Lesson II
Alcohol Dehydrogenase Assay
(estimated duration 1-2 hours)

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Lesson II
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Outline

I. Pre-requisite knowledge

A. Students should be familiar with the basic concepts of graphing, specifically X- and Y- axis location, axis labeling, creating a scale, and graph interpretation.
B. Students should be familiar with proper handling and use of a p200 micropipette.

II. Massachusetts Science and Technology/Engineering Frameworks Compliance

Bio 1.3: Explain the role of enzymes as catalysts that lower the activation energy of biochemical reactions. Identify factors, such as pH and temperature, that have an effect on enzymes.
SIS1: Observe the world from a scientific perspective.
SIS1: Pose questions and form hypotheses based on personal observations.
SIS2: Articulate and explain the major concepts being investigated and the purpose of the investigation.
SIS2: Select required materials, equipment, and conditions for conducting an experiment.
SIS2: Identify independent and dependent variables.
SIS2: Employ appropriate methods for accurately and consistently:
-making observations.
-making and recording measurements at appropriate levels of precision.
-collecting data or evidence in an organized way.
SIS2: Properly use instruments, equipment, and materials (e.g., scales, probeware, meter sticks, microscopes, computers) including set-up, calibration (if required), technique, maintenance, and storage.
SIS4: Review information, explain statistical analysis, and summarize data collected and analyzed as the result of an investigation.
SIS4: Explain diagrams and charts that represent relationships of variables.
SIS4: Construct a reasoned argument and respond appropriately to critical comments and questions.

III. Content to be Taught

A. The liver processes alcohol that enters the body's digestive tract.
B. Rates of enzyme activity can be concentration-dependent and have maximum values.

C. Alcohol dehydrogenase is an enzyme in the human body that breaks down alcohol to acetylaldehyde, NADH, and hydrogen ions.
D. Alcohol dehydrogenase requires the coenzyme NAD^+ in order to function.
E. Addition of PMS in the assay will create a color reaction, and resulting color intensity will be proportional to the amount of NADH produced in the reaction. Since the amount of NADH produced is proportional to the amount of alcohol broken down, the color intensity indicates the amount of alcohol broken down.
F. A spectrophotometer is an instrument that can quantify color by measuring the amount of light it absorbs. The amount of light a solution absorbs is proportional to the intensity of its color, i.e., how dark it is.
G. As substrate concentration (alcohol) increases, overall enzyme activity rate increases to keep up. In our assay, substrate is ethanol (alcohol) and enzyme is ADH.
H. The amount of enzyme is not always sufficient for the amount of alcohol to be broken down. If the enzyme cannot keep up, unprocessed alcohol will accumulate in the body.
I. Accumulation of excess alcohol can be seen in the graph as the straight line (labeled "increasing alcohol concentration" cf. page 5) when it has diverged from the alcohol catabolism curve.

IV. Rationale

In this lesson, students will learn about the breakdown of alcohol in the body and how enzymes work to perform this process. By performing the lab procedure and analyzing the resulting graph, students discover that enzymes cannot always keep up with their substrates and as a consequence alcohol accumulates in the body. Lastly, this lesson develops students' skills to follow a laboratory protocol, use a micropipette, measure solutions using a spectrophotometer, and graph, analyze, and interpret lab-derived data.

V. Goals

Students will understand that alcohol dehydrogenase is an enzyme that breaks down ingested alcohol in the body, and that this enzyme cannot always keep up with the amount of alcohol (substrate) present. Students will learn this by analyzing the graph of their lab data showing that the rate of alcohol breakdown, and therefore overall enzyme activity, levels off as alcohol concentration in the body continues to increase (as one drinks more). Lastly, students will predict that unprocessed alcohol accumulates in the body.

VI. Objectives

A. Predict how alcohol is broken down
B. Learn to use p200 micropipettes.
C. Perform an alcohol dehydrogenase assay.
D. Graph experimental data and interpret findings

VII. Background for Teacher

A. *ADH and the liver*

Alcohol dehydrogenase (ADH) is an enzyme that functions in the liver to break down, or catabolize, alcohol. Other functions of the liver are to produce substances that break down fats, convert glucose to glycogen, produce urea (the main substance of urine), and make certain amino acids (the building blocks of proteins).

REFERENCE: http://www.mamashealth.com/organs/liver.asp

B. *Mechanism of action of ADH*

Alcohol dehydrogenase catalyses the oxidation of ethanol to acetaldehyde in the presence of the coenzyme nicotinamide adenine dinucleotide (NAD^+), according to the following chemical reaction:

$$\underset{\text{(ethanol)}}{CH_3CH_2OH} + NAD^+ \xrightarrow{\text{Alcohol Dehydrogenase}} \underset{\text{(acetaldehyde)}}{CH_3CH{=}O} + NADH + H^+$$

REFERENCE:
http://www.elmhurst.edu/~chm/vchembook/642alcoholmet.html

C. *Alcohol Dehydrogenase assay*

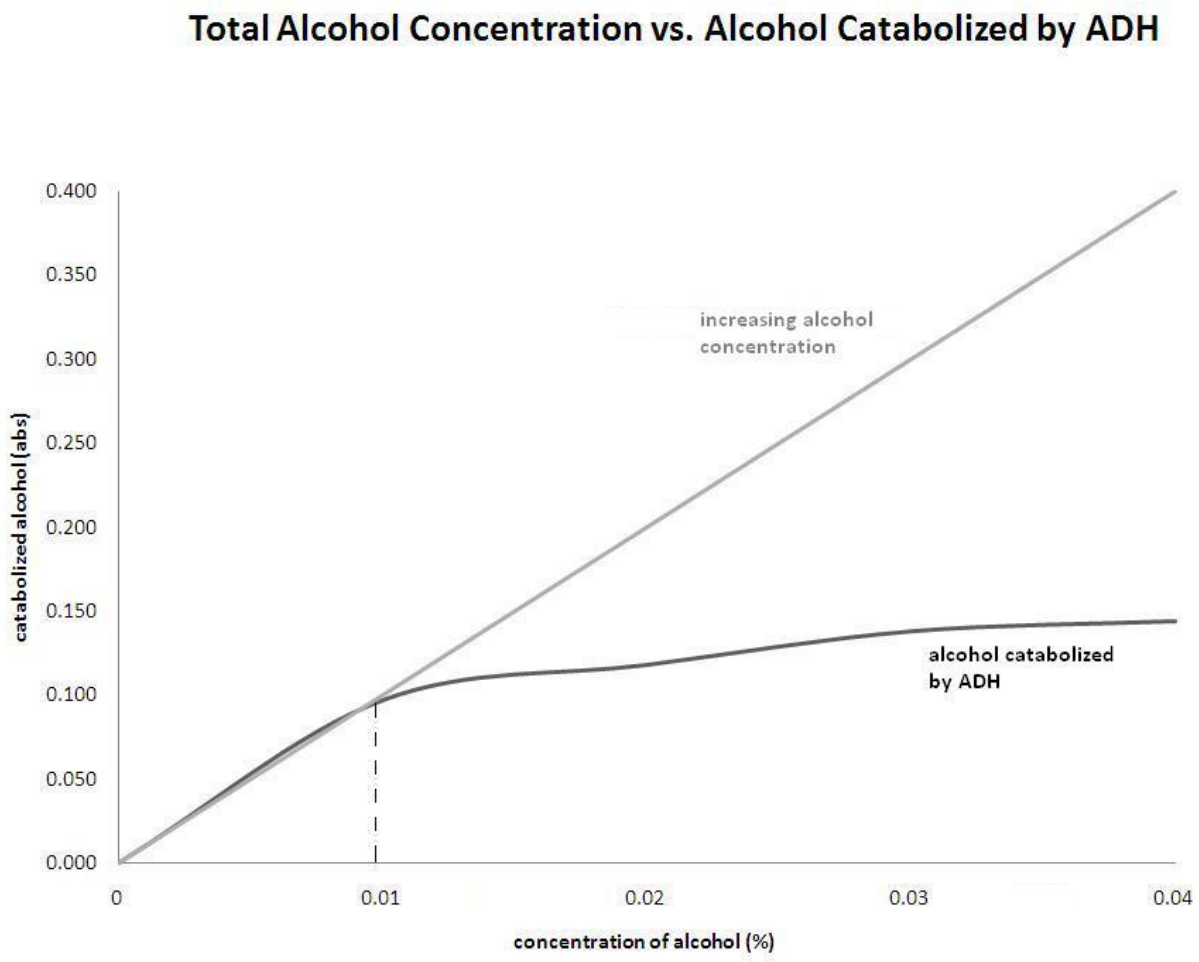
The enzymatic activity of alcohol dehydrogenase can be assayed spectrophotometrically according to a standard assay generally used for all NAD^+ -dependent oxidoreductases, which reduce NAD^+ to NADH. This reduction of NAD^+ is accompanied by a change in absorption of the NAD^+ molecule and thus can be followed spectrophotometrically at 340nm in real time. According to the Lambert-Beer law, the extinction of NAD^+ is proportional to its concentration. The change in concentration of NAD^+ over time will allow the calculation of the activity of alcohol dehydrogenase in the solution, using the following unit definition for alcohol dehydrogenase:

> One unit will convert 1.0 µmole of ethanol to acetaldehyde per minute at pH 8.8 at 25^{o}C. Therefore, one unit will also convert 1.0 µmole of NAD^+ to NADH per minute.

REFERENCE:
Kägi, J.H.R. and Vallee, B.L. (1960) Journal of Biological Chemistry 235, 3188-3192

D. *Explanation*

When the rate of alcohol catabolism cannot keep up with increases in alcohol concentration, unprocessed alcohol begins to accumulate in the system. If the line of constant increasing alcohol concentration is overlaid on an alcohol catabolism curve, this accumulation of alcohol can easily be seen by the separation of the two graphs and is indicated by the dotted line in the graph below. The rate at which ADH can catabolize alcohol has an upper limit, or V_{max}, as seen in the leveling off of the curve in the graph below.



VIII. Materials

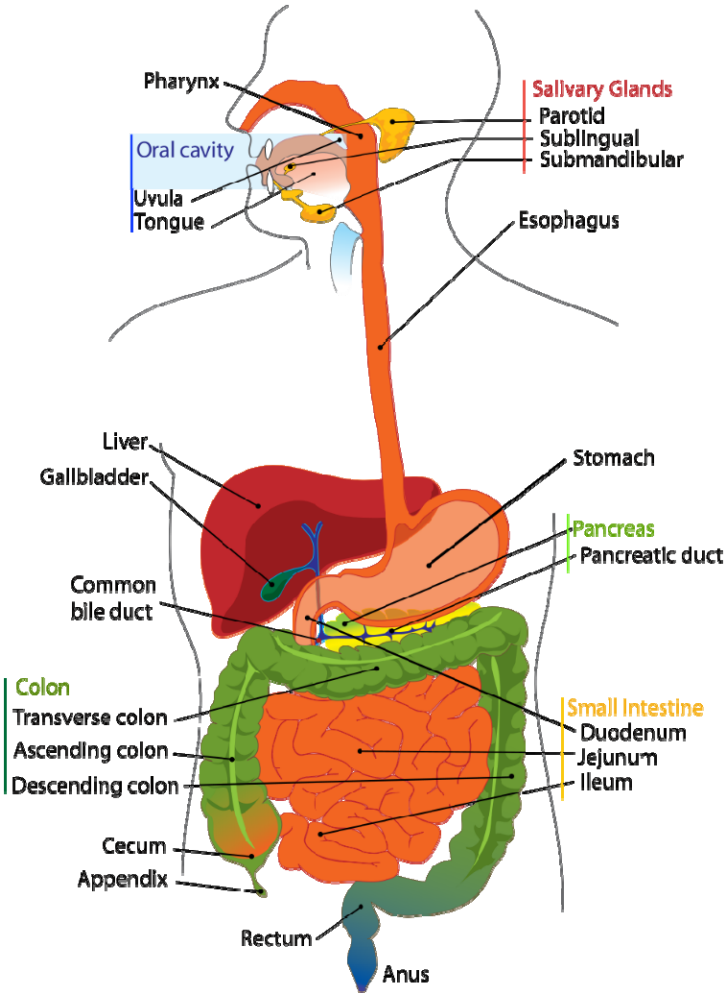
A. See “ADH Lab Preparation” in Appendix 1.

IX. Engagement

A. Introduction to alcohol processing

1. Remind students of the question with which the last investigation ended.
    a. “How does our body cope with alcohol once it is ingested?” (essential question)
    b. “Where does that alcohol go?”
    c. Ask them for their ideas (prior knowledge).

2. Project the following picture (or one comparable):



REFERENCE: http://www.rainbowskill.com/wp-content/uploads/2009/03/digestive-system1.png (Wikimedia Commons)

Emphasize that alcohol is absorbed by the intestines and makes its way to the liver. The liver filters harmful substances from the blood, such as alcohol.

B. Ask, “How does our body cope with alcohol once it is in the liver?”
    1. Think-Pair-Share.
    2. Record ideas on newsprint.

Transition to Exploration

C. Tell the students that we will watch a video that provides some insights into the mechanism that our body uses to deal with alcohol.

X. Exploration 1: Enzyme Activity

A. Show the video of Lucy and Ethel in the chocolate factory (Lucy Lucy Lucy link provided on this website).
B. Lucy and Ethel’s actions are analogous to the activity of alcohol dehydrogenase.
C. Stop the video after 1 min 30 sec.
D. Ask the students to describe how this video provides insights to the mechanism used by the body to break down alcohol.
    1. Ask, “What are the components of this system?”
        a. Lucy, Ethel, chocolates, conveyor belt, boss, wrappers, packages.
    2. Ask, “What are the relationships among the components?”
        a. Lucy and Ethel wrap the chocolates as they pass.
E. Reveal the following analogies:
    1. chocolates in the video represent alcohol.
    2. wrapped chocolates indicate that the alcohol has been broken down (catabolized).
    3. Lucy and Ethel represent enzymes that break down the alcohol.

F. Ask students to predict what will happen when *alcohol (chocolate) concentration increases*. (i.e. More chocolates are put on the conveyer belt.) Phrasing the question as the “conveyer belt speeding up” makes students think that the blood is flowing faster and can lead to confusion.

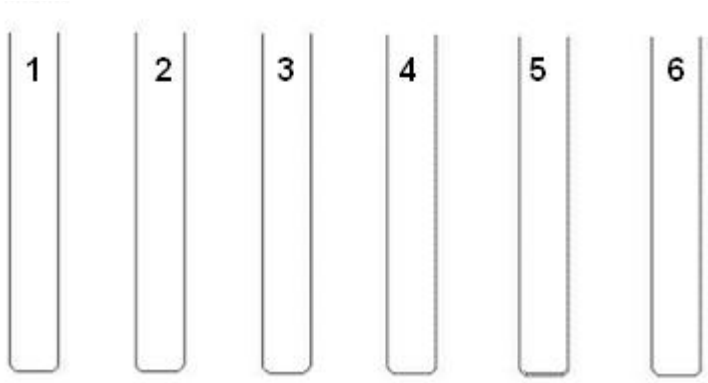
Transition to Exploration 2

Suggest that students test their predictions using real enzymes and alcohol

Xl. Exploration 2: ADH Lab

Alcohol Dehydrogenase Protocol

Each lab team will receive six test tubes with different alcohol concentrations as shown below.



Alcohol Concentration	0%	.005%	.01%	.02%	.03%	.04%

Part 1: Add the Alcohol Dehydrogenase (ADH) enzyme to each solution

Part 2: Stop the ADH activity and create a color reaction that indicates the alcohol that has been broken down

Part 3: Measure the amount of color produced in each tube using a spectrophotometer

Part 4: Graph the results by plotting the absorbances obtained for the alcohol solutions in test tubes 1-6

Alcohol Dehydrogenase Protocol Procedure

Part 1: Add NAD (co-enzyme) and Alcohol Dehydrogenase ADH (enzyme) to each test tube

- ☐ Obtain a solution NAD and ADH from the instructor.
- ☐ Use a p200 to add 100μL of NAD solution to test tubes 1-6.
- ☐ Use a p200 to add 100μL of ADH solution to test tubes 1-6.
- ☐ Use the vortex (speed=3) to gently mix test tubes 1-6 for about 3 seconds.
- ☐ Let test tubes stand for 5 minutes.

Part 2: Stop the Alcohol Dehydrogenase (ADH) activity and create a color reaction that indicates the alcohol that has been broken down

- ☐ Use a p200 to add 200μL of MTT (stop solution) to test tubes 1-6.
- ☐ Use a p200 to add 50μL of PMS (color development solution) to test tubes 1-6.
- ☐ Use the vortex to gently mix test tubes 1-6.
- ☐ Wait 5 minutes.

Part 3: Measure the amount of color produced in each tube using a spectrophotometer set at 460nm

- ☐ Check that the spectrophotometer is set to measure Absorbance (**A**) at 460nm.
- ☐ Set the "blank."
    - o Place test tube number 1 in the spectrophotometer.
    - o Close the lid.
    - o Press the "0 ABS" button and wait for the display to read 0.000.
    - o Record the reading (0.000 Abs) for test tube 1 on the Alcohol Dehydrogenase Data Sheet.

- ☐ Measure the absorbance of test tubes 2 – 6.
    - Place test tube 2 into the spectrophotometer.
    - Close the lid.
    - Record the reading for test tube 2 on the Alcohol Dehydrogenase Data Sheet.
    - Repeat the procedure for test tubes 3 – 6.

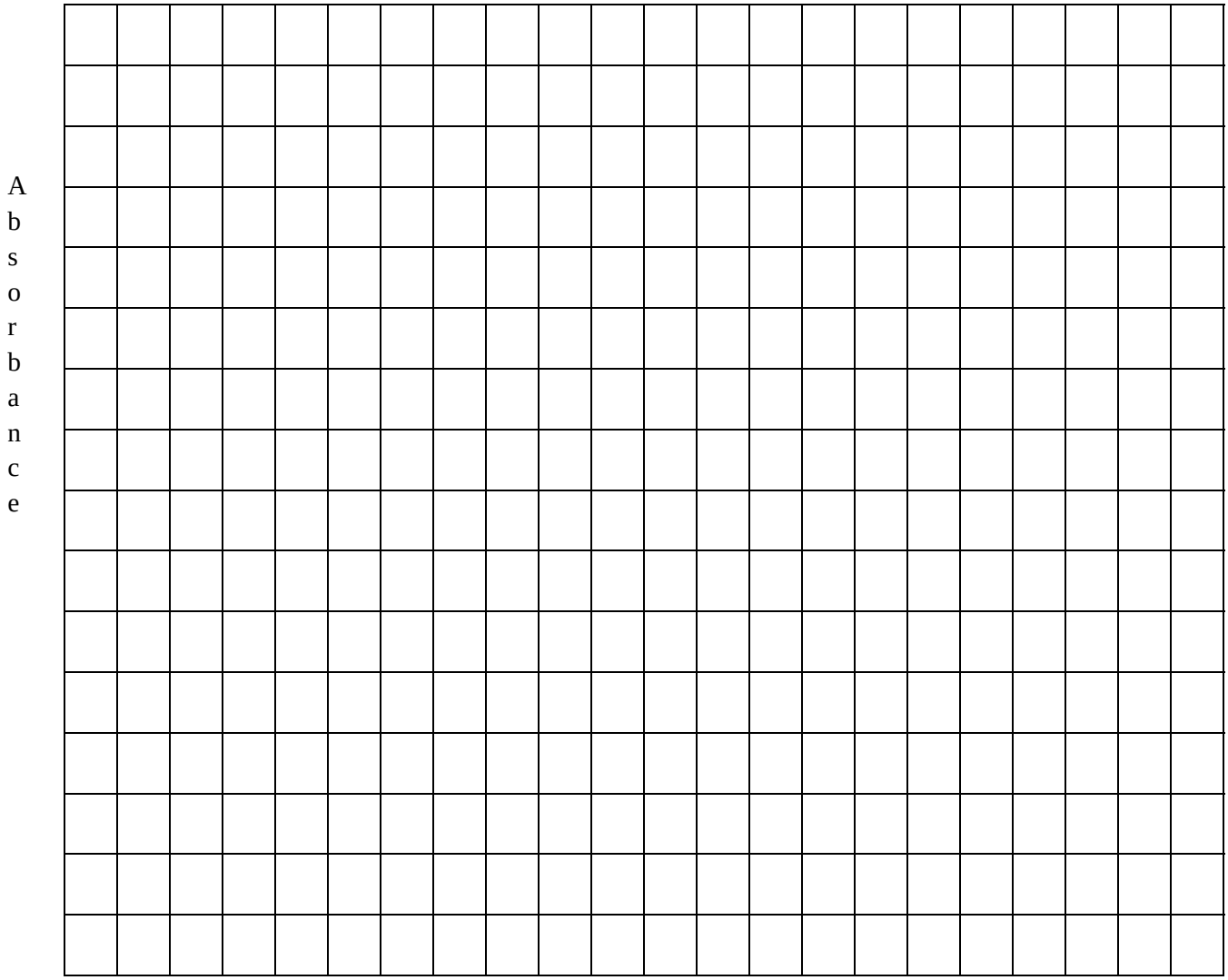
Alcohol Dehydrogenase Data Sheet

Test Tube Number	Concentration of Alcohol	Absorbance
1	0	
2	.005%	
3	.01%	
4	.02%	
5	.03%	
6	.04%	

Part 5: Graph the results by plotting the absorbencies obtained for the alcohol solutions in test tubes 1-6

- ☐ Plot alcohol concentration on the x axis.
- ☐ Use the alcohol concentrations 0% .005% .01% .02% .03% .04%.
- ☐ Plot absorbance on the y axis.
- ☐ Be sure to choose a range that includes all your absorbance readings.

Graph the absorbance data for the six samples on the graph below.

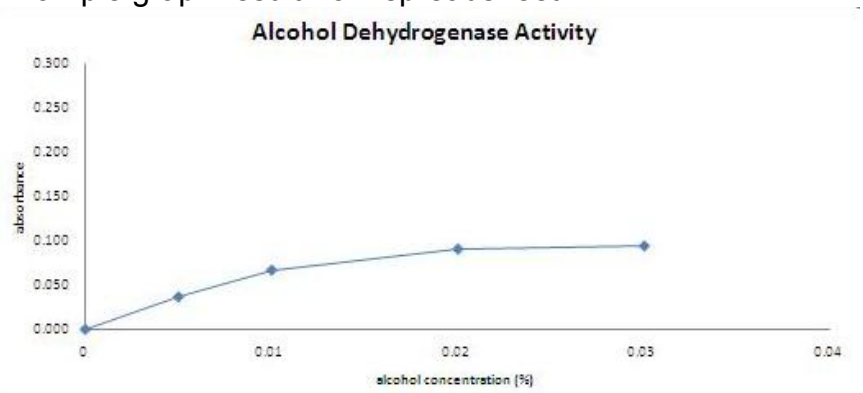


XII. Explanation

A. Ask whether or not the laboratory results support their predictions of what would happen when more alcohol (or chocolate) was introduced into the system.

B. Display the graphs by posting them on newsprint. Optional: enter all data into a spreadsheet (See Class Data Excel File in Appendix 2.). The spreadsheet will generate a graph based on the data entered.

Example graph result from spreadsheet:

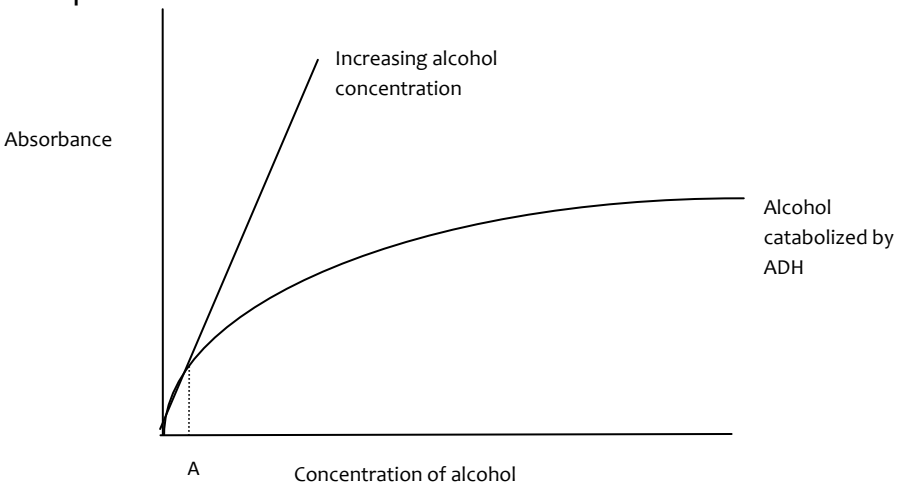


C. Have students share their interpretations of the graph.

D. Project the student curves.

E. Overlay the linear graph of increasing alcohol concentration.

Example:



F. Lead a discussion to explain the graph up until concentration A is reached. The curve represents the rate at which ADH (Lucy and Ethel) can catabolize alcohol (wrap the chocolates). The absorbance represents the amount of alcohol catabolized (wrapped chocolates), which increases with the increase in alcohol concentration (straight line) until about concentration A. This can be seen in the overlap of the curved and straight lines.

G. Ask what happens after concentration A is reached.

The rate of substrate increase (increasing alcohol concentration—straight line) remains constant, but the rate of alcohol catabolysis (curved line—experimental results) begins to increase more slowly. The curved line levels off when the maximum rate of alcohol catabolysis is reached (V_{max}). The explanation is that after alcohol concentration A is reached, ADH (Lucy and Ethel) cannot keep up.

XIII. Evaluation

A. Restate the essential question: "How does our body cope with alcohol once it is ingested?"

B. Ask the students to create a concept map to explain what they know about how the body copes with alcohol, using the following terms:

ADH alcohol rate substrate

C. Close with the following question to prepare for investigation 3: "What happens to the excess alcohol that is not metabolized?"

ADH Lab Prep

Ethyl alcohol (EtOH), 200 proof
distilled water (dH_2O)
graduated cylinder or 50mL disposable pipette for preparing ethanol concentrations
bottles or beakers for mixing and storing ethanol concentrations
glass test tubes
racks for glass test tubes
permanent marker or printed labels for tube marking
1.5mL microcentrifuge tubes
p1000 micropipette for lab prep (making aliquots)
p200 micropipettes for the lab procedure
fridge or freezer for reagent storage
MTT
PMS
containers for storing stock solutions
electronic balance, 1mg accuracy
ADH: to be prepped fresh, before lab procedure
NAD^+: to be prepped fresh before lab procedure
Phosphate buffered saline, pH 7.4
spectrophotometer
diluted food dye in tubes for pipette practice (optional)
necessary chemicals for preparing PBS and pyrophosphate buffers

Lab station setup:

printed lab protocol
test tube rack with MTT and PMS
NAD^+ and ADH tubes on ice
test tube rack with glass test tubes of alcohol concentrations 0%, .01%, .02%, .03%, .04%
p200 micropipette
micropipette tips
waste container
optional: tube of diluted food dye for pipette practice prior to the lab
data sheet and graph paper
spectrophotometer

Reagent preparation:

Ethyl alcohol concentrations
In 5 separate containers, dilute ethanol in distilled water to final concentrations of .04%, .03%, .02%, .01%, and 0% (just water). Save on shelf or in fridge until use.

examples:
for 100mL of .04% EtOH
40uL EtOH
100mL dH_2O
for 100mL of .03% EtOH
30uL EtOH
100mL dH_2O
for 100mL of .02% EtOH
20uL EtOH
100mL dH_2O
for 200mL of .01% EtOH
20uL EtOH
200mL dH_2O

Use ethanol concentrations at room temperature.

For assay, aliquot to glass tubes at 1.5mL (=1500uL) per tube. **Then ADD 100uL pyrophosphate buffer** to each tube before performing assay.

Pyrophosphate buffer, 50mM, pH 8.5
11.15g sodium pyrophosphate
500mL dH_2O
Adjust pH to 8.5 using phosphoric acid.

Store at room temperature.

Add 100uL pyrophosphate buffer to each ethanol tube at the station setups prior to performing the assay.

PBS, pH 7.4 (1 liter)
Dissolve in 800 ml of distilled H_2O:
8 g of NaCl
0.2 g of KCl
1.44 g of Na_2HPO_4 and
0.24 g of KH_2PO_4.
Adjust the pH to 7.4 with HCl.
Add diH_2O to 1 liter.
Store at room temperature.

600µM MTT

To make 10x concentrated solution (6mM), measure using the following ratio:

.1g MTT

40mL PBS, pH 7.4

(Hint: Because it is easier to accurately measure liquids than solids, measure close to .1g of MTT, then adjust PBS volume according to the exact amount of MTT measured.)

Store 10x stock in freezer. Allow room in container for PBS to expand when frozen.

Dilute 1:10 in PBS for final concentration of 600µM and aliquot into 1.5mL microcentrifuge tubes, 1020uL per tube. Store in freezer.

100µM PMS

To make a 100x concentrated solution, measure using the following ratio:

.248g PMS

40mL PBS

(Hint: Because it is easier to accurately measure liquids than solids, measure close to .248g of PMS, then adjust PBS volume according to the exact amount of PMS measured.)

Store stock in freezer and keep away from light. Allow room in container for PBS to expand when frozen.

Dilute 1:100 in PBS for final concentration of 100µM and aliquot into 1.5mL microcentrifuge tubes, 270uL per tube. Store in freezer.

25mg/mL NAD^+ – make fresh

50 mg b-NAD

2 mL diH_2O

Hints: if container has 250mg, add 10ml dH_2O → makes 10ml

one container is sufficient for 19 stations.

Prepare the NAD^+ just prior to performing assay. Aliquot into 1.5mL microcentrifuge tubes, 520uL per tube. Store on ice or in fridge until use.

10mg/mL ADH – make fresh

20 mg ADH

2 mL cold diH_2O

Hints: if container has 15.59mg, add 1.56ml dH_2O → makes 1.56ml

if container has 22 mg, add 2.26ml dH_2O → makes 2.2ml

need 3-5 containers for one class, depending on container size

Prepare the ADH just prior to performing assay. Aliquot into 1.5mL microcentrifuge tubes, 520uL per tube. Store on ice or in fridge until use.

Summary of reagent aliquots:

Reagent	per station:	total necessary for 12 stations (24 students working in partners)
EtOH concentrations	1500µl per tube	20mL each EtOH solution
NAD^+	520µl	8ml
ADH	520µl	8ml
MTT	1020µl	14ml
PMS	270µl	4ml

Product information:

glass test tubes: Kimax glass test tubes, 13x100mm borosilicate glass
Fisher Scientific, cat# 03-341-3, case of 1000 for $89.51
1.7mL microcentrifuge tubes: 500/pk
Denville Scientific, cat# C2170, $14.75
NAD^+: beta-Nicotinamide adenine dinucleotide hydrate
Sigma-Aldrich, cat# N7004- 250mg, $20.50
ADH: Alcohol Dehydrogenase, from *Saccharomyces cerevisiae*
Sigma-Aldrich, cat# A7011 - 7.5KU, $28.60 or cat#A7011 - 7.5KU, $57.30
MTT: Thiazolyl Blue Tetrazolium Bromide
Sigma-Aldrich, cat# M2128, $18.30 for 100mg - $82.70 for 1g
PMS: Phenazine methosulfate
Sigma-Aldrich, cat# P9625, $21.30 for 500mg, $32.90 for 1g

Assay Calculations:

1. How many µmoles of ethanol are we using?
   ethanol = 46.07g/mole
   ethanol = .789g/mL

   In 1.5mL of the .04% solution, there is .6uL ethanol (=.0006mL)

   Grams ethanol?
   .789g/1mL x .0006mL = **.0004734g** ethanol

   µmoles ethanol?
   .0004734g x 1mole/46.07g x 1,000,000µmoles/1mole
   = **10.276µmoles**

We have **10.276µmoles ethanol** in the highest concentrated solution (.04%).
We have **2.569µmoles ethanol** in the lowest concentrated solution (.01%).

2. How many units of ADH are we using in each tube?
   ADH = 10mg/mL in our assay
   If the ADH stock is 331U/mg, then:

   10mg/1mL x 331U/1mg = 3310U/mL.

   We use 100uL, or .1mL in each tube
   = **331U per tube**.

   After 5 minutes of incubation, 331U ADH x 5min = **1655 units ADH**.
   1655U ADH can convert 1655µmoles ethanol.

Why do we use so much ADH? It is difficult to accurately measure less than 10mg, and this seems to work well in the assay. Perhaps in the future, the assay could be designed to use less ADH and give the same results.

3. But how much NAD^+ do we have in each tube?
   NAD^+ = 663.43g/mole (or 663.43µg/µmole

   We are using 25mg/mL and adding 100uL per tube.

   25mg/1mL x .1mL = 2.5mg NAD^+ per tube (=**2500µg**).

   2500µg x 1µmole/663.43µg = **3.768µmoles**.

   There are 3.768µmole NAD^+ per tube.
   In our assay, NAD^+ is the limiting factor.

4. Conclusion: There is enough NAD^+ in the test tubes to break down all of the ethanol in the .01% solution, but not enough for the .04% solution, and this should be seen by a leveling off of the color after the PMS is added.