Name _____

Oregon State University   Department of Chemistry

Experiment 3

Integrated Laboratory Experiment

ENZYME KINETICS. YEAST ALCOHOL DEHYDROGENASE

I. INTRODUCTION

A. *General*

Enzymes are an important class of proteins because they function as biological catalysts which enable many essential chemical reactions to take place in living organisms. Enzyme names are formed from a root indicating the substrate and the type of reaction and the suffix -ase. Thus, "carbonic anhydrase" is an enzyme which catalyzes the reaction $H_2O + CO_2 \rightleftharpoons H_2CO_3$.

Enzymes can influence the rates of reactions considerably and can show extraordinary selectivities with respect to substrates. Measurement of the rate of an enzyme catalyzed reaction is the basis for some important analytical techniques for determination of substrates, activators, inhibitors and enzymes. These determinations are often used for clinical or diagnostic purposes.

The basic formulation for enzyme kinetics was developed by Michaelis and Menten. For more detailed information about the formulation, refer to one of the general references given at the end of this document. The theory is based on the following mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \bullet S \overset{k_2}{\longrightarrow} P + E \tag{1}$$
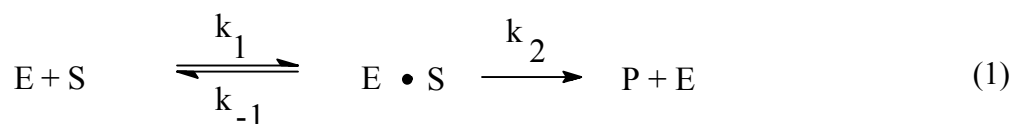
where **E** is the **enzyme**, **S** is the **substrate**, and **P** is the **product**, and E•S is an intermediate enzyme-substrate complex and the k's are rate constants for the respective steps in the mechanism. If the steady state approximation applies to the enzyme-substrate complex, then it can be shown that the initial steady state rate, or $v_0 = d\,[P]\,/\,d\,t$, is given by

$$v_0 = \frac{k_2 [E]_0 [S]_0}{K_m + [S]_0} \tag{2}$$

where $K_m = (k_{-1} + k_2) / k_{-1}$ is the Michaelis-Menten constant,

[E]$_0$ = initial enzyme molar concentration and

[S]$_0$ = initial substrate molar concentration

The units of v_0 are mol $L^{-1}s^{-1}$, of k_2 and k_{-1} are s^{-1}, of k_1 are L $mol^{-1}s^{-1}$, and of K_m are mol L^{-1}.

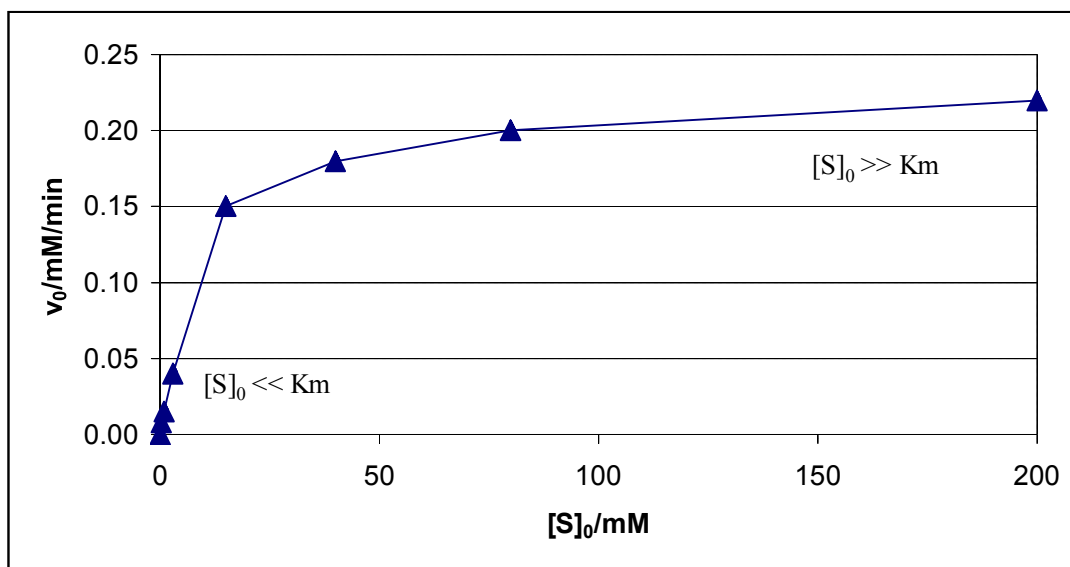A plot of v_0 *versus* $[S]_0$ is shown in Figure 1.

Figure 1. Dependence of the initial rate on substrate concentration, according to equation 5.

Note that two regions in Figure 1 and equation 2 can be identified. At high substrate concentrations, when $[S]_0 >> K_m$, v_0 is independent of $[S]_0$ and **pseudo-zero** order, and equation 2 reduces to

$$v_0 \; = \; v_{max} \; = \; k_2[E]_0 \tag{3}$$

In this case, essentially all the enzyme present is bound with substrate in the E•S complex. At low substrate concentration, when $[S]_0 << K_m$, the rate is linear in $[S]_0$ or **first order** with respect to $[S]_0$, and equation 2 reduces to

$$v_0 \; = \; (k_2/K_m)[E]_0[S]_0 \; = \; (v_{max}/K_m)[S]_0 \tag{4}$$

The definition of v_{max} in equation 3 can be used to simplify equation 2 further. Substitution of v_{max} (eq. 3) for $k_2[E]_0$ (eq. 2) yields the familiar two-parameter Michaelis-Menten equation that is the basis for this experiment:

$$v_0 \; = \; \frac{v_{max}\,[S]_0}{K_m \, + \, [S]_0} \tag{5}$$

Equation 5 shows the experimentally observable v_0 as a function of the initial substrate concentration $[S]_0$ and the two parameters v_{max} and K_m. If experimental data for v_0 and $[S]_0$ are available and plotted as in Figure 1, the value of v_{max} can be determined directly as the plateau

value of v_0, and the value of K_m can be determined from the fact that, according to equation 5, $K_m = [S]_0$ when $v_o = \frac{1}{2} v_{max}$.

Alternatively, equation 5 can be transformed algebraically into a form that allows K_m and v_{max} to be determined through linear regression:

$$v_0 = -K_m \frac{v_0}{[S]_0} + v_{max} \tag{6}$$

A plot of v_0 *vs.* $v_0/[S]_0$ (an Eadie-Hofstee plot) provides a simple means to find K_m from the slope and v_{max} from the intercept.

The mechanism of enzyme kinetic reactions is often much more complex than indicated by equation 1 because two or more substrates, enzyme-substrate intermediates, or products may be involved in the reaction. Nevertheless, it is experimentally found that many reactions obey the Michaelis-Menten rate law indicated in equation 2 or 5 and that the determination of the constants in those equations under a specified set of conditions provides a useful starting point for characterization of enzyme catalyzed reactions.

B. *Alcohol Dehydrogenase*

The enzyme **alcohol dehydrogenase (ADH)** catalyzes the oxidation of ethanol to acetaldehyde. The oxidizing agent, called a coenzyme, is **nicotinamide adenine dinucleotide** (**NAD$^+$**, coenzyme I, diphosphopyridine nucleotide, DPN). Although, as shown in Figure 2, the structure NAD$^+$ is relatively complex, the reduction takes place only in the pyridine ring, as shown in Figure 3. The oxidation of ethanol with NAD$^+$ does not proceed at a measurable rate unless the enzyme is present.

Alcohol dehydrogenase, which is isolated from yeast, is composed of more than 18 different amino acids, has a molecular weight of 1.5×10^5 and has four independent catalytic sites. Zn^{2+} is present in amounts stoichiometric to the number of catalytic sites. Alcohol dehydrogenase will catalyze the oxidation of a number of primary and secondary alcohols, but not those in which there is halogen or amino substitution α- to the hydroxyl group.

An **enzyme activity unit** is often defined as the amount of enzyme that causes transformation of one micromole of substrate per minute at 25 °C under specified measurement conditions. Enzymatic activity is usually measured under conditions where the amount of

enzyme is the rate limiting factor (i.e., equation 3, substrate and cofactor concentrations large enough to be non-limiting). The **specific activity** is the number of enzyme units per mg of protein and provides an indication of a relative purity. For example, the crystalline ADH that we buy for this experiment usually contains between 300 to 400 units per mg, or equivalently, one milligram of the ADH will convert 300 to 400 micromoles of substrate per minute.
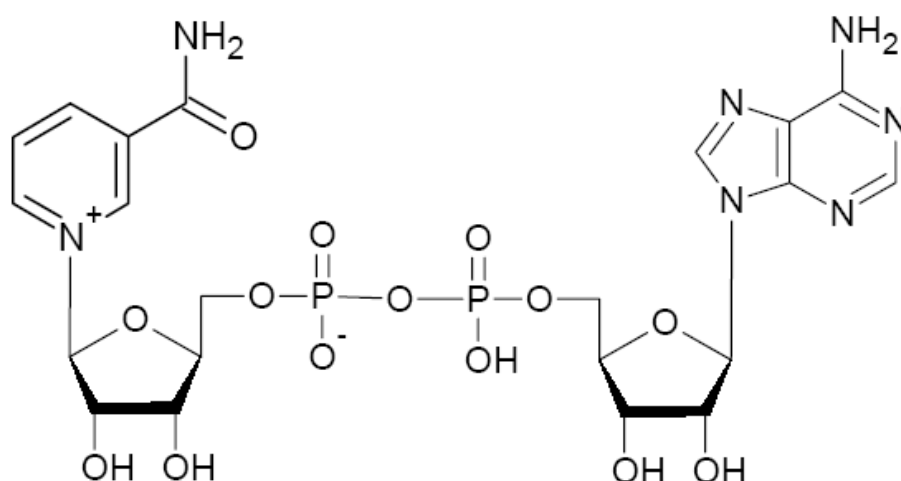
Figure 2. Nicotinamide adenine dinucleotide (NAD $^+$) ($C_{21}H_{27}N_7O_{14}P_2 \cdot 4H_2O$, MW 735.5). The positive sign on NAD$^+$ does not refer to the charge on the entire molecule but rather to the charge on the pyridine ring.
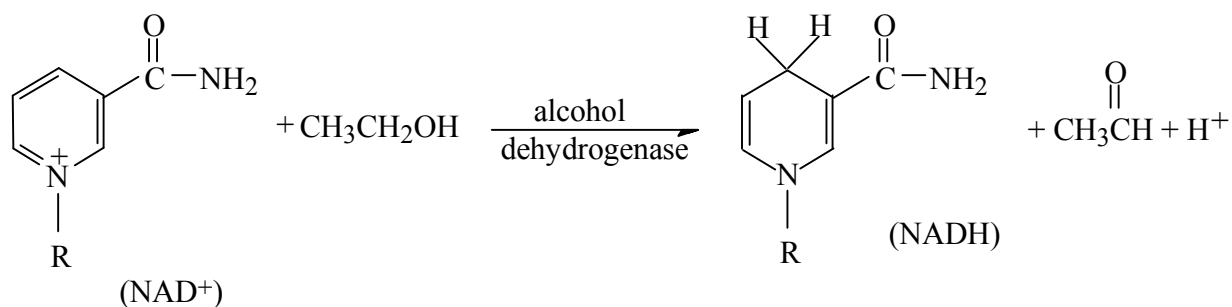
Figure 3. Reaction of NAD$^+$ with ethanol.

C. *Measurement of ADH Kinetics*

To study the kinetics of any reaction, one must have a means to monitor the progress of the reaction with respect to time. Fortunately, in the system described in Figure 3, the reduced coenzyme, **NADH**, has an absorption maximum at a wavelength of 340 nm, at which all the

other species involved exhibit negligible absorption (NAD$^+$ and ADH have absorption maxima at 260 and 280 nm, respectively). Hence the enzyme catalyzed oxidation of alcohol can be monitored with a simple UV-visible absorption spectrophotometer set to 340 nm.

The rate of reaction, v_0, can be determined from the rate of change in the absorbance at 340 nm as shown in equation 7:

$$v_0 = \frac{d[P]}{dt} = \frac{d[NADH]}{dt} = \frac{1}{\varepsilon b}\frac{dA}{dt} = \frac{1}{\varepsilon b}\frac{\Delta A}{\Delta t} \tag{7}$$

where A is the absorbance, ε is the molar absorptivity of NADH and b is the cell path length (Beer's Law (A = εbc)). In this experiment, ε at 340 nm is 6.22 x 10^3 L/mol-cm or 6.22 mL/μmol-cm and b = 1.0 cm.   If v_0 is determined for several standard solutions of substrate [S]$_0$, a curve similar to that in Figure 1 can be constructed. That curve can then be used both to determine the kinetic parameters v_{max} and K_m and, as a calibration curve, to determine unknown values of substrate concentration in sample solutions.

In order to optimize experimental conditions when setting up a kinetic analysis, one needs to know the maximum value expected for dA/dt. The value of v_{max} and the maximum value dA/dt$_{max}$ can be approximated *a priori* from the enzyme activity.   For example, **if** the concentration of the enzyme in the stock solution is 5.0 x 10^{-3} mg/mL and the dilution factor in the reaction cell is 10, the ADH concentration in the reaction cell will be 5.0 x 10^{-4} mg/mL. If the specific activity of the original dry enzyme is 380 μmol/min/mg, then the maximum rate of transformation of substrate will be

v_{max} = concentration of enzyme x specific activity

= 5.0 x 10^{-4} mg/mL x 380 μmol/min/mg = 0.19 μmol/mL/min.

The corresponding maximum value of dA/dt is found from rearrangement of equation 7:

$\Delta A/\Delta t_{max}$ = v_{max} ε b

= 0.19 μmol/mL/min x 6.22 mL/μmol-cm x 1 cm = 1.18 AU / min

at 25°C, at the optimum pH (8.8) and wavelength (340 nm).

D. *Kinetics Methods of Analysis*

In analytical methods based on enzyme catalyzed reactions, substrate concentrations can be found either from the *initial rate of reaction*, in which the initial rate v_0 is related to the substrate concentration [S]$_0$ by equation 4 at low substrate concentrations, or from *final product*

concentration [P] after total conversion of S to P through reaction 1. In this experiment, the *initial rate of reaction* will be used to determine substrate concentration. The reaction rate method is less subject to interference because the change in absorbance is measured. Non-reacting but absorbing species may affect the final absorbance and hence the complete-reaction analysis, but will not affect the accuracy of the kinetic-based method. One disadvantage of kinetic methods of analysis is that very small changes in signals such as absorbance must be measured. The kinetics-based method of analysis is faster -- it takes about 10 min for the reaction in this experiment to reach completion.

E. *Scope of Experiment*

In this experiment, you will determine the initial rate of ethanol oxidation (v_0) in a series of different standard solutions with known ethanol concentrations ($[S]_0$). From these data, you will determine the parameters K_m and $v_{max,}$ and you will calculate the enzyme activity. You will then use the kinetic method of analysis to determine the concentration of ethanol in two real samples, beer and blood.

II. SOLUTIONS

A. *Stock Solutions*

The following stock solutions are available:

Buffer Solution (to control the pH of the reaction mixture)

•   0.024 M pyrophosphate pH 8.8 buffer (made from $Na_4P_2O_7\bullet10H_2O$ and H_3PO_4)

ADH Solution (the enzyme solution)

•   X µg/mL ADH in a 0.01 M pH 7.5 potassium phosphate buffer (you will be provided the value of X which is typically 1 to 15). Dilute solutions of ADH rapidly lose activity upon standing and are quite unstable at pH values below 6.0 or above 8.5. The deactivation rate is reduced by storing the solutions at 0-5 C when not in use and by placing the enzyme solution container in an ice bath during lab work.

NAD⁺ Solution (coenzyme)

•   0.017 g/mL (2.5 mM) in H_2O. This solution is stored in the refrigerator when not in use and in an ice bath during lab work.

Ethanol Solutions

• 0.2500 M (14.58 mL of 100% ethanol (MW, 46.07; density, 0.790) / L of buffer solution)

• 0.1000 M

• 0.01250 M

• 0.003125 M

These stock solutions of ethanol are prepared in buffer which is 0.024 M, pH 8.8 pyrophosphate.

B. *Preparation of Standard Ethanol Solutions*

Rinse all glassware thoroughly as soap can denature the enzyme. Prepare the following standard ethanol solutions from the 0.01250 M (12.50 mM) ethanol stock solution by diluting the appropriate amount of the stock in 100-mL volumetric flasks (note standard solution concentrations are given in millimolar):

Final concentration of EtOH standard solution mM (you calculate exactly)	Nominal volume of 12.50 mM EtOH stock solution from buret mL	Final volume (dilute to mark with pH 8.8 buffer) mL
~0.125	1.XX	100.0
~0.250	2.XX	100.0
~0.625	5.XX	100.0

All these solutions should be diluted to volume with the pH 8.8, 0.024 M, pyrophosphate buffer. Burets have been set up to dispense the 12.5 mM ethanol solution. Record the beginning and ending buret readings (to ±0.01 mL) in your notebook and calculate the exact volumes dispensed and exact concentrations later to be used for analysis. It is very critical that all glassware used in the preparation of the solutions is very clean and that you minimize contamination of the solutions with particulate matter (e.g., dust, hairs, skin).

C. *Preparation of Beer Sample*

The real beer must be diluted by a factor of 2500 and non-alcoholic beer by a factor of 1000 to ensure that the ethanol concentration in the standard solution is in the region where v_0 is proportional to $[S]_0$ (i.e., equation 4 is reasonably valid.)

It is difficult to pipet an exact volume of beer since foam obscures the meniscus. Hence the bubbles (CO_2) must be removed. Place a few milliliters of beer in a test tube, cover with a 1" square piece of Parafilm, shake vigorously, and uncover to release the gas pressure. Repeat a few times until foaming ceases. Alternatively, use a sonicating bath if available.

With a pipet, transfer 2.00 mL of flat beer (or 20.00 mL of non-alcoholic beer) into a 100-mL volumetric flask and dilute to volume with MP water. Transfer 2.00 mL of this solution (cleaned pipet) to another 100-mL volumetric flask and **dilute to volume with pH 8.8**, 0.024 M, pyrophosphate buffer **(do not dilute with water)**.

D. Preparation of Blood Samples and Standards.

Analysis of blood for alcohol is more difficult than the analysis of beer for alcohol because of the rather complex blood matrix. Scattering and absorption by protein in the blood makes reaction rate analysis impossible without pretreatment.

You will be provided with 1 mL of blood which contains ethanol at levels which simulate an ethanol concentration in the blood of a suspected intoxicated driver. The first step is deproteinization with trichloroacetic acid (TCA), which causes the protein to precipitate. After centrifugation, the supernatant can be used in the standard reaction rate procedure. For this part, in contrast to the beer analysis, it is necessary to prepare new standards since the sample pretreatment procedure has some effect on the absolute value of the initial rate.

Blood Samples.   The blood samples and standards will be prepared and run on the second day of the lab.   Rinse glassware thoroughly with DI water as soap can denature the enzyme. Obtain a centrifuge tube from the TA and ask the TA to dispense 1.0 mL of blood into the centrifuge tube. Add 5.0 mL of 6% TCA. With a stirring rod, break up all blood clumps as completely as possible. Centrifuge for 3 min.

Transfer 2.0 mL of the clear supernatant to a 25-mL volumetric flask and add 1 drop of magenta-colored thymol blue (record the number of drops in your laboratory notebook). Add 1 drop of 0.5 M NaOH, shake the contents, wait 10-15 s for a color change. Continue to add single drops of 0.5 M NaOH, shake, and wait until you have added enough to cause the solution to turn from <u>red to yellow </u>(about 13-30 drops total;goes from pH of 1.2 to 2.8). Next use the more

diluted 0.1 M NaOH and add dropwise with a Pasteur pipet (shaking after each drop) until the first faint green-blue color appears and is persistent (pH ~8.8). The pH is now near that for the standard assay procedure. ***Finish dilution with pH 8.8 buffer to the 25-mL mark***. ***Mix these solutions thoroughly.***

     Standards. Use 10-mL volumetric flasks to first prepare two alcohol standard solutions from the stock 0.25 M ethanol solution - one is nominally 0.030 M and the other is 0.060 M. To do this, use the electronic pipettors to add 1.2 mL in one flask and 2.4 mL in the other flask of the stock 0.25 M EtOH and ***dilute each to 10.00 mL with the pH 8.8 buffer***. ***Mix these solutions thoroughly.*** Record the actual values of the 0.25 M EtOH you dispensed and calculate the actual EtOH concentrations later.

     From these two stock standards, make two diluted ethanol standards appropriate for the analysis of blood (i.e., similar in solution composition and pH to the blood samples). To do this, pipet 1.00 mL of one standard you just made above plus 5.00 mL of 6% TCA into a small test tube and mix thoroughly. Next, transfer 2.00 mL of this standard mixture into a 25-mL volumetric flask, add thymol blue, ***adjust the pH as you did for the samples, and finally bring up to volume with buffer, and mix thoroughly***. Repeat this same procedure for the second standard.   Note that after treatment, the concentrations for the two standards are nominally 0.4 mM and 0.8 mM in ethanol (and nominally 0.32 mM and 0.64 mM EtOH for the test solution in the cuvette).

E. *Waste Disposal*

     Dilute ethanol solutions are nonhazardous and can be disposed down the drain. The pellet from the centrifuge is nonhazardous and can be placed in solid waste bottle under the hood.

III. EXPERIMENTAL

A. *Instrumentation*

The PTR components are configured as a spectrophotometer as shown Figure 4 to provide a means of monitoring the kinetics of the enzyme reaction (i.e., to enable the continuous recording of absorbance versus time on a computer via the ADC).
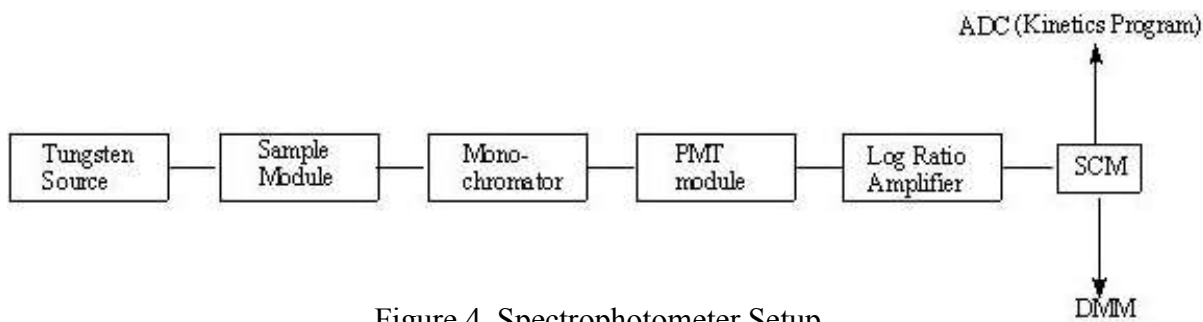

Figure 4. Spectrophotometer Setup

The instrument has been assembled for you (monochromator: 600-μm slits and $\lambda = 340$ nm). The monochromator is placed **after** the sample module so that measurements can be conducted with the sample module cover off. The light source module is bolted to the holes closer to the sample module (as for the emission configuration for exp. 2B). To mix solutions, a small stirring bar driver is inserted beneath the sample cuvette holder. It is connected to a controller to adjust the stirring speed. The use of linear absorbance monitoring is particularly convenient for kinetics experiments.   The electrical connections have been completed as specified in the Appendix. You shouldn't have to adjust anything, but you should verify the PMT signal in Step 5 and the SCM signal in Step 6 before you begin.

B. *Effect of Substrate Concentration and Analysis of Beer*

In this part of the experiment, duplicate initial steady state rates v_0 will be obtained for 7 different standard solutions of ethanol at concentrations from 0.125 - 250 mM. These results will be used both for determination of v_{max} and K_m and as calibration data for the determination of ethanol in beer.

To obtain good data, it is critical that the operations and measurement conditions be reproduced as closely as possible. Take extra care that the correct volumes are added and that the timing of additions and stirring conditions are the same from run to run. Work carefully and as

quickly as you are able. Note that a provision for temperature control of solutions and the sample cuvette holder would improve precision but is not available at the PTR stations.

Initial Preparation

1.   Prepare the three ethanol solutions indicated in section IIB and the one beer solution (either alcohol containing or "alcohol free") in section IIC.

2.   Fill a clean wash bottle half full with the pH 8.8 buffer solution.

3.   Obtain a small screw cap bottle of ADH and of NAD^+ from the refrigerator and place them in a medium sized beaker with ice near your instrument. Keep on ice at all times and keep the caps on tight when not in use (don't let your reagent bottles float!).

4.   Confirm that a 250 μL automatic pipet, some pipet tips, and two sample cuvettes with stirbars are at your station.

Measurement Procedure

1.   Use a clean 2.00-mL volumetric pipet and pipet exactly 2.00 mL of the first standard
        solution shown in Table I (p. 19) into each of the two sample cuvettes. Note that the test
        solutions in the table are set up to make duplicate runs for each ethanol concentration. Be
        sure you rinse the pipet with the next standard solution before switching to the next
        concentration value (e.g., between runs 1B and 2A, etc.). Check that the pipet delivers all
        2.00 mL.

2.   Place a new tip on the automatic pipet and dispense 250 µL of NAD^+ solution into each
        of the cuvettes. When handling the pipet tips, do not touch the tips near the dispensing
        end. **Be absolutely sure you know how to properly use the automatic pipettor** and
        that the pipet tip is securely on. Ask if you have questions.

3.   Insert a small magnetic stirring bar into the first cuvette, place the cuvette in the sample
        holder, and adjust for an even stirring rate. Regular stirring is essential for the success of
        this experiment.   Note the orientation of the cuvette, mark the cuvette, and use the same
        orientation throughout the experiment.

4.   **Set up the Kinetics program to record the data for this experiment**. Choose the
        computer delay time and measurement time specified in the ReadMe notes for Table I (p
        18). Set the gain on the SCM to the value listed in Table I for the particular run and enter
        that value in the Kinetics program. Be sure to save the output filename after the run and
        use a new filename for each run. If you change the delay time, run the software just to
        verify that the Kinetics program is indeed using the new delay time. **Adjust the gain on
        the PMT to make sure that the SCM output at the DMM is about +0.2 V at the start
        of each and every run (yes ..you can change the starting gain on the detector because
        you are measuring the slope, so the absolute starting voltage should not impact the
        resultant slope).**

5.   Place a new tip on the automatic pipet, fill the automatic pipet with 250 μL of ADH solution, check that the stirbar is spinning, and dispense the ADH solution into the cuvette. At the time of injection, start the computer program and observe the reaction. Allow the reaction to proceed at least 30 s. **Measure and record the temperature of run 1A in your lab notebook. Do not repeat the temperature measurement for the other runs.**

6.   Using vacuum aspiration, remove the contents of the sample cuvette and rinse twice with pH 8.8 buffer, being careful not to remove the stirbar. Repeat steps 2 through 5 for run 1B (except for the T measurement). **It is very critical that the sample cuvettes be thoroughly rinsed with buffer between analyses so that no residual alcohol is left from a previous run. Avoid touching the pipet tip with your fingers because proteases on your skin can deactivate the enzyme.**

7.   Now repeat steps 1 to 6 for runs 2A and 2B in Table I and repeat this sequence through run 8B today.

This is the normal stopping place for the first day.

C. *Determination of Alcohol in Blood.*

Run duplicate assays of the two treated blood standards and the TCA /NaOH-treated blood sample using the same procedure as before. That is, add 250 μL of NAD to 2.00 mL of sample or standard added to the cuvette and inject 250 μL of ADH to initiate the runs (through runs 11A & B). Finally return the sample cuvettes with stir bars to a TA.

IV. LAB REPORT

Your lab report should include an abstract, responses to Items A-I below, data Table 1, and your lab notebook copy pages. Also include any raw data that you need to submit as described in Item B below.

A. Prepare Table A showing the exact volumes of the stock 12.5 mM ethanol solution added and the exact ethanol concentrations of the three standards that you prepared and used. **Use these values in subsequent calculations for the rates**. Check that the buret readings are entered on your lab notebook pages.

B. Determine and tabulate the initial rate for all runs from the slope of the linear regression equation reported by the Kinetics program, which is also reported in the saved .csv file for each run. Present this information in a Table B with columns for **run number and rate in A.U./min from the program**.

Note that the raw data and the rates reported by the Kinetics program are adjusted according to the SCM gain value set on the front of the SCM module that you entered in the program before starting the run. If the gain value was not entered in the program correctly, the rates calculated by the program will not be correct- discuss with an instructor if needed.

If the rates reported by the Kinetics program are not consistent for duplicate runs or are not producing a linear region for the lowest standards, then construct plots manually from the .csv files and examine the quality of your raw data to ensure that you have initial rates (AU min^{-1})

that you can use with confidence for the rest of the experiment. Creating the plots and determining the slopes from the raw data is relatively quick and easy if you use the hints in the Appendix.

If the data *are not* smooth and regular, you may have to edit the raw data to eliminate clear outliers and to capture the true rate of the enzyme catalyzed reaction. Editing of raw data should not be undertaken without serious thought; if you have questions, ask. If you do edit the raw data, always keep a copy of the original data (see Appendix) and include in your report a graph with the complete original data set, the data that you retained for calculation of the rate, and your justification for eliminating the points that you did. Be sure the rate reflects the initial stage of the reaction -- if the rate starts to plateau, don't use the plateau, but determine the initial (fastest) rate by drawing a tangent to the initial part of the absorbance vs. time curve where the rate is linear in time (if working in Excel, set a second series for the initial rate points).

C. Prepare Table C. with the data that you will use for the rest of the report. Show a sample calculation for each part (give equations and sample values to demonstrate the calculation and the result). Table C should have 11 rows of data (7 rows for the ethanol standards, 1 for the beer sample, 2 for the blood standards and 1 for the blood sample) and 5 columns labeled as follows:

- **Sample ID** (e.g., std 0.125 mM, beer sample 1, etc.)

- **Exact concentration of EtOH standard (mM) in the cuvette**. This value is $[S]_0$. Values should be calculated from dilution of the volume of EtOH standard delivered from the buret and should be close to those in column 2 in Table 1. For the beer and blood samples, just identify the sample in this column.

- **Average initial rate in ΔA/min**, determine from the average of the rates of the duplicate runs.

- **Average initial rate (v_0) in µmol/mL/min** (from equation 7 and the rate data in the preceding column).

- $v_0 / [S]_0$.

D. **Prepare a Plot D of v_0** *versus* **$v_0/[S]_0$** from 2.5 to 80 mM, report the slope and intercept, and determine the values of K_m and v_{max}. Do not include data from beer or blood samples.

E. Make Plot E of initial rate (μmol/mL/min) versus substrate concentration (i.e., v_0 vs. $[S]_0$) over the ethanol concentration range of 0.5 to 200 mM.

- Do the data suggest a pseudo-zero region referred to in the derivation of equation 3? Explain.

- Estimate v_{max} and K_m by manual extrapolation on the plot and label both of these on the plot. Do not perform a regression fit to the data because the simple unweighted regression does not work well.

- Compare the values v_{max} and K_m with those from part D. Comment on the comparison.

F. From the value of v_{max} calculated above and the known concentration (mg/mL) of enzyme **in the cuvette**, calculate the specific activity of the enzyme in units/mg. Check the course web page for the **exact concentration of the ADH stock solution in the small brown bottle** you worked with (note this is not the concentration in the cuvette !).

G. Make an expanded Plot G of initial rate versus known substrate concentration (i.e., v_0 vs. $[S]_0$) over the standard substrate concentration range of 0 to 0.5 mM in the cuvette.

- Do the data clearly show the first order region referred to in the derivation of equation 4? Mark the region on plot and explain what this means.

- Use the slope and intercept of this plot to estimate the molar concentration of alcohol in the reaction mixture for the diluted beer sample.

- Calculate the molar EtOH concentration in the original beer.

- Calculate the % (w/v) as (g ethanol/100 mL) in the original bottle of beer.

H. Make a separate Plot H of initial rate versus substrate concentration (v_0 vs. $[S]_0$) **for the blood standards** (use the final cuvette values) and assume the origin is one point in the calibration. Comment on how these initial rates compare with the initial rates from the beer standards (runs 1 & 2 and 4). If they are different, why do you think they are different?

1.   Use the slope and intercept from the plot of the blood standards and estimate the molar alcohol concentration in the reaction mixture for the diluted blood sample. Calculate the molar EtOH concentration in the original blood sample and the %(w/v) EtOH (i.e., g/100 mL) in the original blood sample.

2.   In Oregon, an EtOH blood level of 0.08%(w/v) or greater is considered conclusive presumption of intoxication. What does your blood analysis indicate about the chances in court of the driver from whom the blood was supposedly taken?

3.   How many 12 oz bottles of the beer of the type analyzed here would an average person have to drink to bring his/her blood alcohol level up to the value you determined for the blood sample? When calculating the number of bottles needed, consider that out of 40 L of aqueous fluids in an average adult, 5 L are blood. The transfer of ethanol from the blood to various body fluids is very rapid so that the concentration of ethanol in blood and other aqueous bodily fluids is taken to be equal. Assume the number of calculated bottles are consumed in a span of two minutes and that all the alcohol consumed passes directly into the blood stream.

ReadMe Notes on Data Table 1 (table on next page)

1.   The first day you must get through runs 1A/1B through 8A/8B. (A& B are a duplicate set
        of measurements on the same solution).

2.   To prepare to run two measurements on the same solution: setup two cuvettes (A&B) at
        the same time with the EtOH test solution and add the 250 μL of NAD. Put the A cuvette
        in the cell holder and check that the stirbar is rotating <u>before</u> adding the 250 μL of ADH
        which will initiate the reaction run. Record. Repeat for the B cuvette.

3.   If the reaction rates for duplicate runs (e.g., run 1A & run 1B) generated by the program
        don't agree within 5-10% of each other, try another run with the same EtOH
        concentration. If you are having problems achieving this precision, check with one of the
        instructors.

4.   For the Kinetics program, for runs 1A/1B use a 10 s delay and a 30-s measurement time.
        For all other runs, input a 20-s delay time and a 30-s measurement time. Use a 1000 ms
        <u>measurement interval</u> throughout.

5.   Be diligent and set the SCM gain as given in the Table as it changes for different runs.
        Also enter this value for the SCM gain in the Kinetics program. Double check that these
        are correct before you start a run.

6.   Table 1 is posted on the course supplemental page in Excel. Make a local copy and fill it
        in as you proceed through the measurements. Construct the calibration curve while you
        are working.

7.   Note that 2.0 mL of the test solution yields a final cuvette [EtOH] of $2.0/2.5 = 0.8$ X the
        test solution concentration (i.e., the total volume in the cuvette is 2.5 mL).

8.   The Beer Sample test solution is the solution after the 2nd dilution with buffer indicated
        in section IIC.

Table 1. ADH/NAD catalyzed EtOH Reaction Runs
See the ReadMe notes on the previous page and the Electronic Setup (Appendix) before proceeding.

Run	Sample ID	Nominal Final Cuvette[a] [EtOH] (mM)	Nominal Test Solution [EtOH] (mM)	SCM Gain (g)	Rate AU/min
1A	Std 200 mM	200	250	1	
1B	Std 200 mM	200	250	1	
2A	Std 80 mM	80	100	1	
2B	Std 80 mM	80	100	1	
Change The SCM Gain & Enter in Program					
3A	Std 10 mM	10	12.5	5	
3B	Std 10 mM	10	12.5	5	
4A	Std 2.5 mM	2.5	3.125	5	
4B	Std 2.5 mM	2.5	3.125	5	
Change the SCM Gain & Enter in Program					
5A	Std 0.5 mM	0.5	0.625	10	
5B	Std 0.5 mM	0.5	0.625	10	
Change the SCM Gain & Enter in Program					
6A	Std 0.1 mM	0.1xxx	0.125	20	
6B	Std 0.1 mM	0.1xxx	0.125	20	
7A	Std 0.2 mM	0.2xxx	0.250	20	
7B	Std 0.2 mM	0.2xxx	0.250	20	
8A	beer sample[b]			20	
8B	beer sample[b]			20	
Second Day of Lab - SCM and Program Gain of 10					
9A	B Std 0.32 mM			10	
9B	B Std 0.32 mM			10	
10A	B Std 0.64 mM			10	
10B	B Std 0.64 mM			10	
11A	blood sample			10	
11B	blood sample			10	

V. References

Enzyme kinetics are discussed in the following books:

a.   Athel Cornish-Bowden, "Fundamentals of Enzyme Kinetics", Weinheim : Wiley-VCH,
        2012.

b.   C. K. Mathews, K.E. van Holde, D.R. Appling, S.J. Anthony-Cahill, "Biochemistry (4th
        Edition)", Pearson, 2013.

c.   H. R. Mahler and E. H. Cordes, "Biological Chemistry", New York, Harper and Row,
        1972.

d.   M. Dixon and E. C. Webb, "Enzymes", Academic Press Inc., New York, 1958.

e.   D.L. Nelson and M. M. Cox," Lehninger Principles of Biochemistry / Edition 5",
        Freeman, W. H. & Company, 2008.

f.   W. P. Jencks, "Catalysis in Chemistry and Enzymology", McGraw-Hill, New York, 1987.

g.   J. Westley, "Enzymatic Catalysis", Harper and Row, New York, 1969.

h.   M. L. Bender and L. J. Brubacher, "Catalysis and Enzyme Action", McGraw-Hill, New
        York, 1973.

I.   G. C. Guilbault, "Enzymatic Methods of Analysis", Pergamon Press, Oxford, 1970.

Pyridine nucleotide coenzymes are discussed in:

j.   T. P. Singer and E. B. Kearney, Advanc. Enzymol., 15, 79 (1954).

Yeast alcohol dehydrogenase is reviewed by:

k.   H. Snud and H. Theorell, "Alcohol Dehydrogenases", in The Enzymes, Vol. 7, 2nd Ed.,
        P. D. Boyer, H. Lardy, and K. Myrback, eds., p. 25, Academic Press, Inc., New York,
        1963.

The evolution of ethanol from the body is discussed in:

l.   G. V. Calder, J. Chem. Ed., 51, 19 (1974).

Appendix - Electronics Setup

The instrument has already been set up for you according to these instructions --
you should trace through the connections and recheck the PMT dark output in Step 5 and the
SCM zero absorbance in Step 6.

1. Connect the PMT module signal cable to the input of the log amplifier (A/K mode) to provide direct absorbance readout.

2. Connect the output of the log amplifier to the signal conditioning module set initially for a gain of 20 and a 0.3-Hz cutoff frequency.

3. Connect the output of the SCM to channel 0 of the ADC.

4. Initially, connect the DMM to the input of the log amp so you are monitoring the PMT module output. Fill the sample cuvette with water and insert in the sample holder. Open the light shutter and adjust the PMT bias voltage to about 350 V. Adjust the PMT module gain or PMT bias voltage for about a 1 V signal on the DMM. Now adjust the lamp position to maximize the signal and then readjust one of the gains for about a 1 V signal.

5. **With the DMM still connected to the input of the log amp so your are monitoring the PMT output, close the shutter and check that the PMT signal is about +2 mV when the shutter is closed (slightly positive).** If not, adjust with the PMT module zero control.

6. **Move the DMM leads to the output terminal of the SCM module, open the shutter, and check that the signal is near 0 V.** Adjust if necessary with the PMT module gain controls. Because the log ratio amplifier outputs 1 V/A.U. and the SCM gain is 20, 20 mV at the SCM output corresponds to 0.001 A.U. Note, you cannot make a measurement with the shutter closed because the output of the log amp or SCM will go to the limit (`-14 V).

7. Choose the "Kinetics" shortcut icon from the course folder (see next page of appendix for details).

Computer Program - Kinetics

The Kinetics computer program for calculating the rate is based on the data acquisition program you have used throughout the term. The user inputs a delay, measurement time, and the SCM gain. When the user signals to start the measurement cycle (ENTER), the computer times the delay chosen so that the reagents can mix and the reaction rate can reach full velocity. After the delay time, the computer takes and stores a voltage reading every second for the measurement time chosen. Next the program runs a linear regression and reports the slope and intercept.   You should then save the results of the regression and the raw data to a .csv file. Each student in the group should have access to the full set of .csv files. Check the units for time and make sure that you are using minutes in the calculations.

Creating Plots from Similar Sets of Data in Excel

Graphing Multiple runs using a template in Excel: Constructing these plots is relatively quick and easy if you take the first data set (Run 1A), make the graph, add title, labels, etc. and a trend line with slope and intercept displayed.

Method 1:   Then save this spreadsheet as Run01A.xls and then save it again as Run01B.xls. Then open the data for Run 1B, copy columns A and B, paste them into columns A & B of Run01B.xls and save as Run01B.xls. Et cetera. This way you don't have to waste any time reconfiguring the graphs.

Method 2: Alternative method to copy: right click on the tab name at the bottom and select "move or copy" then check the box for "create a copy". Once copied, right click on the new spreadsheet page tab and rename it to Run01B, and proceed as above.