in carrying out the experiment.

II. OBJECTIVES

This is an integrated experiment that includes topics from physical chemistry and biochemistry. It is designed to introduce students to the basics of:

- Studying the effects of reaction environment, including temperature, and varying substrate concentration on the rate of an enzyme-catalyzed reaction.
- Combining physical chemistry and biochemistry, principles and theory, with the goal of determining various biochemical and biophysical constants for the enzyme catalase.
- How to acquire experimental kinetic data for an enzyme catalyzed reaction.
- Learning how to perform data manipulation in order to extract out information such as rate constants from experimental kinetic data.
- Correct handling of UV-VIS Spectroscopy

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[1] This experiment was synthesized by John J. Dolhun by synthesizing various contributions from course textbooks, current literature, and others affiliated with course 5.310 and 5.301, and updated again on (Jun, 2019).

[2] Apparatus adapted and modified from: Lewis, M. E.; Levine, R. M.; York, J. T.; Grubbs, W. T., *Journal of Chemical Education*, (2009) 86, 1227-1230.

[3] Adapted from: Massachusetts Institute of Technology, Department of Chemistry, 5.301 Chemistry Laboratory Techniques, *Protein Assays and Error Analysis*, 2009.

- Operating an Eppendorf Centrifuge and a Pipettman
- Preparation of standard solutions

III. EXPERIMENT BACKGROUND

General References:

SFH: Steinfeld, J. I., Francisco, J. S. and Hase, *Chemical Kinetics and Dynamics*, 2nd Ed., Prentice Hall, (1999). (Available on request from the Chemistry Department Undergraduate Lab Reading Room in 4-449b. See John Dolhun)

• Definition of the Rate of a Chemical Reaction	**SFH** pp. 1-3
• Order and Molecularity of a Reaction	**SFH** pp. 3-6
• Reaction Mechanisms	**SFH** pp. 17-18
• Enzyme-Catalyzed Reactions	**SFH** pp. 159-163
• Measuring Mass and Volume	**MHS** pp. 38-46
• Heating and Cooling Methods	**MHS** pp. 49-58
• Rates of Chemical Reaction	**SWH** pp. 879-884
• Enzyme-Catalyzed Reactions	**SWH** pp. 885-892

Videos: Digital Techniques Manual

1. Volumetric Techniques (pipette, volumetric flask)
2. Balances

1. INTRODUCTION CHEMICAL KINETICS

One of the main goals of chemical kinetics is to understand the steps by which a reaction takes place and how reaction conditions such as temperature and concentration affect the rate at which reactions occur. The series of elementary steps is the reaction mechanism. Understanding the mechanism allows us to find ways to manipulate the reaction. The rate of a chemical reaction depends on the temperature, pressure, pH and concentration of the reactants. From a study of the rate of a reaction, information can be obtained about the steps by which reactants are transformed into products. For a typical reaction:

$$aA + bB \rightarrow cC + dD$$

the rate of the reaction, v, can be written as:

$$rate = v = -\frac{1}{a}\frac{d[A]}{dt} = -\frac{1}{b}\frac{d[B]}{dt} = \frac{1}{c}\frac{d[C]}{dt} = \frac{1}{d}\frac{d[D]}{dt}$$

The rate law, or differential rate law, can also show how the rate of a reaction is a function of the concentrations of the reactants:

$$v = k\ [A]^x\ [B]^y$$

where in this rate equation, k is the rate constant, and x and y represents the order of the reaction with respect to reactants A and B. Please note that x and y are not related directly to the stoichiometric coefficients a and b in the balanced equation but are independent coefficients that must be determined experimentally.

2. THE DECOMPOSITION OF HYDROGEN PEROXIDE

Hydrogen peroxide is decomposed by the enzyme catalase to water and oxygen:

$$2H_2O_2(aq) \xrightarrow{catalase} 2H_2O(l) + O_2(g)$$

The rate of the decomposition of hydrogen peroxide, the rate at which oxygen is generated and the overall rate of the reaction are related by the stoichiometry of the reaction. The rate law for the above reaction is defined as:

$$-\frac{1}{2}\frac{\Delta H_2O_2}{\Delta t} = \frac{\Delta O_2}{\Delta t} = \text{rate of reaction}$$

For us, it is much easier to determine the amount of oxygen produced rather than the amount of hydrogen peroxide that remains in solution so for our purposes we will study the kinetics of this reaction by monitoring the rate that oxygen forms. We can do this in one of two ways, by measuring the volume of oxygen that is produced at a constant pressure or by measuring the pressure of oxygen produced under conditions of constant volume. We will use the latter method and follow the rate of the catalase decomposition of hydrogen peroxide by measuring the pressure of O_2 produced under conditions of constant volume. One method of measuring the pressure of O_2 produced is to attach a pressure sensor to the reaction flask and measure changes in pressure with time. From this data, we can obtain a graph of the pressure of oxygen generated versus time. The rate of the oxygen formation is just the slope of the curve on the plot $\Delta P/\Delta t$. The slope will be

changing throughout the course of the reaction and is not constant because it depends on the temperature and the amount of hydrogen peroxide remaining in solution at any given time. In the case of O_2 formed, the pressure measured can be readily converted back to concentration using the ideal gas equation[4].

$$PV=nRT$$

that is:

$$P = \frac{n}{V} RT = [O_2]RT$$

In terms of the Kinetics of the reaction and rate of oxygen gas production:

$$rate = \frac{d[O2]}{dt} = \frac{1}{RT} \frac{d[P]}{dt}$$

Plotting P v. Time and from the increase in oxygen pressure with time we can relate that back to the concentration of O_2 present (M/s).

[4] Raymond Chang, *Chemistry*, 9th Edition, McGraw Hill, (2007) pp. 550-551.

3. ENZYME KINETICS[5]

An Enzyme or high molecular weight protein is synthesized in living cells and is made up mainly of long chains of amino acids connected by peptide bonds.
Enzymes are biochemical catalysts that can accelerate reactions by lowering the activation energy, thereby, speeding up the reaction without being used up or changed. Initially, an enzyme combines temporarily with a substrate molecule to form an enzyme-substrate complex, which proceeds to break up and release the product(s) plus the unchanged enzyme. The released enzyme then locates another substrate molecule to continue its catalytic process. A reaction for a typical substrate and enzyme is illustrated below showing the intermediate enzyme-substrate complex, the products and the released enzyme.

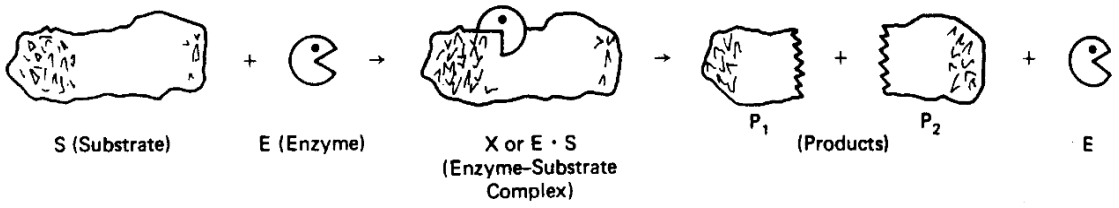

Figure 1. A Schematic Representation of an Enzyme-Catalyzed Mechanism[6]

Enzyme Kinetics involves the study of enzyme-catalyzed reactions, which may involve the study of the reaction rate and its dependence on substrate concentration, temperature, pH or a host of other variables. The enzyme-substrate complex is an intermediate in the reaction, and generally its presence in low concentrations makes it difficult to isolate.

In 1913 two researchers Michaelis and Menten proposed a theoretical way to study the mechanism of enzyme kinetics. The theory proposes that when an enzyme acts upon a substrate molecule, an enzyme-substrate complex forms and that this complex yields the product(s) in addition to the original unchanged enzyme. If the substrate is present in excess over the enzyme, the enzyme would get used up and form enzyme-substrate complexes. The rate in producing products becomes constant when a condition of steady state is reached.

Many one-substrate reactions follow this simple kinetic scheme, knows as the Michaelis-Menten mechanism.

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[5] Kinetics discussion adapted and modified from: Massachusetts Institute of Technology, Department of Chemistry, 5.310 Laboratory Chemistry, *Kinetics Of An Enzyme – Catalyzed Reaction*, Spring, 1993.
[6] Steinfeld, J. I., Francisco, J. S. and Hase, *Chemical Kinetics and Dynamics*, 2nd Ed., Prentice Hall, (1999) pp. 159-163.

4. CATALASE

Catalase was chosen for this study because of its presence in the aerobic cells of most living organisms. It's easy to qualitatively detect its presence in our blood if we get cut. When we apply hydrogen peroxide to the cut, we can see bubbles forming at the interface of the cut and our skin. This is the result of the enzyme catalase present in our blood breaking the hydrogen peroxide down into water and oxygen on the surface of our skin. We all produce a small amount of hydrogen peroxide as a by-product of our metabolism in a cellular membrane called peroxisome. During the normal course of oxidation/reduction reactions that take place in the human body, electrons are given up and some of those electrons promote the reduction of oxygen to hydrogen peroxide. Catalase present in our cells then catalyzes the breakdown of this hydrogen peroxide into oxygen and water in a disproportionation reaction. If the catalase were not present, the hydrogen peroxide produced in this normal metabolic process would build up and cause oxidative damage to our sensitive cellular components.

Catalase is a large quaternary protein, a tetramer, consisting of four large subunits. Each active subunit contains over 500 amino acids with a heme, a prosthetic group consisting of a protoporphyrin ring with a central iron at its center, similar to other quaternary type proteins such as hemoglobin.

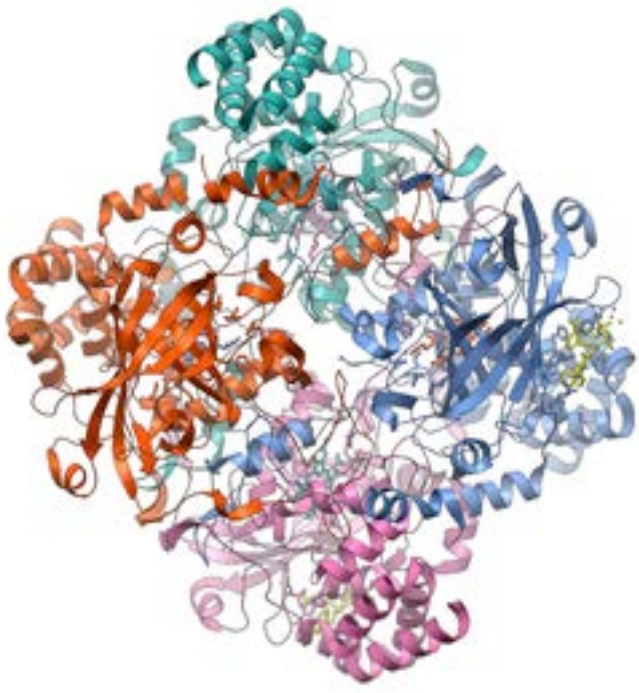

Figure 4. Three-Dimensional Structure of Catalase from PDB[7]

[7] Diagram taken from: Worldwide Protein Data Bank (PDB): www.wwpdb.org/docs.html

III SAFETY[8]

Students will handle a number of chemicals during this experiment, many of which must be treated with care in order to avoid harm. None of the chemicals listed here should be ingested or allowed to come in contact with your skin or eyes. The TAs will provide additional safety information and procedures during the weekly pre-laboratory lectures for this module.

1. **Sodium dihydrogen phosphate monohydrate:** An irritant. Target organs include eyes, skin and respiratory tract. Can produce delayed pulmonary edema.

2. **Catalase from bovine liver culture:** Classified as non-hazardous. However, do not breathe the dust. Treat it as a potential biological hazard. Incompatible with strong oxidizing agents. Air and moisture sensitive.

3. **Bovine Serum Albumin (BSA):** No dangerous reactions known. No adverse health effects. Large doses may cause gastro-intestinal upset.

4. **MOPS Buffer:** May be harmful if swallowed, inhaled or absorbed through the skin. May cause irritation to skin, eyes, and respiratory tract.

5. **Coomassie® Dye:** Potential and acute chronic health effects are unknown. Avoid contact with eyes, skin and clothing. No known significant hazards.

6. **30% Hydrogen Peroxide:** A dangerous strong oxidizer. Contact with other material may cause fire. Corrosive. Causes severe burns to skin, eyes and respiratory tract. Harmful if swallowed or inhaled. Use gloves when pouring.

7. **Tris HCl Buffer:** May cause irritation to the respiratory tract. Symptoms may include coughing and shortness of breath. Skin irritant producing redness and pain. Chronic dermatitis may follow skin contact.

[8] Various Material Safety Data Sheets: Malinckrodt Chemicals, J. T. Baker, Phillipsburg, NJ; Fischer Scientific, MSDS, Pittsburgh, PA

Day #1: A Study of the Kinetics of the Enzyme Catalase in the Decomposition of Hydrogen Peroxide

In Day #1 of the experiment the catalytic decomposition of hydrogen peroxide by the enzyme catalase is investigated:

$$2H_2O_2(aq) \xrightarrow{catalase} O_2(g) + 2H_2O(l)$$

The catalase hydrogen peroxide reaction will be studied and the order of the reaction will be determined with respect to hydrogen peroxide. We will use the order of the reaction determined in Day 1 to calculate the rate constant k on Day 2 when we conduct a temperature study to determine the energy of activation for the reaction from an Arrhenius plot. The reaction will be followed by monitoring the pressure in a reaction tube using a low-cost computer interfaced gas pressure sensor by which we can monitor the pressure increase inside the closed off reaction tube. The initial rate of the reaction is simply the maximum slope of the curve resulting from the plot of pressure of oxygen generated versus time immediately after the reaction begins. The rate during the first minute or so should appear to increase at a relatively constant rate giving the graph a linear appearance. We will determine the rate by finding the maximum slope of this line, which should represent a good approximation of the initial rate.

The order of the reaction will be determined by running a series of experiments varying the concentration of hydrogen peroxide and finding the initial rates for each of five reactions. We will do a graphical plot of the data from a series of five reactions to determine the order of the reaction (a) as represented in the rate equation below:

$$rate = k[H_2O_2]_0^a$$

$$\ln(rate) = a\ln([H_2O_2]_0) + \ln k$$

A plot of the natural log of the rate versus the natural log of the initial hydrogen peroxide concentration from each of the five trials should give us a straight line with the slope = a the order of the reaction with respect to hydrogen peroxide.

The units for rate will initially be in (M/s) [oxygen] generated per second. Using the stoichiometry of the equation and the Volume of gas (V_g) and Volume of solution (V_s) we can readily convert this into M/s in terms of hydrogen peroxide decomposing.

In order to determine the value for the rate constant (k) the units for the rate and the units for the concentration of hydrogen peroxide have to have some consistency. Once the

units of the rate and concentration are consistent a value for k can be calculated as follows:

$$k = \frac{rate}{[H_2O_2]_0^a}$$

In general, for a first order reaction we can write an expression for the differential rate law as follows:

$$A \rightarrow \text{Pr}oducts$$
$$Rate = -\frac{\Delta[A]_t}{\Delta t} = k[A]_0$$

Using calculus, we can derive the integrated rate law:

$$\ln\frac{[A]_t}{[A]_0} = -kt$$

Note the units for k for a first order reaction:

$$k = \frac{rate}{[A]_0} = \frac{M/_s}{M} = \frac{1}{s}$$

Similarly, for a second order reaction we can write an expression for the differential rate law:

$$A \rightarrow \text{Pr}oducts$$
$$Rate = \frac{\Delta[A]_t}{\Delta t} = k[A]_0^2$$

Using calculus, we can derive an integrated rate law:

$$\frac{1}{[A]_t} = kt + \frac{1}{[A]_0}$$

Note the units for k for a second order reaction:

$$k = \frac{rate}{[A]_0^2} = \frac{M/_s}{M^2} = \frac{liter}{mole - \text{sec}}$$

In both of the above cases its important to note that the units for rate are M/s to make sense in calculating the rate constant.

Prior to the start of the lab, the TA will prepare the following solutions:

1. **TAs prepare Stock Phosphate Buffer Solution**—Dissolve 27.2 grams of NaH2PO4 x H2O in 2 Liter of distilled water. Adjust the pH to 6.8 by slowly adding concentrated NaOH with stirring. This should yield a 2 Liter pH=6.8 Phosphate Buffer Solution approximately 0.1 M. TAs should plan on about 0.75 Liter of Stock Buffer Solution for each student team for the Day 1 & 2 Experiments.
2. **TAs prepare Stock Catalase Enzyme Solution**—Dissolve approximately 35 mg of purified catalase from bovine liver culture into 200 mL of the above phosphate buffer stock solution. This solution must be prepared fresh at the start of each experiment, as catalase is known to decompose via aerobic oxidation. TAs should calculate on having about 15 mL per student team on Day 1 and 15 mL per student team on Day 2. Enzyme should be prepared in the air-conditioned catalase room and kept there for the duration of the lab.

Experiment Outline:

Preparation of Working Solutions[9]:

1. **Student prepares working solution of H_2O_2**—Pour out 13.3 mL of 30% hydrogen peroxide solution and dilute to 100 mL in a volumetric flask with the above phosphate buffer solution. This solution must be prepared immediately on the day of the experiment. This should yield 100 mL of a 4% H_2O_2 stock solution.
2. **Student prepares working solution of Phosphate Buffer**—33 mL of distilled water is mixed with 217 mL of the above stock phosphate buffer solution, yielding 250 mL of a working buffer solution containing the same concentration of phosphate as the stock H_2O_2 solution.

Equipment[10]:

Each Team will set up an apparatus similar to that shown in Figure 4 below. The apparatus consists of a glass closed pressure tube connected to an electronic pressure sensor that is interfaced to the student / lab computer. The gas pressure tube where the reaction takes place can be submerged in a large beaker of room temperature water to keep the reaction mixture at a constant temperature. The volume of the reacting mixture occupies about a third of the entire volume of the tube so that the reaction can be stirred

[9] Solutions modified from supplement,
cited in Lewis, M. E.; Levine, R. M.; York, J. T.; Grubbs, W. T., *Journal of Chemical Education*, (2009) 86, 1227-1230.

[10] Equipment adapted and modified from supplement,
cited in Lewis, M. E.; Levine, R. M.; York, J. T.; Grubbs, W. T., *Journal of Chemical Education*, (2009) 86, 1227-1230.

vigorously with a small stir bar to help promote the rapid evolution of O_2 (g). In this experiment, we will use a Vernier gas pressure sensor (#GPS-BTA) connected to a Go-Link computer interface, the interface is connected directly to a computer, which will be fitted with Logger Pro data acquisition software.

Method:

Each team as assigned by the TA will set up the apparatus shown in Figure 5.

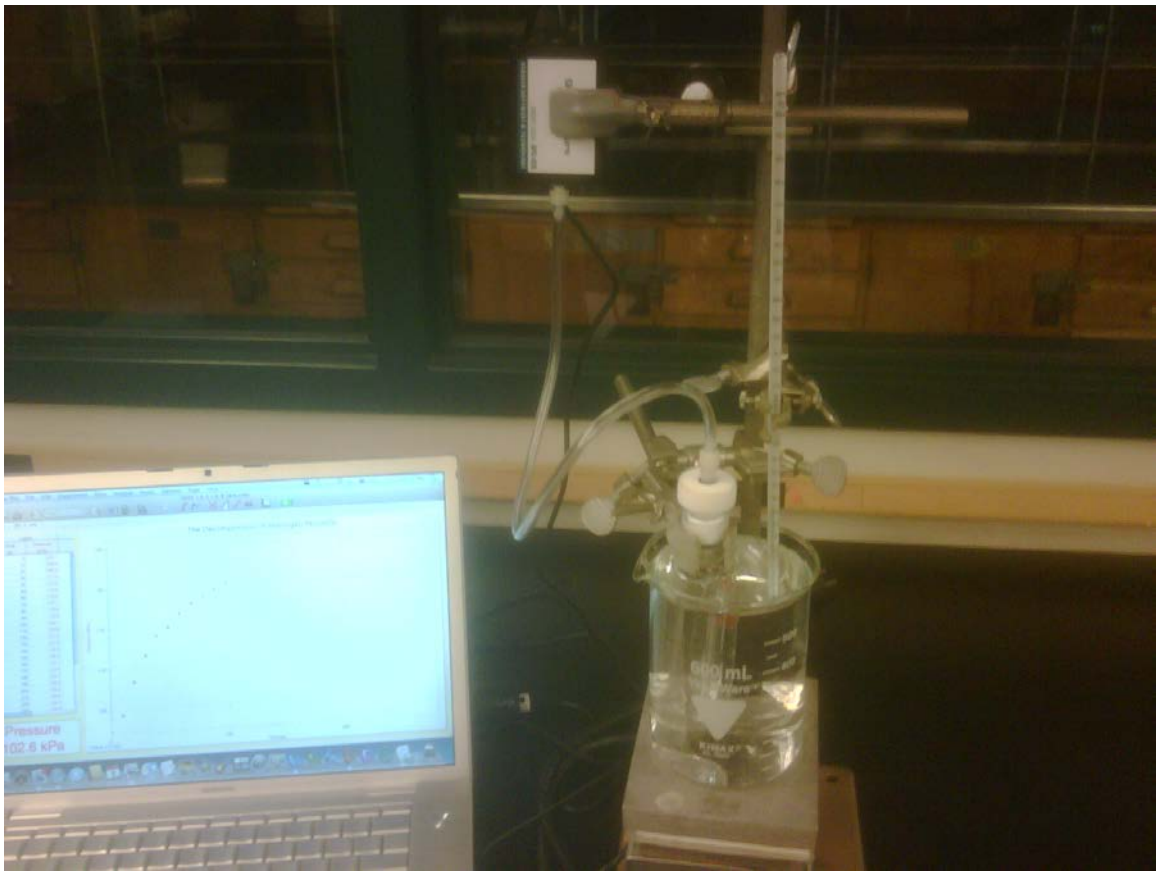

Figure 5. Apparatus for Measuring Kinetics of Catalase-H_2O_2 Decomposition[11]

The reaction vessel consists of a glass pressure tube that can be sealed quickly by spinning on a Teflon threaded plug that is fitted with a rubber O-ring. The Teflon plug has another threaded outlet that is custom fitted with two O-rings and connects directly to

[11]Adapted and modified from: Lewis, M. E.; Levine, R. M.; York, J. T.; Grubbs, W. T., *Journal of Chemical Education*, (2009) 86, 1227-1230.

the computer interfaced gas pressure probe. About 80-90% of the pressure tube can be submerged in a 600-mL beaker of water during the collection of data to facilitate a constant temperature during each of the kinetic runs. Obtain room temperature water and set up the bath in a 600-mL beaker. Use a thermometer to measure the temperature of the water bath. Record this temperature into your laboratory notebooks. You should monitor the temperature of the water bath constantly throughout the entire experiment. A stir bar and magnetic stir plate is used to vigorously stir the reaction contents inside the pressure tube to promote the rapid evolution of O_2 (g). The stir bar rate of stirring should be set before adding the enzyme and should not be changed during the collection of data. The Gas Pressure sensor is connected to a Vernier computer interface Go-Link connector that plugs directly into any computer. Connect the gas pressure sensor to the top of the Teflon® threaded interface. Start the Logger Pro data-monitoring program on computer and open the appropriate collection file called Module 12 Peroxide in the Advanced Chemistry folder.

Each team will run five trial runs, which vary the concentration of the hydrogen peroxide keeping the concentration of the enzyme constant. The following data Table 1 illustrates suggested volumes of stock reagents, which can be used in each trial. The individual teams could vary these concentrations slightly then calculate the corresponding concentration for the hydrogen peroxide solutions used based on the stock reagents tabulated.

Solution I		Solution II			
Stock H_2O_2 mL	Phosphate Buffer mL	Stock Enzyme mL	Total Volume mL	$[H_2O_2]$ mol/L	Reaction Rate Ms^{-1}
0.25	23.75	1.00	25.00		
0.50	23.50	1.00	25.00		
1.00	23.00	1.00	25.00		
1.50	22.50	1.00	25.00		
2.00	22.00	1.00	25.00		

Table 1 Suggested Volumes of Stock reagents for the Kinetics Decomposition of H_2O_2.

To begin, remove the pressure tube from the water bath and unscrew the threaded Teflon® plug and seal, now, insert the stir bar into the pressure tube along with the first volume of Solution I which includes the stock H_2O_2 (add this volume to the pressure tube using a digital pipette) anted the Phosphate buffer (add this volume to the pressure tube using a 25 mL glass pipet). Now carefully screw the Teflon plug onto the pressure tube and place it into the apparatus 600 mL beaker water bath clamping it near the top edge of the white Teflon threaded plug so that the pressure tube is as far down into the 600 mL beaker as you can get close to the magnet for effective contact with the stir bar. Start your stir bar for a constant stir rate. The rate of stirring must not change during the entire

experiment otherwise, you will experience drops in pressure that will cause dips in your graph. You should try to use the same stir bar rate for each trial keeping the dial set at that particular rate. Practice setting the stir rate, making sure it remains fixed for the duration of each run. The next part must be done quickly. Using a digital pipette, inject 1.00 mL of stock solution of catalase Solution II directly through the hole in the luer lock on the Teflon plug using an automatic 1000 μL pipettor, the hole is then quickly closed by connecting the luer lock fitting to the Vernier gas probe with a gentle turn. When you attach the luer lock from the pressure sensor to the luer lock in the Teflon plug be careful not to twist tighten otherwise you will end up stripping the connection and the tube will have a leak. Make sure that you set the stir rate for maximum stirring before you inject the enzyme and leave it constant throughout the entire reaction. When you inject the enzyme, the pipette must be held firmly straight with the tip placed into the open luer lock, then, a quick injection of the enzyme. If the pipette is not firmly in place or crooked the enzyme will shoot out all over the outside of the reaction vessel and you will need to repeat. Practice with water several times to make sure you are comfortable injecting the enzyme into the reaction tube. As soon as the enzyme has been added to the pressure tube and you have snapped on the gas probe connector click collect to begin data collection. The entire process of injecting the enzyme, closing the luer lock and pressing start on the computer should take place in 1-2 seconds. I believe the software is designed to run for 5 minutes. If your pressure curve is not rising during the run you may have a leak and you should call this to the attention of your TA and figure out where the leak may be coming from. After collecting the data when the time has elapsed, carefully release the luer lock connection and unscrew the Teflon® cap to relieve the pressure in the tube. Remove the tube from the water bath and dispose of the contents into the appropriate waste collection flask for this module, which the TAs will set up on the lab bench. Save the data set to a disc drive or appropriate file on the computer. Rinse and clean the pressure tube with distilled water and prepare for the second thru the fifth trials as above. At the end of running the five trials you can begin to analyze the graphs with the data analysis options included with the software. Carefully examine the graph, and select a point just beyond the initial flat upward inflection portion and use the data analysis software to determine the slope of the tangent line (m) corresponding to the steepest point in your pressure v. time curve. You can click the Linear Regression button on the software program to calculate the best-fit line equation that fits the graph at the steepest slope. The slope of the tangent line is your initial rate of your reaction and should be recorded into your laboratory notebooks.

Analysis and Discussion

1. Using the volumes of stock solutions, in Table 1, calculate the molar concentrations for the H_2O_2 in each trial.
2. Calculate the air space volume above your reacting solution in the pressure-tube you used (at the temperature of your water bath) to do this mass the empty tube and Teflon cap, fill the tube with water and close the Teflon® threaded cap, then mass the tube again filled with water, take the temperature of the water and use the density of water to calculate the total volume of water that filled the tube, subtract the 25.00 mL you used for your reactions and you should now have the volume of the airspace above your reacting solution. Be sure to include the volume of air space in the tube that is connected to the pressure sensor (3.62 mL). The total air space volume calculated represents the volume of O_2 (g) collected V_g.
3. Calculate the initial rate of oxygen formation for each trial, which is simply the maximum slope of the curve (m) in your plot of Pressure v. Time. This slope, will have units of kPa/sec, and is the initial rate for the reaction.
4. Calculate the initial rate of reaction for each trial in units of M/s by dividing the observed slope of the tangent line (m) from (3) above by RT. (R=8.314 L-kPa/mole-K). You should end up with the rate of formation of Oxygen $d[O_2]/dt$ in (M/s).
5. Using the Vg, Vs, and the stoichiometry of the reaction convert the above rate in (4) into the rate hydrogen peroxide used up ($- d[H_2O_2]/dt$) (M/s).
6. Construct a table of your results include the mass of water in your pressure tube (g), the temperature of the water (^{0}C), density of water from Chemistry and Physics Handbook (g/mL), Volume of the Pressure tube (mL), Volume of solution V_s, (mL), Volume of Gas, V_g (mL), Room Pressure, 4% H_2O_2 volumes (mL), $[H_2O_2]$ (M), Temperature for each trial (^{0}C) & (K), Rate (kPa/s), Rate O_2 M/s, Rate O_2 mole/s, Rate H_2O_2 mole/s, Rate M/s with respect to H_2O_2.
7. Do a plot of ln(Rate) v. $\ln[H_2O_2]_0$ and determine the order for the reaction.

$$\text{rate}_o = k[H_2O_2]_o^a$$

$$\ln(\text{rate}_o) = \ln(k * [H_2O_2]_o^a)$$

$$\ln(\text{rate}_o) = a * \ln([H_2O_2]_o) + \ln(k)$$

$$y = m * x + b$$

Day #2: Determination of the Activation Energy for the Catalase-H_2O_2 Complex[12]

Enzymes principally act as catalysts increasing the rates at which reactions take place making the conversion of reactants to products go faster. One way that enzymes do this is by lowering the activation energy necessary for a spontaneous reaction to proceed on its own by helping to bring the reactants closer together. An important part of any kinetic analysis is the determination of this activation energy E_a. Activation Energy is the spark of energy necessary to help a spontaneous reaction get started so that it can proceed to form products on its own without any further input of energy. Molecules must have enough effective collisions to make it over an energy hump that needs to get the reaction going. This is the activation energy (E_a) we are talking about. An uncatalyzed reaction would require higher activation energy than a catalyzed reaction.

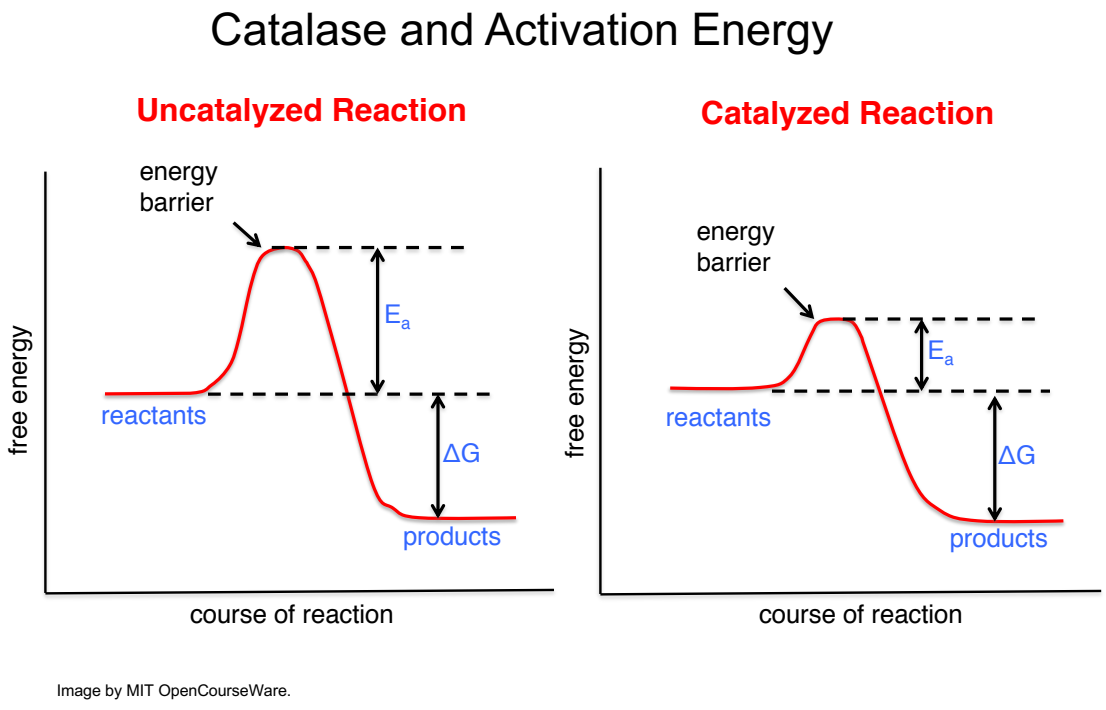

Image by MIT OpenCourseWare.

Figure 5. Free Energy diagram monitoring the progress of a catalyzed and uncatalyzed reaction[13].

[12] Equation derivations: Raymond Chang, *Chemistry*, 9th edition, McGraw Hill, 2007, p. 571.

The dependence of the rate constant on activation energy and temperature can be expressed in the Arrhenius Equation:

$$k = A\ e^{-Ea/RT}$$

Ea = activation energy KJ/mole
R = universal gas constant 8.314 J/K-mol
T = absolute temperature in Kelvin (K)
A = collision frequency factor

Rate constants for most chemical reactions follow the above equation. Given the temperature this equation allows us to determine the activation energy required to initiate a reaction. The form of the above equation can be re-written in logarithmic form which provides a useful working equation that relates k and T. Taking the natural logarithm of both sides of the above we arrive at the following:

$$\ln k = \left(-\frac{E_a}{R}\right)\left(\frac{1}{T}\right) + \ln A$$

The equation now resembles an equation of a straight-line y = mx + b.
Using excel to plot ln k vs. 1/T yields a straight line with a slope m = $-E_a/R$. The activation energy for the reaction can be determined from the slop of this line.

To determine the activation energy, we will measure the rate constants taken at seven different temperatures. We will monitor the reaction at different temperatures, while keeping the initial concentrations of the reactants identical for each trial. This will allow us to study and measure the effect of changing temperature on our rate of reaction. From the data obtained the student will be able to calculate the activation energy, E_a, for the reaction.

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[13] Diagram modified from: Donald & Judith Voet, *Biochemistry*, 3rd. edition, John Wiley and Sons, Inc., 2004, p. 477 (showing the effect of a catalyst on the transition state diagram of a reaction).

Method[14]:

Each team as assigned by the TA will set up the apparatus used on Day 1 as illustrated in Figure 4. The reaction vessel consists of a glass pressure tube that can be sealed quickly by spinning on a Teflon threaded plug that is fitted with a rubber O-ring. The Teflon plug has another threaded outlet that connects via a luer lock directly to the computer interfaced gas pressure probe. About 80-90% of the pressure tube is submerged in a 600 mL beaker of water during the collection of data to facilitate a constant temperature during each of the kinetic runs. First, heat the water bath up to a temperature between 35 to 40 ^{0}C in the 600 mL beaker. Use a thermometer to measure the temperature of the water bath. Record this temperature into your laboratory notebooks. We will assume that the water bath temperature remains constant throughout the entire experiment. You will need to add ice and heat as necessary to maintain the constant temperature. A stir bar and magnetic stir plate is used to vigorously stir the reaction contents inside the pressure tube to promote the rapid evolution of O_2 (g). The stir bar setting should be set at the same rate for all four trials. Connect a Gas Pressure sensor to the Go-Link computer interface connector and connect that to the computer. Connect the gas pressure sensor to the luer lock connection at the top of the Teflon® threaded interface. Start the Logger Pro data-monitoring program on your computer and open the appropriate collection file, which we will be found under Advanced Chemistry Module 12 Peroxide.

Each team will run seven trial runs, which vary the temperature of your reaction vessel and you can pick any temperature within the following ranges, 5 to 10 ^{0}C, 10 to 15 ^{0}C, 15 to 20 ^{0}C, 20 to 25 ^{0}C, 25 to 30 ^{0}C, 30 to 35 ^{0}C, 35 to 40 ^{0}C, then, choose a stock H_2O_2 of 1.00 mL and a phosphate buffer of 23.00 mL with stock enzyme of 1.00 mL for a total volume of 25.00 mL. Or, choose another stock solution that you will keep constant throughout the temperature study. We will call this our standard stock solution.

After the temperature is stabilized in the range of 35 to 40 ^{0}C, remove the pressure tube from the water bath and unscrew the threaded Teflon® plug and seal, now, insert the stir bar into the pressure tube along with the first volume of Solution I which includes the stock H_2O_2 and the Phosphate buffer. You should allow Solution I time to equilibrate to the temperature in the pressure tube. The easiest and quickest means to handle the next part is to screw the Teflon plug onto the pressure tube then, inject 1.00 mL of stock solution of catalase Solution II directly through the luer lock hole in the Teflon plug using an automatic 1000 μL pipettor. Make sure you hold the pipette tightly in the luer lock hole so that when you inject you don't have any backflow out of the connection. Connecting to the Vernier gas probe then quickly closes the hole. Make sure that you set the stir rate for maximum stirring before you inject the enzyme and leave it constant throughout the entire reaction and all of the subsequent temperature studies. As soon as the enzyme has been added to the pressure tube and you have snapped on the gas probe connector, click collect to begin data collection. The software is designed to run for approximately 5 minutes, however, during the run click on "Experiment" at the top of the

[14] Use of the pressure tube technique adopted from: Lewis, M. E.; Levine, R. M.; York, J. T.; Grubbs, W. T., *Journal of Chemical Education*, (2009) 86, 1227-1230.

screen and then click extend run to 450 seconds as this will give you the best data for your file. After collecting the data when the time has elapsed, carefully unscrew the Teflon® cap to relieve the pressure in the tube. Remove the tube from the water bath and dispose of the contents into the appropriate waste collection flask for this module. Save the data set to a disc drive or appropriate file on the computer. Rinse and clean the pressure tube with distilled water and prepare for the second thru the seventh trials as above. At the end of running the seven trials you can begin to analyze the graphs with the data analysis options included with the software. Carefully examine the graph, and select a point just beyond the initial flat upward inflection portion. Use the data analysis software to determine the slope of the tangent line (m) corresponding to the steepest point in your pressure v. time curve. Click the Linear Regression button on the software program to calculate the best-fit line equation that fits the graph at the steepest slope. The slope of the tangent line is your initial rate of your reaction and should be recorded into your laboratory notebooks. You need to convert this rate, which is KPa/sec oxygen formed into M/sec of hydrogen peroxide decomposing. This is critical because you will be using this rate to calculate the rate constant k for each trial. You may if you prefer, use Microsoft Excel to do your data analysis.

Analysis and Discussion (Be sure to include/discuss these in your report)

1. Using the volume of stock solution, you used from Table 1 calculate the molar concentration of H_2O_2 in your trials.
2. Calculate the initial rate of oxygen formation for each trial, which is simply the maximum slope of the curve (m) in your plot of Pressure v. Time. This slope, will have units of kPa/sec, and is the initial rate for the reaction.
3. Calculate the initial rate of reaction for each trial in units of M/s by dividing the observed slope of the tangent line (m) from (2) above by RT. (R=8.314 L-kPa/mole-K). You should end up with the rate of formation of Oxygen $d[O_2]/dt$ in (M/s).
4. Using the V_g, V_s, and the stoichiometry of the reaction convert the above rate in (3) into the rate hydrogen peroxide used up ($- d[H_2O_2]/dt$) (M/s).
5. Construct a table of your results include the mass of water in your pressure tube (g), the temperature of the water (^{0}C), density of water from Chemistry and Physics Handbook (g/mL), Volume of the Pressure tube (mL), Volume of solution V_s, (mL), Volume of Gas, V_g (mL), Room Pressure, 4% H_2O_2 volumes (mL), $[H_2O_2]$ (M), Temperature for each trial (^{0}C) & (K), Rate (kPa/s), Rate O_2 M/s, Rate O_2 mole/s, Rate H_2O_2 mole/s, Rate M/s with respect to H_2O_2.
6. Calculate the rate constant for each trial by dividing the rate in M/sec with respect to H_2O_2 by the concentration of H_2O_2 raised to the power representing the order of the reaction that you calculated from Day 1.

$$k = \frac{rate}{[H_2O_2]_o^a}$$

7. Using Excel or Log Pro, create a graph of ln k vs. 1/T. Add a trendline to your plotted data, and obtain both the equation of the trendline and R^2 value. Be sure to include a printout of this graph in your Appendix of the lab report. Use the graph and equation generated to determine the activation energy, Ea in kJ/mole, and the frequency factor A, for this reaction. Show your calculations.
8. Briefly comment on what impact you think the catalyst had on the activation energy of this specific reaction. Discuss and explain.
9. What is the effect of changing the temperature of the solution on the reaction rate? Explain.
10. Compare your activation energy with other values in the literature and analyze the equipment used here and elsewhere. How accurate do you think the value that you calculated compares with other methods that have been published? What are the advantages of the equipment we used with respect to what has been done? What are the limitations?

Day #3: Protein Assay of a Sample of Catalase from Bovine Liver Culture

In this part of the experiment, the student will learn about pipetting with a pipetman and also calibration of the pipetman (see Appendix 1). In addition, each student will prepare a standard curve, practice a serial dilution, and operate the UV-VIS spectrometers.

Students will receive a sample solution of catalase enzyme from bovine liver culture. Students will use the Coomassie Plus Modified Bradford Protein Assay to determine the concentration of the catalase in the sample.

Each student works solo assembling a tray of microcentrifuge tubes: five tubes containing 50 micro liters each of unknown catalase solution, and seven empty tubes for mixing standard solutions the zero standard will serve as your blank. Students will be provided with a bottle of 40mM Tris HCl buffer, (pH 8.2).

Experimental

Calibration of the Pipetman

For proper operation of the pipetman please see (Appendix 1). Calibration of the pipetman is as simple as dialing up a volume on the instrument. Drawing up that amount of water from a beaker and pipetting it into a tared weighing vessel. Then, reading off the mass from the balance and knowing the density of water 1.00 g/mL, calculating the volume. Compare this to the volume dialed up on the instrument. This will indicate the accuracy of the instrument. From our experience with this experiment, most pipetman are accurately calibrated and in cases where they are not, they are generally off by no more than $\pm 1 \mu L$.

Coomassie Protein Modified Bradford Method

The basic principle of the modified Bradford method works as follows: The Coomassie Plus dye in its acidic red-brown form absorbs at 465 nm (A_{465}). When the dye forms a complex with the protein, it is converted to an anionic blue form with the absorbance shifting to 595 nm (A_{595}). The absorbance is directly proportional to the concentration of protein present. Students first generate a standard curve using the protein Bovine Serum Albumin (BSA) by measuring the absorbance at 595 nm of a series of seven standards of known concentrations. Next, student will measure the A595 of the student's five samples and determine their concentration by interpolating the reading at 595 nm against the standard curve.

Experiment #4: Catalase Kinetics

Instructors will provide the following solutions:

Buffer 40mM Tris HCl (pH 8.2): Dissolve 4.8456 grams Tris in 1 liter Milli-Q water acidified with HCl to pH 8.2.

Coomassie Plus Protein Assay Reagent: 950 mL Product #1856210 Thermo Scientific

Albumin Standard (BSA): 2 mg/mL, 50 mL bottle, Product #23210 Thermo Scientific

Catalase: Worthington Biochemical Corporation, Catalase 0.22μm Filtered Code: CTS 10 mL vial of catalase with exact concentration. Instructor should dilute with 10 mL 40 mM Tris HCl buffer to create the unknowns for student samples. Worthington Product Code #LS001896

1. Preparation of BSA standards[15]

Prepare a set of seven Bovine serum albumin (BSA) protein standard solutions by diluting the 2.0 mg/mL BSA stock solution as illustrated in Table 1. When you have finished making A & B, snap-lock the tubes mix well and centrifuge them for 30 s. Then make up Standards C & D from A & B as below, snap-lock all four tubes mix well and centrifuge them for 30 s. Now make up Standard E from D, snap-lock both tubes mix well and centrifuge for 30 s, finally make up F from E, and G. Snap-lock mix well then centrifuge for 30 s. Follow the procedure steps indicated below. **The centrifuge machine must be balanced please do not operate it if not balanced or the motor will burn up. If you are not sure about how to balance the centrifuge please check with your TA.**

Table 1: Preparation of BSA Standards

Volume/(μL) of the BSA to Add	Volume/(μL) of Diluent (Buffer) to Add	Final Volume/ (μL)	Final BSA Concentration/(□g /mL)
375 of Stock	125	500	**A** - 1500
325 of Stock	325	650	**B** - 1000
175 of A	175	350	**C** - 750
325 of B	325	650	**D** - 500
325 of D	325	650	**E** - 250
325 of E	325	650	**F** - 125
0	325	325	**G** - 0

2. Preparation of the Coomassie Plus Protein Assay Reagent

[15] Adapted from: Dolhun, J. J., *5.301 Chemistry Laboratory Techniques, January IAP, 2012.* (Massachusetts Institute of Technology: MIT OpenCourseWare). http://ocw.mit.edu/courses/chemistry/5-301-chemistry-laboratory-techniques-january-iap-2012/labs/biochemistry/ (accessed Mar 27, 2013). License: Creative Commons BY-NC-SA.

Obtain a bottle of Coomassie Plus reagent from the refrigerator. Mix the Coomassie Plus reagent by gently inverting the bottle twice. Pour out the amount of reagent that you need for the lab. Each pair of students will need about 25 mL. Allow the poured Coomassie Plus reagent to come to room temperature 40 minutes prior to adding it the samples and standards.

3. Procedure

1. Pipette 50 μL each of six BSA standards (B thru G) and 50 μL each of five unknown catalase samples (supplied by TA) into eleven labeled microcentrifuge tubes.
2. The BSA 'G' Standard above will also serve as your blank.
3. To each of the six standards and five unknown samples, working quickly, add 1.5 mL of Coomassie Plus reagent. Snap lock the microcentrifuge tubes and gently mix by inverting them several times. Allow the tubes to sit for 2 minutes at room temperature for color to develop.
4. Transfer standards, unknown samples and blank to separate 1.5 mL plastic disposable UV cuvettes by gently mixing then, opening the microcentrifuge tubes and pouring each by hand into the 1.5 mL plastic disposable UV cuvettes. Immediately proceed to the UV-VIS spectrometer to measure the absorbance at 595 nm for each standard and sample *vs.* blank. **It's important that you run your samples within 10 minutes of adding the first drop Coomassie dye. No more than 15 minutes should elapse between the time you add the Coomassie dye and the complete running of all the standards and samples. Generally, it takes about 2.5 minutes to add the dye to all 11 standards and samples. Snapping the tubes shut and mixing takes another 2 minutes. Opening and pouring the tubes into UV cuvettes takes 2 minutes. At 9 minutes, you should be at the UV instrument running your zero blank, followed by loading the standards and unknowns. When your stop watch reads 10 min press the start button running your standards and unknowns this takes about 2 min, then take out the standards and load your samples you should be at about 13.5 min, press start and run the samples finish at 15 minutes.** Follow the guidelines for starting the program and operating the UV-VIS Spectrometer (see Appendix II).
5. Chart a linear standard curve using the BSA standards in the range of 125 $\mu g ml^{-1}$ to 1000 $\mu g ml^{-1}$ by exporting data to Microsoft Excel and do a linear regression curve fit by plotting the average blank corrected absorbance 595 nm reading for each BSA standard *vs.* its concentration in μg /ml. Do not force the computer generated linear regression through zero. A Bradford Assay has three linear regions when BSA is used as a standard. One of the linear ranges is from 0 to 125 $\mu g ml^{-1}$ another is from 125 $\mu g ml^{-1}$ to 1000 $\mu g ml^{-1}$ or 125 $\mu g ml^{-1}$ to 750 $\mu g ml^{-1}$ [Test these to see which gives the steepest slope] a third is from around 1000 $\mu g ml^{-1}$ to 2000 $\mu g ml^{-1}$. This is beyond the UV capabilities when absorbance values start to exceed 1.0. The value of the sample absorbance reading determines

which linear range is used to calculate the protein present in the sample. As absorbance increases the accuracy of the value decreases. It is advisable to assay using protein concentrations that fall on the linear line with the largest slope. Please verify this using either 125 μgml^{-1} to 1000 μgml^{-1} or 125 μgml^{-1} to 750 μgml^{-1} To determine the unknown protein concentration, interpolate the absorbance values from each unknown sample against the standard curve.

Clean-Up:

Empty all of the leftover solutions into the appropriate Catalase waste container. The empty UV cuvettes must be disposed of in the plastic waste container. All the plastic pipette tips and microcentrifuge tubes should also be disposed of in the plastic waste container. If you are not sure, please ask your TA. DO NOT PLACE THE PLASTIC UV CUVETTES OR THE PLASTIC PIPETTE TIPS IN THE BROKEN GLASS WASTE BOX.

Analysis and Discussion

1. Determine the protein concentration of your unknown sample. Discuss reasons in general why performing a protein assay is important.
2. Do a detailed error analysis on your unknown concentrations including standard deviations, error of the mean, and 95% confidence levels for the results.
3. Include a line of best fit for the data in the laboratory appendix that clearly shows a correlation coefficient (R value).
4. Discuss any random and or systematic error that could be present and any other sources of error that are present.

Day #4: A Modified Ferrozine Method for the Determination of Protein-Bound Iron in Catalase[16]

Students are given a sample solution of catalase enzyme from bovine liver culture. From the previous lab, the protein concentration of your sample is known. Students will now use a modified ferrozine method to determine the concentration of protein-bound iron in the sample.

Experimental

Iron Assay

Ferrozine is a bidentate chelating ligand. It complexes with Fe (II) creating an octahedral complex that absorbs light at 562 nm. Comparing this absorption against a calibration curve of Fe standards, allows one to determine the concentration of iron in the original protein sample.

Instructor will provide the following solutions:
Fe standard (100.00 μg/mL; prepare 1000 mL)
TA prepares a ferrous iron stock solution (100.00 mg/L Fe^{+2}) by dissolving 0.7022 grams of ferrous ammonium sulfate hexahydrate, in Milli-Q water and diluting to 1000 mL in a volumetric flask. Must be prepared fresh immediately before experiment.

Buffer 40 mM Tris HCl (pH 8.2): Dissolve 4.8456 grams Tris in 1 liter Milli-Q water acidified with HCl to pH 8.2.

4M Methanesulfonic acid: 38.442 g concentrated acid diluted to 100 mL using Milli-Q water

Iron Complex Mix: 6.5mM Ferrozine 80 mg, 15.4mM neocuproine 80 mg, 1M Ascorbic Acid 4.4g, 2.5M Ammonium Acetate 4.8g, all in 25 mL of Milli-Q water—Should be prepared fresh on the day of the experiment. Best to prepare in a 50 mL Polypropylene graduated conical tube with large opening. Shake well to dissolve it may take a few minutes. Each student will need approximately 4 mL of the reagent. 25 mL will be enough for 5 groups.

10 N sodium hydroxide solution: 40g/100mL

[16] Adapted from: Davis, M. D.; Kaufman, S.; Milstien S. *J. Biochem. Biophys. Methods* **1986,** *13*, 39-45.

1. Preparation of Iron Standards

1. Prepare a fresh set of seven iron standards in pre-labeled 2 mL microcentrifuge tubes, as illustrated below. The zero standard will also serve as your blank. Pipette 200 μL each of five catalase samples into five more labeled microcentrifuge tubes.

Table 2: Preparation of Iron Standards

Fe AA standard Volume/(μL) (100.00 μg/mL)	Volume /(μL) of Diluent (Buffer) to add	Final Volume / (μL)	Final Concentration/ (μg/mL)
0.00	200	200	0.00
1.00	199	200	0.50
2.00	198	200	1.00
4.00	196	200	2.00
8.00	192	200	4.00
12.00	188	200	6.00
16.00	184	200	8.00

2. Procedure

1. After addition of 800 μl of 4M methanesulfonic acid to each standard and sample; the microcentrifuge tubes were snapped shut.
2. Place the twelve closed microcentrifuge tubes containing the standards and samples into a micro centrifuge-heating block sitting on top of a hotplate and pre-heated to 103-104 ^{0}C for 40 minutes (Do Not Exceed 105 ^{0}C). Set your stop-watch and start it as soon as you begin placing the tubes into the heat-block. Please do not adjust the temperature during the experiment (initially the temperature may drop when tubes are placed into the pre-heated heat block, this is normal and the block will re-adjust itself). WARNING!! Exceeding 110 ^{0}C may cause excessive pressure to build up in the tubes, which could result in tubes popping open and blowing the contents out of the tubes out with loss of the samples. It is critical that the temperature stay below 105 ^{0}C. The Safe Seal micro-centrifuge tubes specified are rated to +121 ^{0}C.
3. After that, the tubes are removed from the heat block using long tweezers and allowed to cool for 5 minutes, then, take the five samples and seven standards and centrifuge at 12,500 rpm for 1 minute, then collect the supernatant condensate from each tube. The samples are transferred to 12 new, labeled microcentrifuge tubes. **[If no precipitate forms, then proceed directly below to step 4 in the**

same old tubes. No precipitates have been evident at this step using the recommended catalase source and protocol]

4. Add 320 µl of 10N NaOH to each standard and sample (all twelve tubes).
5. Add 300 µl of the iron complex mixture to each standard and sample (6.5 mM Ferrozine, 15.4 mM neocuproine, 1M Ascorbic Acid, and 2.5M Ammonium Acetate in 25mL Milli-Q water). Snap-lock the tubes and give each one a few gentle shakes. Allow the tubes to sit for 15 minutes for the color to develop.
6. Centrifuge the microcentrifuge tubes at 12,500 rpm for 2 minutes and then, pipette out 1000 µl of the supernatant condensate from each tube directly into 1.5 mL disposable plastic UV cuvettes. The solutions should be magenta/purple in color. **[Students should practice on a microcentrifuge tube filled with water to get comfortable taking off the top 1000 µl of supernatant condensate prior to performing the operation on the samples].** This operation requires that you use a new pipette tip for each of the 12 microcentrifuge tubes.
7. Follow the instructions for operating the UV-VIS spectrometer and measure the absorbance of each standard and sample at 562 nm *vs.* the blank (it is best to blank against your 0 standard).
6. If the computer software on the UV machine is not programmed to generate a standard curve then, chart a standard curve by exporting data to Microsoft Excel and do a linear regression curve fit by plotting the average blank corrected absorbance 562 nm reading for each Iron standard *vs.* its concentration in □g /ml)
8. To determine the unknown iron concentrations, interpolate the absorbance values from each unknown sample against the standard curve.

Clean-Up:

Empty all of your leftover solutions into the appropriate labeled catalase waste container. The empty UV cuvettes must be disposed of in the plastic waste container. All the plastic pipette tips should also be disposed of in the plastic waste container. If you are not sure, please ask your TA. DO NOT PLACE THE PLASTIC UV CUVETTES OR THE PLASTIC PIPETTE TIPS IN THE BROKEN GLASS WASTE BOX.

Analysis and Discussion

1. Generate a calibration curve of A_{562} vs. [Fe] from the standards.
2. Determine what type of correlation exists between the absorbance at 562 nm and the amount of iron present in solution.
3. Determine the iron concentration of the unknown catalase samples.
4. Do an error analysis on the results including average, standard deviation, error of the mean, and 95% confidence levels.
5. From the results of both days, calculate the ratio of the moles of iron per mole of catalase. Compare the calculated mole ratio of iron per catalase molecule with the expected theoretical value. Please explain any discrepancies and discuss the reasons.

Appendix I
Operation of the Pipettman[17]

The Pipettman, an adjustable digital variable volume microliter pipettor, is to be used only for dispensing the enzyme and solutions in this experiment. The volume indicators consist of three numbered dials and are read from top to bottom. Carefully read the volume of the pipettor you are using since the pipettors we have in the lab are available in several standard volumes from 20 μl to 1000 μl. The digits represent milliliters or microliters depending on which model unit you are using.

1. To set the volume, hold the Pipettman in one hand and turn the volume adjustment knob with the other hand until the correct volume shows on the digital indicator. If you pass the desired setting, turn the dial a few digits above the desired setting and reset the volume. The lab pipettors are remarkably free of mechanical backlash, however, it is considered good practice to consistently overset the actual volume required and ensures that the best accuracy is obtained.
2. Attach a new disposable tip to the pipet shaft. Press firmly to ensure a positive airtight seal using a slight screwing action. NEVER USE THE PIPETTOR WITHOUT A TIP ATTACHED.
3. Depress the plunger to the FIRST STOP. This part of the stroke is the calibrated volume displayed on the digital volume indicator.
4. Holding the pipettman vertically, immerse the disposable tip into the sample liquid to the proper immersion depth generally 3-5 mm into the sample being tested.
5. Allow the pushbutton to return SLOWLY to the up position. NEVER LET IT SNAP UP!!
6. Wait a few seconds to ensure that the full volume of sample is drawn into the tip.
7. Withdraw the tip from the sample liquid slowly. Should any liquid remain on the outside of the tip, wipe it carefully with a lint-free tissue, taking care not to touch the tip orifice. DO NOT WIPE THE TIP WITH A TISSUE.
8. To dispense samples, touch the tip end against the sidewall of the receiving vessel about midway down, not at the very bottom, and depress the plunger slowly to the FIRST STOP. Wait 2-3 seconds. Then press the plunger to the SECOND STOP (bottom of stroke), expelling any residual liquid in the tip.
9. With the plunger fully depressed, withdraw the Pipettman from the vessel carefully, with the tip sliding along the wall of the vessel.
10. Allow the plunger to return to the UP position.
11. Discard the tip by depressing the tip ejector button most of the pipettors we have in the undergraduate lab do not have ejector buttons so you can pull the tip off with gloves and dispose into the disposable container designated by the TA. A fresh tip should be used for each sample to prevent sample carryover.

[17] Adapted from: VWR brand Pipettor Instruction Manual, VWR Scientific Products.

APPENDIX II
CARY 60 UV-VIS__5.310
OPERATING INSTRUCTIONS

1. Turn on UV-VIS then Turn on computer
2. Logon USER: student PASSWORD: student
3. Click Method on Screen either Charles River Phosphate Determination, Catalase Protein Concentration or Catalase Iron Quantitation depending on the experiment you are doing.
4. Wait until the zero is highlighted in left margin.
5. Click ZERO button in left column.
6. CELL LOADING GUIDE appears. Open cell changer drawer and place Zero Blank into Position 1 as shown in cell loading guide on screen.
6. Close cell changer drawer on UV VIS.
7. Click OK on Cell Loading Guide machine now scans blank which is also your zero standard.
8. Remove the zero blank.
9. Press START on computer located at top.
10. Standard Sample Selection Guide appears standards and unknowns should be listed under selected for analysis.
11. Click OK on Standards / Sample selection guide.
12. CELL LOADING GUIDE appears load standards and unknowns as per loading guide. Load standards from low to high concentration then load samples into positions 8 to 12.
13. Close the UV lid and click OK on Cell Loading guide.
14. Click PRINT Button bottom left to print data.
15. Print to local laser printer default or select Microsoft Print to PDF and click OK

Insert personal USB and browse to USB and list a file name and save to USB

16. If calibration fails, you will get a warning click OK. Click OK again to continue with your analysis. You will see no results for your unknowns in the concentrations. You can click RECALCULATE and the standards pop up with an option to ELIMINATE a particular bad standard. Double click the YES next to the standards you want to eliminate and it turns to a NO, you can also change the minimum R2 value to a lower number this will permit the calculations of the curve. Then click OK.

5.310 Laboratory Chemistry

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