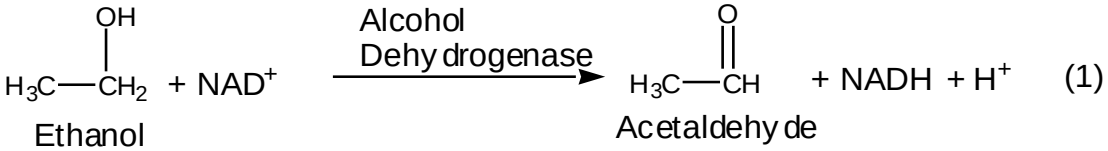
Kinetics of ADH-Catalyzed Metabolism of Ethanol

Abstract: We will study the oxidation of ethanol *in vitro* (in laboratory glassware), with the help of the enzyme Alcohol Dehydrogenase (ADH). The same reaction happens in our body when we consume alcohol. The order of the reaction will be determined. We will also see how the color reagent is reoxidized by air and recovers its color in absence of ethanol. The ability of several aliphatic alcohols to serve as substrates for the reaction will be investigated during the first week of experiments. The effects of ethanol concentration on the rate of this enzymatic reaction will be explored during the second week. The effect of pH on the rate of the reaction will be investigated during the third week. The kinetics of this reaction will be followed using a spectrophotometer.

Introduction:

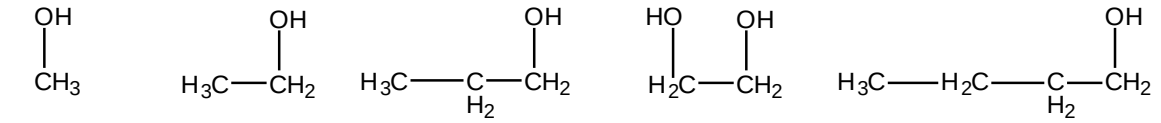
As we will see, there are many different alcohols, but ethanol (ethyl alcohol) is the specific alcohol contained in alcoholic beverages, such as beer, wine, or liqueurs. It causes drowsiness, mental excitement or depression, loss of coordination, impaired perception, and, at high doses, may lead to nausea, vomiting, coma, and even death. Human organisms fight the effects of ethanol by removing it in the following reaction:



The enzyme Alcohol Dehydrogenase (ADH) catalyzes this reaction, in which nicotinamide adenine dinucleotide (NAD^+), an important metabolic intermediate derived from vitamin niacin, oxidizes ethanol. The genetic makeup of a person defines of how much and what kind of Alcohol Dehydrogenases (ADH's) a person has. People who have decreased activity of ADH in their body remove ethanol slower and get drunk faster than people with high ADH activity. The product of this reaction, acetaldehyde, is several times more toxic than ethanol so its removal is essential. The reaction of acetaldehyde with available amines and the dehydration of organism that follows ingestion of ethanol cause headaches that we refer to as hangovers. The removal of acetaldehyde is facilitated by another enzyme, called Aldehyde Dehydrogenase. Alcoholics are sometimes administered inhibitors of Aldehyde Dehydrogenase. If, despite the warning, they consume alcohol, ADH's in their body produce acetaldehyde, and since the removal of acetaldehyde is inhibited, the consumption of drinks punishes alcoholics by making them very sick.

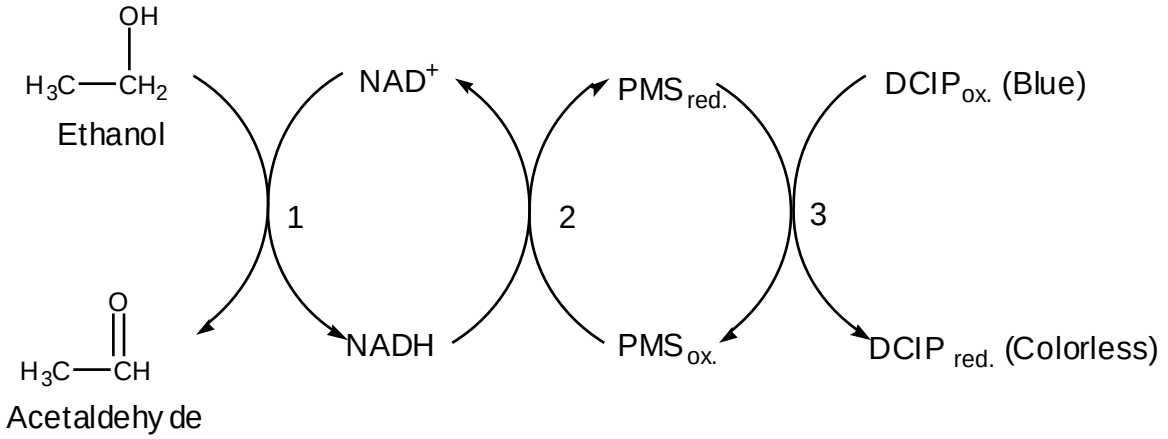
Even if both reactants are present, reaction (1) will not proceed at a measurable rate in the absence of the enzyme. While the reaction is spontaneous in the thermodynamic sense (it is not at equilibrium), it has a high activation energy. Enzymes are very powerful catalysts that speed up reactions millions of times as compared to uncatalyzed reactions. They do that by bringing reactants (which are called ***substrates*** when they bind to the enzyme) close together into ideal orientation for the reaction to occur in the enzyme's active site. Enzymes bind to the substrates loosely but bind to the reaction transition state very tightly, and lower the activation energy of the reaction in this way. Enzymes are most often polypeptides (polymers of amino acids). Their 3D structure is maintained by weak forces such as hydrophobic interactions, ion-ion interactions, hydrogen bonding, and Van der Waals forces. Thus, the structure of proteins is very sensitive to the

changes of the environment. Sudden changes in the temperature or pH of surrounding media and/or presence of certain salts or solvents might "denature" proteins, that is, destroy their catalytic activity by modifying the enzyme's active site in some way. For example, enzymes usually exhibit the highest activity at certain pH (ADH is most active at pH 9), and have much lower activity at either higher or lower pH values. The active site of an enzyme is so specific that it can recognize very definite substrates. If chemicals are very similar, the enzyme might use them as substrates but often the reaction slows down significantly. We will try different substrates for the ADH reaction:



Two of these, methanol (used as a denaturing agent in denatured alcohol) and ethylene glycol (used in antifreeze fluid) are occasionally ingested by people and cause drowsiness along with more severe effects such as blindness and death. Death can be caused by as little as 30 mL of methanol. Since the methanol molecule is slightly smaller than ethanol (a natural substrate of this enzyme), ADH consumes methanol significantly more slowly. The product of this reaction is a very toxic compound- formaldehyde. One of the ways of saving a person's life in this situation is to immediately give him/her large volumes of ethanol to drink. Thus, by providing ADH with its favorite substrate (ethanol) the reaction with methanol is slowed down even more, and the kidneys remove methanol, and its metabolic product, formaldehyde, while the patient is very drunk from ethanol.

The ADH reaction (1) produces NADH which allows us to follow the kinetics of this reaction. NADH is NAD^+ which has been reduced by addition of electrons and H^+. We use two additional chemicals, phenazine methosulfate (PMS) and 2,6-dichloroindophenol (DCIP) to help us with the task of monitoring the reaction. NADH reduces PMS_{ox} to PMS_{red}, then the PMS_{red} reduces $DCIP_{ox}$ (which is blue) to $DCIP_{red}$, which is colorless:



The disappearance of the blue color can be followed visually or by use of a spectrophotometer (also called a spectrometer, photometer, or colorimeter). This instrument measures the percentage of light that goes through the solution in a cuvette. Most spectrometers measure the percent transmittance (%T) but convert it to "Absorbance" (A) of the solution, because the absorbance is directly related to the concentration of the colored species. It is defined as A = - log (%T/100%). That is, if all light goes through the solution, and transmittance is 100%, the absorbance is 0, if 10% of light goes trough the cuvette, the A = -log (10%/100%) = - log (10^{-1})= 1, and if only 1% of light goes trough (which means the solution is very dark), then A=2. According

to Beer's Law,

A = a b C

the absorbance of a solution is proportional to the concentration C of the blue dye DCIP in our solutions. Since the reactions 2 and 3 are significantly faster that reaction 1, the rate of disappearance of the blue dye is directly proportional to the rate of consumption of ethanol. Thus, the kinetics of the enzymatic reaction can be followed in the visible region of spectra by measuring rates as the change of absorbance per minute in the reaction mixtures with different ethanol concentrations.

Zero order reaction

The rate of a "zero order" reaction is independent of reactant concentration:

$$\text{Rate} = \frac{\Delta[Substr]}{\Delta t} = k * [\text{Substrate}]^0 = k$$

A plot of substrate concentration ([Substrate]) vs. time will have a slope of $\frac{\Delta[Substr]}{\Delta t}$, which is the rate. Since the rate is constant for a zero order process, the slope will be constant, and the plot should be a straight line. In cases where the absorbance is directly related to the concentration, a plot of absorbance, A, vs. time should also be a straight line.

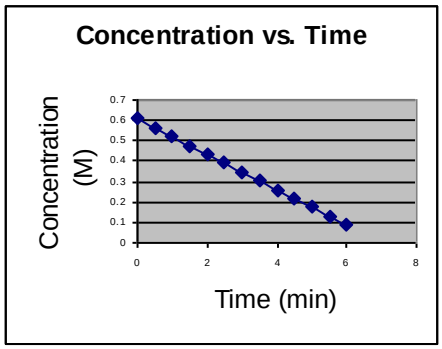


Figure 1

First order reaction

The rate of reaction increases as the concentration of reactant increases:

$$\text{Rate} = \frac{\Delta[Substr]}{\Delta t} = k * [\text{Substrate}]^1$$

Since the rate of a first order process is directly proportional to the concentration of the reactant, it decreases as the reaction progresses as the reactant is consumed. A plot of [Substrate] vs. time for a first order process would be a curved line of decreasing slope (a so-called exponential decay).

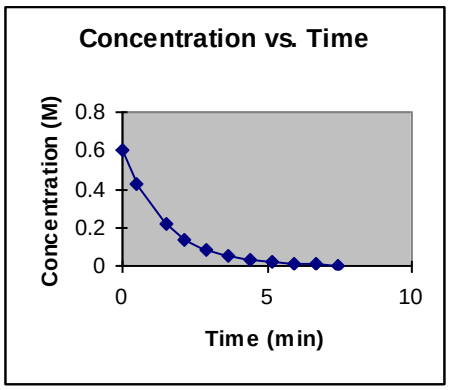


Figure 2

Michaelis-Menten kinetics

Enzymatic kinetics follows a model that incorporates both first and zero order reactions. The mechanism involves one substrate molecule (S) docking on one molecule of the enzyme (E), and making a molecule of a product (P) while regenerating the enzyme,

Step 1: $E + S \rightleftharpoons ES$

Step 2: $ES \rightarrow E + P$

Three reasonable assumptions are made to simplify the model. The rate limiting reaction is the production of P in the second step, since the equilibrium of making ES is reached very quickly. The total concentration of enzyme and the concentration of enzyme- substrate complex ES are assumed to be constant. The reaction of product formation is non-reversible.

The net effect of the above assumptions is that the rate simplifies to first order kinetics with respect to substrate at relatively low substrate concentrations, where k' includes the constant enzyme concentration:

Low [S]: Rate = $\frac{\Delta[S]}{\Delta t}$ = k[E][S]^1 = k'[S], because [E] is constant

But as the concentration of substrate increases, the enzyme becomes saturated with substrate, and the reaction cannot proceed any faster as more substrate is added. The process is then "pseudo" zero order; that is, it proceeds at a constant, maximum rate, R_{max}, in spite of increasing substrate concentrations.

High [S]: $R_{max} = \frac{\Delta[S]}{\Delta t}$ = k' [S]^0 or R_{max} = k" = k' S_{max},

where S_{max} is constant and proportional to the total enzyme concentration. An overall rate law that includes both the zero, and first order cases is called the Michaelis-Menten equation,

$$\text{Rate} = R_{max}\left[\frac{[S]}{k+[S]}\right]$$

To get good data to demonstrate Michaelis-Menten enzyme kinetics, the concentration of substrate should be varied widely. We expect to see the rate of reaction slowly rise as [S] is increased, and then reach a plateau where it no longer increases as [S] is increased. That means that the rate of alcohol metabolism increases as the concentration of ethanol in our body is increased up to a certain level. But as the concentration of ethanol increases beyond that level, metabolism can go no faster and added alcohol is metabolized at the same rate, so ethanol builds up faster. An analogy one might use here is the one of a brick layer. Let's say you ask a brick layer to lay 2 bricks per minute. He/she will be able to do it efficiently. The same will happen with 3 or 5 bricks. His/her rate will increase until let's say about 12 bricks per minute, and then it becomes physically impossible to do more than 12. If you give 50 or 100 bricks, it does not matter, the bricklayer will lay 12 bricks per minute as a maximum. You will be able to determine the order of reaction in two concentration regions after you complete your first experiment.

When we study the rate of the reaction as a function of ethanol concentration, we may observe plots like Figure 3. The initial part "a" indicates an initially very low rate, then the rate increases in segment "b". This is where the PMS and DCIP reactions are "catching up" with the ethanol oxidation. The main segment "c" appears to be a straight line through most of the run, *even when the concentration of ethanol is too small to saturate the enzyme*. Under these conditions, the reaction should be first order in substrate, and the plot should look like Figure 2. The reason that it doesn't is that the change in ethanol concentration over the time of our experiment is very small. You will note that if you look at any <u>small</u> segment of the curved line in Figure 2, it is difficult to distinguish from a straight line. In order to see the curvature in the first order plot, the reaction has to proceed over several half lives (where the initial concentration is cut in half several times). This does not happen in our experiment. DCIP needs to be at only about 70 μM (70 x 10^{-6} M) to give an absorbance close to 1, so all of the DCIP is consumed stoichiometrically when 70 μM of ethanol is consumed. This is an insignificant change in the initial concentration, so the rate changes insignificantly, and the plot appears to be a straight line. The rates calculated as the slope of any segment of this line are virtually indistinguishable, so an average slope can be used to represent a single rate for the reaction at the initial ethanol concentration. Of course at concentrations of ethanol high enough to saturate the enzyme, segment "c" is truly a straight line, indicating the zero order kinetics. In regions "d" and "e", the DCIP concentration decreases to a very low level and the rate of bleaching approaches zero.

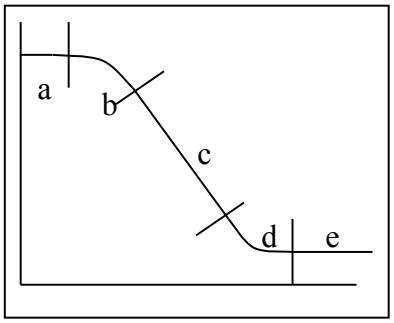


Figure 3

When we study the rate of reaction as a function of pH or temperature, we will intentionally

compare the rates in the region of zero order kinetics. That is because we hope to minimize the effects of other variables like concentration, on the rate, so we will see only the effects of pH or temperature. In the zero order region, concentration has no effect at all on rate, so any changes we see are the result of changes in pH or temperature.

You are also asked to let one of your reactions run for some time. There is something peculiar that you might observe in this cuvette. The ethanol you add to the reaction acts as a source of reducing power, reducing blue $DCIP_{ox}$ to colorless $DCIP_{red}$. Once the ethanol is exhausted, $DCIP_{red}$ is slowly reoxidized by oxygen in the air and becomes dark blue again. This observation has meaning for the living systems in the thermodynamic sense. All animals on this planet receive their energy as food in the form of reduced substances like ethanol. Intake of food keeps us in a steady state, where the food is oxidized gradually, and the organism never reaches equilibrium with available air. If the food/energy supply is exhausted for a living system, the system dies as oxidation by air reaches equilibrium. We will observe the same in our system.

During the first week of experiments, you will examine the specificity of the enzyme towards different substrates. We will note and compare how fast reactions with different alcohol substrates run. How fast are poisonous methanol and ethylene glycol consumed?

During the second week of experiments, you will perform a kinetic analysis of the enzyme with its optimal substrate in order to determine the Michaelis-Menton parameters K_m and V_{max} In order to determine these parameters, you will calculate the enzymatic activity at several different concentrations of substrate. You will then plot the reciprocals of the enzymatic activity versus substrate concentration, a common technique known as a Lineweaver-Burke plot. The slope of this line will be equal to K_m/V_{max} the x-intercept will be $1/K_m$ and the y-intercept will be $1/V_{max}$.

During the third week of experiments you will vary the pH of the reaction. This will influence the reaction (1) itself because H^+ ions are produced in this reaction. In addition to this, the pH change will affect the shape of the enzyme's active site and the stability of the enzyme. Protons (H^+ ions) bind to the amino acids that constitute the enzyme, changing the charges of various sites on the enzyme and frequently altering the intramolecular bonding that gives rise to the tertiary structure of the enzyme, and changing its activity.

Week 2: The effect of substrate on the ADH catalyzed reaction

Objective:

The objective of the second week of this experiment is to complete a Control run and a Standard Run at 20ºC, pH 9, and ethanol substrate at 0.9 M concentration. We will compare the results of this run to other reactions run with different aliphatic alcohols. All data must be tabulated in an Excel spreadsheet. When you get into the Lab, discuss which person will be doing each trial when several are specified, because data will be pooled to complete the tables on the spreadsheet.

Calibration:

Calibrate the spectrophotometer at 635 nm with distilled water according to the directions for the Spec-20 in your laboratory manual.

Control Run:

In a clean glass spectrophotometer cuvette (rinsed with dH_2O), add 1.0mL of the DCIP, PMS, NAD^+ solution, 1.0mL of the 0.9 M Ethanol solution, 0.5mL of the 0.06M Tris HCl buffer pH 9.0, and 1.0mL of dH_2O. **Remember to always use a different syringe for each solution as to avoid contamination.** Briefly mix the solution by aspirating it with a syringe, start the stopwatch, put the cuvette in the spectrometer and record the absorbance every 10 seconds for 4 minutes.

Substrate Reactions:

You will need to obtain 50 mL beakers filled with each of the available substrates (methanol, ethanol, 1-propanol, 1-butanol and ethylene glycol) and bring them to your lab bench. In a clean glass spectrophotometer cell (rinsed with dH_2O), add 1.0mL of the DCIP, PMS, NAD^+ solution, 1.0mL of the 0.9M alcohol solution, 0.5mL of the buffer of the appropriate pH, and 1.0mL of the ADH enzyme. **Remember, once the enzyme is added, the reaction begins!!!** Briefly mix the solution by aspirating it with a syringe, start the stopwatch, put the cuvette in the spectrometer and record the absorbance every 10 seconds for 4 minutes. You should run each substrate in triplicate so repeat this procedure two more times.

Data Analysis:

Enter your data for each trial and each substrate into an Excel spreadsheet. Average the absorbencies for the three trials at each substrate concentration. Next, graph absorbance vs. time (s) using the average absorbance for each time period for each substrate. You should have one graph with the date from all five substrates plotted. The reaction rate, $\Delta A/\Delta t$, is determined by the slope of the linear part of the curve. The linear part of the curve may be different for each substrate. Compare the rates for each substrate by generating a bar graph.

Week 3: Determination of Michaeils-Menton parameters for ADH

Objective:

The objective of the third week of this experiment is to complete a control run, a standard experimental run, and several runs at different ethanol concentrations. All data must be tabulated in an Excel spreadsheet.

Calibration:

Calibrate the spectrophotometer at 635 nm with distilled water according to the directions for the Spec-20 in your laboratory manual.

Control Run: (Each group should do one run)

In a clean glass spectrophotometer cuvette (rinsed with dH_2O), add 1.0mL of the DCIP, PMS, NAD^+ solution, 1.0mL of the 0.9 M Ethanol solution, 0.5mL of the 0.06M Tris HCl buffer pH 9.0, and 1.0mL of dH_2O. **Remember to always use a different syringe for each solution as to avoid contamination.** Briefly mix the solution and start data collection as last week.

You should not use the data from the Control run that you carried out last week. A control always gives you a starting point for a new experiment. Although you are continuing the experiment from last week, you never know…something may be different this week. It is simply a good protocol to have controls done every time you do an experiment.

Standard Experiment: (Each student should do one run to check repeatability)

In a clean glass spectrophotometer cuvette (rinsed with dH_2O), add 1mL of the DCIP, PMS, NAD^+ solution, 1.0mL of the 0.9M Ethanol solution, 0.5mL of the .06M Tris HCl buffer pH 9.0, and 1.0mL of the enzyme solution. Note that the concentration of ethanol in the reaction mixture will be 0.257 M, enough to saturate the enzyme. **It is important that the enzyme is ALWAYS added last!!!** Again, use different 1.0mL syringes for each solution. Briefly mix this solution by aspirating it with a syringe and start the stopwatch. **Remember, the reaction begins as soon as the enzyme is added, so for best results, little time should be wasted between the addition of the enzyme and the collection of data**.

Since we are working as a group for these experiments, some differences between data collected by different members of the same group. See how similar (or dissimilar) your results are. If there is a significant difference, you may want to discuss what was done differently between you, as this is the most likely source of error. A normal run should exhibit absorbance data that decreases from ~1.000-0.700, to 0.100-0.050 in two to three minutes.

Effect of Ethanol concentration (Michaelis-Menten kinetics)

Record the data for the control run and for one Standard Experiment (with 0.257 M ethanol) into your laboratory notebook. The rate of the reaction is to be measured at 0.006M, 0.014M, 0.028M, 0.060M, 0.086M, 0.171M, 0.257M concentrations of ethanol runs.

In a clean glass spectrophotometer cell (rinsed with dH_2O), add 1.0mL of the DCIP, PMS, NAD^+ solution, 1.0mL of the appropriately labeled ethanol solution (0.021M, 0.048M, 0.099M, 0.300M, 0.600M, 0.900M, 1.20M), 0.5mL of the 0.06M Tris HCl buffer pH 9.0, and 1.0mL of the enzyme. Quickly mix and begin the collection of data as usual. Transfer the collected absorbance data into your laboratory notebook.

Data Analysis

Enter your data for each trial and each substrate into an Excel spreadsheet. Average the absorbencies for the three trials at each ethanol concentration. Next, graph absorbance vs. time (s) for each ethanol concentration. At this point, you should have 8 graphs. Next, determine the

reaction rate for each concentration by determining the slope over the linear part of the graph. The linear part of the curve may be different at each concentration. Once you have determined the reaction rate for each concentration, make a plot of rate ($\Delta A/\Delta t$) vs. concentration of ethanol (M). In order to explore the effect of ethanol concentration of reaction rate, you will generate a Lineweaver-Burk plot. Generate a plot of the reciprocal of reaction rate vs. the reciprocal of ethanol concentration. The slope of this line will be equal to K_m/V_{max} the x-intercept will be $1/K_m$ and the y-intercept will be $1/V_{max}$.

Week 4: The effect of pH on the ADH catalyzed reaction

Objective:

The objective of the forth week of this experiment is to complete a Control run and a Standard Run at 20ºC, pH 9, and ethanol substrate at .3 M concentration. We will compare the results of this run to others with different pH values (pH = 6, 8, 9, 10, 12).

Calibration:

Calibrate the spectrophotometer at 635 nm with distilled water according to the directions for the Spec-20 in your laboratory manual.

Control Run:

In a clean glass spectrophotometer cuvette (rinsed with dH_2O), add 1.0mL of the DCIP, PMS, NAD^+ solution, 1.0mL of the 0.9 M Ethanol solution, 0.5mL of the 0.06M Tris HCl buffer pH 9.0, and 1.0mL of dH_2O. **Remember to always use a different syringe for each solution as to avoid contamination.** Briefly mix the solution and start the stopwatch.

You should not use the data from the Control run that you carried out last week. A control always gives you a starting point for a new experiment. Although you are continuing the experiment from last week, you never know…something may be different this week. It is simply a good protocol to have controls done every time you do an experiment.

pH 6, 8, 9, 10, and 12:

In a clean glass spectrophotometer cuvette (rinsed with dH_2O), add 1.0mL of the DCIP, PMS, NAD^+ solution, 1.0mL of the 0.9M Ethanol solution, 0.5mL of the buffer of the appropriate pH, and 1.0mL of the ADH enzyme. **Remember, once the enzyme is added, the reaction begins!!!** Quickly mix and begin the collection of data as usual.

Data Analysis

Enter your data for each trial at each pH into an Excel spreadsheet. Average the absorbencies for the three trials at each pH. Next, graph absorbance vs. time (s) using the average absorbance for each time period for each pH. You should have one graph with the date from all five substrates plotted. The reaction rate, $\Delta A/\Delta t$, is determined by the slope of the linear part of the curve. The linear part of the curve may be different for each pH. Compare the rates for each substrate by generating a bar graph.

Prelaboratory Questions:

Week 2:
1. What effect do you think carbon chain length of the alcohol substrates will have on the observed reaction rate and why?
2. Draw the structures of each of the alcohols used in the experiment. For the structures of 1-propanol and 1-butanol

Week 3:
1. Sketch a graph of Substrate Concentration vs. Time for a zero order process. Invent values for [S] and t for two points on the graph and calculate the slope. What does the slope represent?
2. From the description of Michaelis-Menten kinetics, make a rough sketch of how you would expect the plot to look like if you graphed Rate vs. Concentration of Substrate.
3. From the concentration of the stock ethanol solution used to prepare the solution for the Standard Experiment, calculate the concentration of ethanol in the solution used for the kinetics run.
4. Why is the enzyme added to the reaction mixture last?
5. Why do you use the same cuvette for all experimental trials?
6. Why do you use a spatula before collecting data beginning the reaction?
7. If the rate of reaction is independent of substrate concentration, what is the order of reaction?
8. What is the proof value of the 0.9 M ethanol? Hint: convert $\frac{0.9\,mol}{L}$ to $\frac{Vethanc}{Vtot}$ and multiply by 9. The density of ethanol is 0.79 g/mL and the density of the solution is ~1.0 g/mL.

Week 4:
1. Why do you run a control in both weeks of the experiment?
2. Considering the structures of other potential substrates provided in the introduction, do you expect the same rate for all reactions? Why?
3. Why is the effect of pH studied by comparing rates measured in the zero order region of the kinetics runs?
4. If the "regular runs" shared between you and your partners are strongly dissimilar, why should you not precede any further?

Equipment & Supplies:
Spectrophotometer
Four 1 mL syringes/ group of students
The following reagents in several 50 mL beakers each:
0.2mM DCIP, 0.08mM PMS, 0.75mM NAD^+
0.1% BSA (store at 4°C, keep on ice during the lab)
0.1M NaH_2PO_4 pH 7.5
Stock ADH: 1mg ADH per 1mL of 0.1M NaH_2PO_4 pH 7.5 (good for 3 weeks if stored at 4°C, keep

on ice during the lab)
ADH for use in experiment: dissolve 50 µL of 1mg/mL stock ADH in 50mL 0.1% BSA (good for 5 days if stored at 4°C, keep on ice during the lab)
0.9M Methanol
0.9M 1-Propanol
0.9M 1-Butanol
0.9M Ethylene Glycol
0.06M Tris pH 9.0
0.06M NaH_2PO_4 pH 12.0
0.06M Glycine pH 10.0:
0.06M Tris pH 8.0
0.06M Sodium acetate pH 6.0
1.2M Ethanol, 0.9M Ethanol, 0.6M Ethanol, 0.3M Ethanol, 0.21M Ethanol, 0.099M Ethanol, 0.048M Ethanol, 0.021M Ethanol, and 0.02M Ethanol

Literature:

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