BASTYR UNIVERSITY

COURSE INFORMATION FOR STUDENTS

Winter Quarter 09

<table>
<thead>
<tr>
<th>COURSE NUMBER</th>
<th>BC3131</th>
</tr>
</thead>
<tbody>
<tr>
<td>COURSE TITLE</td>
<td>Biochemistry for Life Science 1</td>
</tr>
<tr>
<td>INSTRUCTOR</td>
<td>Tess Cabasco-Cebrian</td>
</tr>
<tr>
<td>CLASS TIME</td>
<td>Section A: 8:00 -12:00 PM  Section B: 1:00 – 5:00 PM alternating Fridays (1,3,5,7,9)  Section C: 8:00 -12:00 PM  Section D 1:00 - 5:00 PM alternating Fridays (2,4,6,8,10)</td>
</tr>
<tr>
<td>CREDITS</td>
<td>1</td>
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<tr>
<td>STUDENT ADVISING HOURS</td>
<td>Friday 12 to 1 Please call for an appointment.</td>
</tr>
<tr>
<td>PHONE</td>
<td>(W)(206) 587-4075  FAX (206) 587-3837</td>
</tr>
</tbody>
</table>

Students are responsible for knowing and adhering to Academic Policies and Procedures as outlined in the Student Handbook.

** There is lab make up. Only excused absences will be exempted from 20% point deduction for each lab missed. Absences due to illness must have a doctor’s note to be excused.

1. Table of Contents
   - Course Overview
   - Course objective
   - Requirements
   - Grading
   - Report Format
   - Sample Lab Report
   - Course Outline and Time Schedule
   - Lab Rules
2. **Course Overview**

The course is designed as an introduction to biochemistry laboratory techniques. Experiments include acid/base and buffer analysis, chromatography and spectrophotometry. Computer technology will be used for data analysis.

The experiments will vary in degree of difficulty. As the quarter progresses the experiments will be more challenging and more interesting. It is important to come to lab prepared, on time and with a positive attitude. Remember the definition of “experiment.” Although the experiments have been tried and are known to work, sometimes they do not. Failure of an experiment is just as important a learning tool as one that works. It allows you to examine the procedure more thoroughly to figure out what went wrong. A degree of enthusiasm and the willingness to learn and work hard are the key to a successful lab experience.

3. **Course Objectives**

- Learn techniques utilized in Biochemistry
- Develop data analysis skills
- Learn to write complete, clear and concise lab reports
- Maintain a laboratory notebook
- Utilize Excel for data analysis.

4. **Requirements**

1. Complete 5 experiments. **There is no lab makeup.**
2. Each absence will be 20% deduction from total score.
3. Experiments will be performed in groups of no more than 2 students.
4. A lab notebook is required for the course. **All notes must be written in the notebook, not on loose pieces of paper.** Each student must have a notebook and keep their data and observation. Write in **pen** only. Your lab notebook will be checked during the quarter.
5. The pre-lab assignment for each of the five experiments is to prepare a flow chart of the experiment. The purpose of this exercise is to allow you to understand the procedure clearly and to ensure your preparedness. **Prelab assignments must be written on your notebook.** You will not be allowed to start the lab until prelab assignment is completed.
6. Lab reports and any post lab questions are due by 1:00 PM the following Friday (Friday that you do not have lab). Report can be turned in electronically as a word attachment (tcabasco-cebrian@sccd.ctc.edu) or as hard copy. A 10-point deduction per week will be applied for late report. Lab reports can be faxed on or before the due date. **Only one (1) set of lab report and post lab questions are required per partner if done collaboratively. Partners also have the option of turning in individual lab reports; if so please indicate on the report that it is an individual report.**

5. **GRADING**

Your lab grade will be based on the following

- Prelab assignment 10pts each
- Lab performance*, Formal lab reports and questions 100pts each
- Notebook 10pts each

*Laboratory performance will be an evaluation of lab technique, lab preparedness, lab etiquette; which includes cleaning after oneself, keeping track of lab glassware, putting away chemicals,
effort given to make for a successful experiment, and most important, following safety
instructions. Courtesy to lab instructor and classmates is expected.

**Grading will be as follows:**

<table>
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<th>Grade</th>
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<tr>
<td>95-100%</td>
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<tr>
<td>90-94.9</td>
<td>A-</td>
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<tr>
<td>87-89.9</td>
<td>B+</td>
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<td>83-86.9</td>
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<td>80-82.9</td>
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<td>77-79.9</td>
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<tr>
<td>73-76.8</td>
<td>C</td>
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6. **FORMAL LAB REPORT**

**TITLE**

**AUTHORS**

1. The person who did the work and wrote the paper is generally listed as the first author
   of a research paper.
2. For published articles, other people who made substantial contributions to the work are
   also listed as authors. Ask your mentor's permission before including his/her name as co-
   author.

**INTRODUCTION**

What question did you ask in your experiment? Why is it interesting? The introduction
summarizes the relevant literature so that the reader will understand why you were
interested in the question you asked. End this section with a sentence explaining the
specific question you asked in this experiment or the experiment’s objectives.

**MATERIALS AND METHODS**

1. There should be enough information here to allow another scientist to repeat your
   experiment. Look at other papers that have been published in your field to get some idea
   of what is included in this section.
2. If you had a complicated protocol, it may helpful to include a diagram, table or
   flowchart to explain the methods you used.
3. Do not put results in this section. You may, however, include preliminary results that
   were used to design the main experiment that you are reporting on. ("In a preliminary
   study, I observed the owls for one week, and found that 73 % of their locomotor activity
   occurred during the night, and so I conducted all subsequent experiments between 11 pm
   and 6 am.")
4. Mention relevant ethical considerations. If you used human subjects, did they consent
to participate? If you used animals, what measures did you take to minimize pain?

**RESULTS**

1. This is where you present the results you've gotten. Use graphs and tables if
   appropriate, *but also summarize your main findings in the text*. Do NOT discuss
   the results or speculate as to why something happened; that goes in the Discussion.
2. Use appropriate methods of showing data. Don't try to manipulate the data to make it
   look like you did more than you actually did.
TABLES AND GRAPHS

1. If you present your data in a table or graph, include a title describing what's in the table ("Enzyme activity at various temperatures", not "My results"). For graphs, you should also label the x and y axes.

2. Don't use a table or graph just to be "fancy". If you can summarize the information in one sentence, then a table or graph is not necessary.

DISCUSSION/CONCLUSION

1. Highlight the most significant results, but don't just repeat what you've written in the Results section. How do these results relate to the original question? Do the data support your hypothesis? Are your results consistent with what other investigators have reported? If your results were unexpected, try to explain why. Is there another way to interpret your results? What further research would be necessary to answer the questions raised by your results? How do your results fit into the big picture?

Word process all formal lab reports.
Sample lab report

SIMULATION OF A 40-YEAR CLIMATIC TIME SERIES TO ILLUSTRATE A RANDOM TREND

Andrew C. Comrie

Introduction
Many climate studies have examined trends in quantities such as temperature, precipitation, and carbon dioxide (CO₂) based on time series of data collected over the last 50 to 100 years (e.g., Cayan et al., 1998; Peterson and Vose, 1997; Keeling and Whorf, 1998). These studies frequently include time-series plots showing, for example, increases since the middle of the twentieth century. In some cases, these figures include trend lines or smoothed curves to highlight the nature of a particular trend.

The statistical strength or weakness of any such trend is usually detailed in the paper. However, it is not uncommon for a graph of an especially newsworthy trend to be reproduced in the media. Figure 1 shows two examples of this phenomenon, the annual Mauna Loa CO₂ curve and the annual mean minimum temperature for Tucson, Arizona. While trends published in scientific articles have undergone review for scientific and statistical robustness, it is easy for the untrained eye to see apparent trends in other similar, relatively short time series that may not be real.

![Figure 1. Two examples of climatic time series and trend lines for (a) Mauna Loa CO₂ data from Keeling and Whorf (1998) and (b) mean minimum temperature data at Tucson International Airport from Peterson and Vose (1997).](image)

The aim of this paper is to examine the apparent trend in a simulated annual climatic time series using random numbers. Any trends present in the data will have occurred by chance, and will highlight the level of caution required for interpretation.

Material and Methods
A series of 40 random numbers was created in a spreadsheet (Microsoft Excel) to simulate annual temperature, representing an arbitrary climatic variable. For each data point, the software returns an evenly distributed random number greater than or equal to 0 and less than 1. The long-term trend in the data was examined by calculating and plotting a straight-line regression. Shorter-term trends approximating a decadal time scale were examined by calculating a 9-year moving average to smooth the annual data (i.e., year 1 to year 9 average plotted at year 5, year 2 to year 10 average plotted at year 6, etc.). The data and results were plotted to enable visual comparison.

To illustrate the likelihood of the observed trend having occurred by chance, 100 versions of the random series were generated for comparison. Slope coefficients were calculated for each series and tabulated by frequency of occurrence.
**Results**

Figure 2 illustrates the simulated time series and results. Visual inspection shows a clear upward trend in the 40-year series, although there is noticeable annual and decadal variability within the overall trend. The regression line has a calculated slope of 0.0089 and an intercept of 0.401, and it explains about 16 percent of the variance in the data ($R^2 = 0.1616$). The moving average highlights two apparent cycles of rising and falling simulated annual temperatures, with a decrease in the middle of the series that lasts for more than a decade. While the overall spread of data covers the range between 0 and 1, the higher values tend to fall (randomly) in the middle and later part of this particular series, thereby leading to an apparent upward trend.

![Figure 2](image-url)

*Figure 2. Random time series of 40 simulated annual temperatures, showing the raw annual values, the smoothed series using a 9-year moving average to highlight decadal variability, and the best-fit regression line highlighting the apparent long-term trend.*

If these data were actual temperatures, this would be the point to consider explanations for the observed trends in the data. However, these are randomly generated data that are known to have occurred by chance. To examine the likelihood of the strong apparent trend in Figure 1, the frequency distribution of slope values representing the long-term trends from 100 simulated data series is provided in Table 2. It can be seen that the chance of the 0.0089 slope in Figure 1 is about 2 percent, or 1 in 50 occurrences.

**Table 1: Percentage frequency of slope coefficients in 10 equal sized categories from 100 series of 40 years each.**

<table>
<thead>
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<th>Slope</th>
<th>Frequency (%)</th>
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<tbody>
<tr>
<td>&lt; -0.008</td>
<td>0</td>
</tr>
<tr>
<td>-0.008 to -</td>
<td>5</td>
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<td>Interval</td>
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<td>-----------</td>
</tr>
<tr>
<td>-0.002 to 0</td>
<td>18</td>
</tr>
<tr>
<td>0 to 0.002</td>
<td>18</td>
</tr>
<tr>
<td>0.002 to 0.004</td>
<td>14</td>
</tr>
<tr>
<td>0.004 to 0.006</td>
<td>13</td>
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<tr>
<td>0.006 to 0.008</td>
<td>5</td>
</tr>
<tr>
<td>&gt; 0.008</td>
<td>2</td>
</tr>
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</table>

**Discussion and Conclusion**

The results show that a remarkably strong apparent trend occurs in this example of a 40-year random time series of simulated temperature. There are also strong apparent shorter-term trends visible in the data. While these trends are real in the sense that they exist for these specific data, the more refined question is to what degree the observed long-term trend might have occurred by chance. The simulation of 100 time series provides an answer to this question, and it mimics what would normally be calculated with some basic statistics.

For these 100 trials, 67 percent of the slopes fell between -0.004 and 0.004 (coinciding with a quantity known as the standard deviation). There are few slope values near the upper and lower tails of the frequency distribution, which is said to be normal (or bell shaped), and it appears that the trend in this example is relatively unusual. Slopes of this magnitude occur with a frequency of only about 2 percent. This may seem small, but to put it in perspective, if individual random time series were assigned to each member of a class of 25 students there would be a 50 percent chance of someone having a series displaying a trend as strong as this example.

Notice also that there is an equal chance of any simulated temperature in the series (or the temperature for the next year, 41) being between 0 and 1. Yet, the slope values calculated from the time series are normally distributed, and they have a much greater chance of being near zero.

In conclusion, this paper has examined trends in a simulated annual climatic time series using random numbers. The study identified a clear long-term trend in an example series that is known to have occurred by chance, and it highlights the caution that should be used when interpreting trends in situations where the underlying processes are not fully understood.

Source: Dept. of Geography and Regional Development
University of Arizona, Tucson, AZ 85721, U.S.A.
7. Course Outline & Time Schedule

<table>
<thead>
<tr>
<th>Week</th>
<th>DATE</th>
<th>TOPIC</th>
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<tbody>
<tr>
<td>1 &amp; 2</td>
<td>1/11 &amp; 1/18</td>
<td>Digestion of Food</td>
<td>1/9 1/16</td>
</tr>
<tr>
<td>3 &amp; 4</td>
<td>1/25 &amp; 2/1</td>
<td></td>
<td>1/23 1/30</td>
</tr>
<tr>
<td>5 &amp; 6</td>
<td>2/8 &amp; 2/15</td>
<td>The Study of Carbohydrates</td>
<td>2/6 2/13</td>
</tr>
<tr>
<td>7 &amp; 8</td>
<td>2/22 &amp; 2/29</td>
<td>Cellular Respiration</td>
<td>2/20 2/27</td>
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<tr>
<td></td>
<td></td>
<td>Trans-esterification of LA and GLA prep for GC</td>
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</tbody>
</table>

8. Lab Rules

**THINK SAFETY**

- Wear your goggles at all times. NO IFS AND/OR BUTS!
- Absolutely NO food or drinks in the lab.
- Keep coats, books and other belongings away from the workbench or in your keep them in personal lockers.
- Wipe you desk before and after each experiment. Remember that this lab is also used for microbiology.
- Follow chemical waste disposal instruction given by you instructor. In general, all organic solvents are disposed into the organic solvent jar. Acid and bases also go into waste jars.
- Discard all broken glass, except thermometers; in the BROKEN GLASS BUCKET-NOT the garbage can.
- **PREVENT CONTAMINATION! DO NOT RETURN CHEMICALS INTO REAGENT BOTTLES.** Excess chemicals are treated as waste chemicals.
- Read labels carefully. Pay attention to the Safety stickers on the reagent bottles.
- DO NOT use droppers or pipets in liquid reagent bottles. Use only CLEAN spatulas for solid chemicals.
- FIRST AID: Rinsing with cold water and then an ice pack is the first aid for chemical spills on your self.
- Chemical spillage on the desktop or floor can be mopped up. Acid and base spills must first be neutralized before they are cleaned.
- Come to lab prepared. Read and understand your experiment ahead of time. To use the laboratory time efficiently, your must have a clear plan of action.
- Rinse all glassware and place them in the cart designated for dirty dishes.
LOCATIONS:

A, B, C, D, I, J - HOODS
E, P - INCUBATORS
F, H - DISHWASHING SINKS
G - ALL GLASSWARE AND METALWARE
K, L - EYEWASH STATION
M - FIRE BLANKET
N - MISCELLANEOUS SUPPLIES (PIPETS, PENS, RULERS)
O - FIRE EXTINGUISHER
R - FIRST AIDE KIT
The Digestion of Food

Food and drink must be digested into smaller molecules of nutrients before they can be absorbed into the blood and carried to cells throughout the body. Digestion involves mixing food with digestive juices, moving it through the digestive tract, and breaking down large molecules of food into smaller molecules. Digestion is completed in the small intestine.

The digestive glands that act first are in the mouth—the salivary glands. Saliva produced by these glands contains the enzyme, amylase that begins to digest the starch from food into smaller molecules. The next set of digestive glands is in the stomach lining. They produce stomach acid and an enzyme that digests protein. A thick mucus layer coats the mucosa and helps keep the acidic digestive juice from dissolving the tissue of the stomach itself. In most people, the stomach mucosa is able to resist the juice, although food and other tissues of the body cannot.

After the stomach empties the food and juice mixture into the small intestine, the juices of two other digestive organs mix with the food. One of these organs, the pancreas, produces a juice that contains a wide array of enzymes to break down the carbohydrate, fat, and protein in food. Other enzymes that are active in the process come from glands in the wall of the intestine.

The second organ, the liver, produces yet another digestive juice—bile. Bile is stored between meals in the gallbladder. At mealtime, it is squeezed out of the gallbladder, through the bile ducts, and into the intestine to mix with the fat in food. The bile acids dissolve fat into the watery contents of the intestine, much like detergents that dissolve grease from a frying pan. After fat is dissolved, it is digested by enzymes from the pancreas and the lining of the intestine.

Digestion of carbohydrates starts with when mixed with the saliva containing salivary amylase which catalyzes the hydrolysis of starch to smaller polysaccharide such as maltose and free glucose. Starch passes through the stomach to the small intestine where digestion begins. The pancreatic amylase secreted in the small intestine catalyses the complete hydrolysis of starch to maltose. Maltase completes the conversion of maltose to glucose.

The digestion of protein start in the acidic environment of the stomach where the protein is denatured, exposing several peptide bonds that are then catalyzed in the presence of pepsin. The mixture of digested proteins, peptides, polypeptides, and free amino acids enters the small intestines where hydrolysis of the remaining peptides are catalyzed by enzymes; try sin, chymotrypsin and peptidases.

Included in dietary lipids are triglycerides, cholesterol and polar lipids. Cholesterol does not require digestion and is absorbed directly in the lymph system from the small intestines. The triglycerides trigger the gallbladder to pass bile into the small intestine where its digestion begins. The bile’s function is to emulsify the triglyceride into small droplet and aide in the absorption of fat soluble vitamins from the small intestine. The pancreas secretes lipases which catalyze the hydrolysis of triglycerides into glycerol and fatty acids. The hydrolysates are absorbed though the intestinal membrane and then re-synthesized into triglycerides and phospholipids, which combines with protein form
lipoprotein complex and are transported in the lymph system until they enter the blood. In the liver, the triglyceride is stored. The remaining lipoprotein is transported to the cells where the protein is removed and the triglyceride is used for energy.

Summary of Digestive Enzymes.

In this experiment you will observe several reactions that occur during digestion. Reactions will be compared to the control which contains all reactants except the digestive enzymes. Do part C first because this part requires one hour incubation.

Digestion of Carbohydrate
Part A  Salivary Amylase
Starch is a polymer of glucose that forms a complex with blue-black complex when reacted with iodine solution. When digested starch is broken into the disaccharide, maltose and a small amount of glucose. While maltose and glucose do not react with iodine, it does give a positive reaction to the Benedict test. In the Benedict test, maltose and glucose reduces the CuSO₄ to copper I oxide, a red precipitate.

Caution: You will collect saliva for this part of the experiment. Only the student whose saliva is collected will handle the saliva and wash the glassware from this section.
1. Collect about 10 mL of saliva into a 50 mL beaker. The saliva will be the source of salivary amylase.
2. Label 4 test tubes 1, 2, 3 and 4. Add 40 drops of 2 % starch into each of the test tubes.
3. Add 4 mL of DI water to test tubes 1 and 2.
4. To test tubes 4 and 5, add 4 mL of saliva.
5. Cover all the test tubes with Parafilm and mix using the vortex mixer. Place all four test tubes in the 37°C water bath for 30 minutes.
6. Remove the test tubes from the water bath and add 2 drops of iodine solution to test tubes 2 and 4. Save test tubes 1 and 3 for the Benedict’s test in Part 2.

Part B  **Hydrolysis of starch by Pancreatic Amylase**

The pancreas also makes amylase (alpha amylase) to break down dietary starch into di- and trisaccharides. The α-amylases are calcium metalloenzymes, completely unable to function in the absence of calcium. By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates.

For this part of the experiment, pancreatic amylase (pancreatin) will be tested at the pH found in the small intestine by adjusting the pH with sodium carbonate solution and at acidic environment of the stomach by mixing HCl solution with the pancreatin solution.

The presence of starch will again be tested using the iodine solution.

1. Label 4 test tubes 5, 6, 7 and 8. Add the following to each test tube
   - Test tube 5: 4 mL DI water
   - Test tube 6: 2mL of 5% pancreatin solution and 2 mL DI water
   - Test tube 7: 2mL of 5% pancreatin solution and 2 mL 0.5% Na₂CO₃
   - Test tube 8: 2mL of 5% pancreatin solution and 2 mL 0.2 M HCl.

   Dip a stirring rod into test tube 5 and immediately touch the moist stirring rod into a pH paper. Note the pH. Rinse and wipe the stirring rod and determine the pH of the solution in test tubes 6,7 and 8.

2. To each test tube add 2 mL of 2% starch solution and cover with a Parafilm. Use the vortex to mix.

3. Incubate the test tubes in a 37°C water bath for 30 minutes. Fill a 400 mL beaker with 300 mL of water and heat to boil.

4. Label 4 new test tubes 5a, 6a, 7a, and 8a. After incubation, transfer 40 drops from each test tube into appropriate “a” tubes.

5. Add 2 drops of iodine solution to each of the “a” tubes and record observations.

6. Add 5 mL of Benedict’s reagent to test tubes 1 and 3 from Part A and test tubes 5, 6, 7 and 8.

7. Place the 6 test tubes in the boiling water for 7 to 10 minutes. A red precipitate indicates the presence of maltose and/or glucose.

Part C  **Protein Digestion**

Pepsin is expressed as a proenzyme, pepsinogen, an inactive enzyme precursor. In the stomach, chief cells release pepsinogen. This proenzyme is activated by hydrochloric acid (HCl), which is released from parietal cells in the stomach lining. The hormone gastrin and the vagus nerve trigger the release of both pepsinogen and HCl from the stomach lining when food is ingested. Hydrochloric acid creates an acidic environment which allows pepsinogen to unfold and cleave itself in an autocatalytic fashion, thereby generating the active form, pepsin. Pepsin functions best in acidic environments and is often found in an acidic environment, particularly those with a pH of 1.5 to 2.
Pepsin’s activity will be determined in different pH environment.

1. Label 3 test tubes 9, 10, and 11. Add 5 mL of 2% pepsin to test tubes 10 and 11.
2. To test tube 9 add 5 mL of DI water and 1 mL 0.1 M HCl, this is your control.
3. In test tube 10, add 1 mL of 0.2 M HCl, the pH in the stomach.
4. Add 1 mL DI water to test tube 11 which will give a neutral pH.
5. Add a small piece of boiled egg white (albumin protein) and incubate for 1 hour.
6. Record observations between the control and the two other samples.

Part D Digestion of Triglycerides

While other macromolecules begin to be digested and broken down in the stomach, triglycerides are for the most part unchanged until they reach the small intestine. Bile excreted from the liver and pancreatic lipase is transported into the small intestines when food is present. Bile emulsifies the fat and the lipase hydrolyzes it. The bile salts continue to emulsify the products after hydrolysis for two reasons. First, the hydrolysis of triglycerides is a highly reversible chemical process. To prevent hydrolyzed fatty acids and monoglycerides from reforming triglycerides, the products must be separated from the vicinity of the other. Bile salts separate the products by emulsifying them in globules called micelles. The micelles consist of 20-50 bile salt molecules with the hydrophilic ends facing outward to allow the globule to dissolve in the water-based intestinal chyme. The hydrophobic ends of the bile salts surround the fatty acids or monoglycerides on the inside of the globule. The second reason bile salts from micelles is to transport the triglyceride products to the intestinal walls to be absorbed into the blood stream. The hydrolysis of a single triglyceride produces glycerol and three fatty acids. Fatty acids are weak acids and will change the pH of choleate, a bile salt, will be added to triglyceride. A change in pH indicates digestion of the triglyceride.

1. Label 3 test tubes, 12, 13, and 14. Add the following to each test tube:
   - To test tube 12, add 2 mL of 5% sodium choleate, 2 mL 5% pancreatin, 10 drop universal indicator and mix.
   - To test tube 13, add 2 mL of 5% sodium choleate and 10 drops of universal indicator.
   - To test tube 14, add 2 mL of 5% pancreatin and 10 drops of universal indicator.
2. Add 0.1 % NaOH drop by drop while shaking the test to mix to tubes 13 and 14. Stop when the pH reaches 7. Add DI water to each of the tubes 13 and 14 until the volume equals that of test tube 12.
3. Add 5 drops of corn oil (triglyceride) to each of the three test tubes. Incubate at 37ºC for 10 minutes. Observe the change in the color and record the pH.
### Data Table

#### Part A

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#### Part B

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#### Part C

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#### Part D

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Factors that Affect Enzyme Activity

Background
Enzymes are protein molecules that function as biological catalysts. Catalysts alter the rate of chemical reactions without undergoing any change themselves. It is estimated that the human body has at least 50,000 enzymes to catalyze the enormous number of biochemical reactions that take place. Enzymes are necessary for most bodily reactions. Some reactions that would otherwise require years for completion can be made to occur almost instantly with the aid of enzymes. For example, the enzyme catalase can effect the decomposition of hydrogen peroxide, \( \text{H}_2\text{O}_2 \).

Most enzymes are complex molecules that often contain non-protein parts. Metal ions such as Zn\(^{+2}\) and Mg\(^{+2}\) are essential to the enzyme's activity and are known as co-factors. Another non-protein portion is the coenzyme, a small organic molecule. The protein component of an enzyme that lacks an essential cofactor is called an apoenzyme. Intact enzymes with their bound cofactors are holoenzymes. The composition of an enzyme can be viewed as in the following equation:

\[
\text{Protein part} + \text{Nonprotein part} = \text{Enzyme (holoenzyme)}
\]

The active site of an enzyme is the portion that binds the substrate, the substance that is undergoing the catalysis (i.e., the reactant). The binding that occurs between the enzyme and its substrate usually consists of non-covalent interactions: London forces, hydrogen bonds, and dipole forces. The outcome of the enzyme-substrate complex is the product of the reaction and the enzyme. Remember that the enzyme is a catalyst and comes out of the reaction environment unchanged.

Enzyme activity must be regulated in the body. If regulation does not take place in the body, enzymes would continually work and use up all of their substrates. This type of situation is often undesirable. Inhibition occurs when an enzymatic process is slowed or stopped. When this happens for regulatory purposes, the inhibition is considered positive. Conversely, some cases of inhibition halt a desirable reaction. When poisons enter the body, they change enzymatic activity in a negative manner. The first type of inhibition is referred to as competitive inhibition. This occurs when a substance other than the substrate competes for the active site of the enzyme. The competitive inhibitor is able to fit into the active site of the enzyme. Since competition occurs between two substances, enzymatic
activity slows down, as opposed to being disrupted altogether. Also, this process is reversible. When all of the inhibitor is used up, the normal enzymatic process can continue. The second type of inhibition is known as non-competitive inhibition. Unlike competitive inhibition, the foreign substance binds on the enzyme at a spot other than the active site. When this binding occurs, the entire conformation of the enzyme is altered. A change in the structure of the enzyme usually leaves it nonfunctional. Heavy metals often work as non-competitive inhibitors.

In this experiment, you will observe the various factors that influence the enzyme activity such as temperature, pH, substrate concentration and enzyme concentration. You will examine different enzyme inhibitors. Today you will isolate polyphenoloxidase, an oxidase that catalyzes the transfer of hydrogen from some compound to molecular oxygen. Polyphenoloxidase is a copper containing compound enzyme that catalyzes the oxidation (hydrogen removal) dihydroxyphenols to the corresponding quinones. The reaction is easily observed because quinones are colored compounds. The reaction above is responsible for the browning of peeled potatoes. The source of the polyphenoloxidase for today's experiment will be a potato.

**Experiment**  
**Part A Preparation of Enzyme Extract**

1. Obtain about 10 g of peeled potato.  
2. Cut the potato into small pieces and place the pieces into a mortar. 
3. Add 5 mL of distilled water and about 5 g of clean sand to the mortar. 
4. Grind the mixture thoroughly and rinse it into a 100-mL beaker using 30 mL of 2% sodium fluoride (NaF) solution. 
5. Let the mixture stand for 2 minutes and then pour it into a 100-mL beaker through 4 layers of cheesecloth held in a funnel. The liquid potato extract contains polyphenoloxidase.  
6. Rinse the used cheesecloth and potato residue in funnel with tap water and discard in the wastebasket.

**Part B Enzyme Specificity**
Enzymes are often quite specific in their activity, catalyzing a reaction only if the proper substrate is present. In this exercise, you will study the specificity of polyphenoloxidase by providing various substrates that have structural similarities.

1. Prepare four labeled 10-cm test tubes as follows:
   **Tube**  |  **Contents**
   A         |  20 drops of distilled water  
   B         |  20 drops of 0.01 M catechol solution  
   C         |  20 drops of 0.01 M phenol solution  
   D         |  20 drops of 0.01 M 1,4-cyclohexanediol solution  

The tube containing water will serve as a control in which no reaction takes place.  
2. Place all the tubes into a water bath maintained at 37°C. 

3. Obtain four new 10-cm test tubes and put 30 drops of potato extract into each of the four tubes. Place these tubes into the 37°C water bath.
4. After the tubes with the extract have been in the water bath for 5 minutes, quickly empty a test tube of potato extract into each labeled tube. Mix with a vortex shaker, if available, and leave the labeled tubes in the bath for another 5 minutes.

5. After 5 minutes, remove the labeled tubes from the water bath. Compare the color of test tubes B, C, and D to the color of test tube A. The best way to detect and compare colors is to hold the tubes vertically over a piece of white paper, and look down into the tubes. Record the intensity of brown color that develops in each tube, using the following scale:

<table>
<thead>
<tr>
<th>Most intense (darkest)</th>
<th>Next most intense</th>
<th>Least intense change</th>
<th>No color when compared to Test tube A</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

If more than one tube shows the same intensity, assign them the same number. The development of a brown color is visual evidence of the oxidation process that was catalyzed by polyphenoloxidase.

6. Discard all contents of the test tubes into the waste jar.

Part C Substrate Concentration

The rate of an enzyme-catalyzed reaction increases as the substrate concentration increases until a limiting rate is reached. Beyond this point, the rate is independent of increases in the substrate concentration.

1. Obtain four 10-cm labeled test tubes, and check them to make sure they have the same diameter and or name brand.
2. Label the four test tubes to indicate the number of drops of catechol solution they will contain from the following list, and then put the appropriate amount of catechol solution in each tube.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Contents</th>
<th>Relative Substrate Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>40 drops of 0.01 M catechol solution</td>
<td>8</td>
</tr>
<tr>
<td>B</td>
<td>20 drops of 0.01 M catechol solution + 20 drops of water</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>10 drops of 0.01 M catechol solution + 30 drops of water</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>5 drops of 0.01 M catechol solution + 35 drops of water</td>
<td>1</td>
</tr>
</tbody>
</table>

The liquid level in each tube should match the liquid level in test tube A.

3. Place these four labeled tubes into a 37°C bath.
4. Obtain four more 10-cm test tubes and put 20 drops of potato extract into each one. Place these tubes into the 37°C bath for 5 minutes.
5. After the extract-containing test tubes have been in the bath for 5 minutes, quickly empty a test tube of potato extract into test tubes A through D. Mix and leave the labeled tubes in the water bath for another 2 minutes.
6. After 2 minutes, remove the labeled tubes from the water bath. Note and record the intensity of color that has developed in each tube. Use the scale and numbers given in Part B.

**If no difference in intensity is observed, the enzyme is too concentrated. Dilute enzyme solution prepared in Part A 2:1 (8 mL enzyme + 4 mL 2% NaF). If this is still too concentrated, dilute the original enzyme solution 1:1 with the NaF solution.**

Once the optimum concentration is achieved, repeat Part C. Use the same enzyme concentration for the remaining parts of the experiment.

7. Discard all contents into the waste jar.

**Part D Enzyme Concentration**

In most cases, the enzyme concentration is much lower than the concentration of the substrate. As a result, the rate of an enzymatic reaction is always directly dependent on the enzyme concentration.

1. Obtain three 10-cm test tubes, and check them to make certain they are identical in terms of their diameter and brand name.

2. Label the three test tubes to indicate the number of drops of potato extract they will contain from the following list, then put the appropriate amount of potato extract in each tube.

<table>
<thead>
<tr>
<th>Tube Contents</th>
<th>Relative Enzyme Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A  15 drops of potato extract</td>
<td>15</td>
</tr>
<tr>
<td>B  5 drops of potato extract + 10 drops of water</td>
<td>5</td>
</tr>
<tr>
<td>C  1 drop of potato extract + 14 drops of water</td>
<td>1</td>
</tr>
</tbody>
</table>

The liquid level in tubes B and C should match the liquid level in test tube A.

3. Place these three tubes into a 37°C water bath.
4. Put 10 drops of 0.01 M catechol solution into each of three other 10-cm test tubes. Place these tubes into the water bath for 5 minutes.
5. After 5 minutes, quickly pour the catechol solution from one 10-cm tube into each of the labeled tubes. Agitate to mix and leave the labeled tubes in the water bath for another 5 minutes.
6. After 5 minutes, remove the labeled tubes from the water bath. Note and record the intensity of any color that develops. Use the scale and numbers given in Part B.
7. Discard all contents into the waste jar.

**Part E Effect of pH**
The pH exerts a significant influence on enzyme activity. Most enzymes are active over a relatively narrow pH range. The pH at which the enzyme activity is at its maximum is called the optimum pH for the enzyme. Activity falls off sharply when the pH changes to a value on either side of the optimum value.
1. Prepare four labeled 10-cm test tubes with contents as follows:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20 drops of 0.1 M hydrochloric acid (HCl)</td>
</tr>
<tr>
<td>B</td>
<td>20 drops of pH 4 buffer</td>
</tr>
<tr>
<td>C</td>
<td>20 drops of pH 7 buffer</td>
</tr>
<tr>
<td>D</td>
<td>20 drops of 0.1 M sodium carbonate solution (Na₂CO₃)</td>
</tr>
</tbody>
</table>

These give pH values of approximately 1, 4, 7, and 10, respectively.

2. Add 10 drops of 0.01M catechol solution to each tube.
3. Add 10 drops of potato extract to each tube.
4. Agitate to mix well, then place the tubes in a 37°C water bath.
5. Wait 10 minutes, and then examine each tube for color changes in the solution.
6. Record the results, using the scale and numbers of Part B.
7. Discard the test tube contents into the waste jar.

**Part F Effect of Temperature**

For many chemical reactions, a 10°C rise in temperature approximately doubles or triples the reaction rate. To a certain extent, this is also true for enzymatic reactions. After a certain point, however, an increase in temperature causes a decrease in the reaction rate because the enzyme begins to denature. The temperature of maximum enzyme activity is called the optimum temperature. Most enzymes of warm-blooded animals have optimum temperatures near the normal body temperature of about 37°C.

1. Label three 10-cm test tubes A, B, and C.
2. Add 10 drops of potato extract to each tube.
3. Place the tubes in the following temperature baths for 10 minutes:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0°C (an ice bath)</td>
</tr>
<tr>
<td>B</td>
<td>37°C</td>
</tr>
<tr>
<td>C</td>
<td>70°C</td>
</tr>
</tbody>
</table>

4. Put 10 drops of 0.01 M catechol solution into each of three other 10-cm test tubes. Place one of these tubes into each of the water baths. Leave them there until the labeled tubes have been in for the proper amount of time (approximately 10 minutes).
5. Quickly, combine and agitate the contents of the two tubes in each bath. Leave the tube containing the final mixture in each temperature bath.
6. After 5 minutes, examine each tube for the development of a color. Record the results, using the scale and numbers of Part B.
7. Discard all contents into the waste jar.

**Part G Inhibitors**

Inhibitors decrease, or destroy, the ability of an enzyme to catalyze reactions. Since enzymes are proteins, substances that denature proteins inhibit them. In addition, any agent that combines with a necessary cofactor can function as an inhibitor. For this part, the effect of the following inhibitors will be demonstrated:

- **Trypsin** - An enzyme that digests proteins (including other enzymes)
- **Phenylthiourea** - Combines with the copper ion cofactor
Lead nitrate - A heavy metal denaturant

1. Label four 10-cm test tubes A, B, C, and D.
2. Add 10 drops of potato extract to each tube.
3. Add the following to the tubes:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10 drops of distilled water</td>
</tr>
<tr>
<td>B</td>
<td>10 drops of 5% trypsin suspension</td>
</tr>
<tr>
<td>C</td>
<td>10 drops of phenylthiourea (saturated solution)</td>
</tr>
<tr>
<td>D</td>
<td>10 drops of 5% lead nitrate solution, Pb(NO$_3$)$_2$</td>
</tr>
</tbody>
</table>

4. Place the four tubes into a 37°C water bath and leave them there for a minimum of 10 minutes.
5. After 10 minutes, add 10 drops of 0.01 M catechol solution to each tube and mix well. Leave the labeled tubes in the water bath for another 5 minutes.
6. Examine the tubes for the development of a color in the solution. If a precipitate forms, allow it to settle before observing the color. Record the results, using the scale and numbers of Part B.
7. Discard all waste into the assigned waste jar.

Data Analysis:
- Plot the data for parts C to F with enzyme activity score of 1 to 4 in the y axis and variables used for each part in the x axis (conc. of substrate, conc. of enzyme, pH and temperature).
- Connect the points in each graph to form a graphical representation of enzyme behavior.
- Interpret the graphs in the discussion portion of your report

Postlab Questions:
1. According to the data, which structural features of the compounds tested appear to be necessary in a substrate for polyphenoloxidase? Explain.
2. Did the experiment reach the optimum substrate concentration? Explain.
3. Based on the graphed result of enzyme activity vs. enzyme concentration, how would the enzyme activity change if the relative enzyme concentration was increased to 20? Explain.
4. In part G, if precipitation occurred, it is likely to be:
   a. denatured enzyme
   b. digested enzyme
   c. inhibitor combined with cofactor.
   Explain.
Cellular Respiration

A series of exergonic chemical reactions, collectively known as cellular respiration, enables the living cell to release the energy "stored" in the chemical bonds of the various substances of protoplasm. The energy then is temporarily stored as molecules of ATP. These reactions, each catalyzed by a specific enzyme, serve to degrade (break down) glucose by one of two paths.

1. Anaerobic respiration (Embden-Meyerhof pathway), in which glucose is broken down in the absence of oxygen (Figure 1).
2. Aerobic respiration, in which glucose is completely oxidized only in the presence of oxygen.

Part 1
Anaerobic Respiration

Fermentation

Yeast cells in the absence of oxygen are able to convert glucose to ethyl alcohol and carbon dioxide in a process known as fermentation. ATP provides the "spark" to start the reaction sequence of fermentation by phosphorylating glucose. The glucose 1-phosphate is isomerized to glucose 6-phosphate.

![Fermentation Reaction Diagram](image)

Glucose 6-phosphate, in turn, is isomerized to fructose 6-phosphate, which is then phosphorylated by another molecule of ATP to form fructose 1,6-diphosphate. Two molecules of ATP are therefore consumed during the initial reactions of fermentation.
Fructose 6-phosphate \[ \text{ ATP } \rightarrow \text{ ADP} \]

Fructose 6-phosphate \[ \text{ ATP } \rightarrow \text{ ADP} \]

Fructose 1,6-diphosphate then splits into two molecules of triose phosphate (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate).

Glyceraldehyde 3-phosphate is then oxidized with the addition of phosphate (oxidative phosphorylation) to 1,3-diphosphoglyceric acid.
FIGURE 1  The Embden-Meyerhof pathway.
1,3-Diphosphoglyceric acid becomes, upon the loss of a high-energy phosphate group (to ADP) during a substrate-level phosphorylation, 3-phosphoglyceric acid, which is then isomerized to 2-phosphoglyceric acid.

\[
\begin{align*}
\text{O} &= \text{C} \quad \text{O} \sim \text{P} \\
\text{2 H} &= \text{C} \quad \text{OH} \\
\text{H} &= \text{C} \quad \text{O} \sim \text{P} \\
\end{align*}
\]

\[
\begin{align*}
2 \text{ADP} &\quad 2 \text{ATP} \\
\end{align*}
\]

\[
\begin{align*}
\text{O} &= \text{C} \quad \text{OH} \\
\text{2 H} &= \text{C} \quad \text{OH} \\
\end{align*}
\]

\[
\begin{align*}
\text{H} &= \text{C} \quad \text{OH} \\
\end{align*}
\]

2-Phosphoglyceric acid loses water to form phosphoenolpyruvic acid (PEP), and the PEP becomes converted to pyruvic acid, with substrate-level phosphorylation again generating ATP.

\[
\begin{align*}
\text{O} &= \text{C} \quad \text{OH} \\
\text{2 H} &= \text{C} \quad \text{O} \sim \text{P} \\
\text{CH}_{2} \text{OH} &\quad \text{enolase} \quad 2 \text{H}_{2} \text{O} \\
2 &= \text{C} \quad \text{O} \sim \text{P} \\
\text{CH}_{2} &\quad 2 \text{ADP} \quad 2 \text{ATP} \\
2 &= \text{C} \quad \text{CH}_{3} \\
\end{align*}
\]

Carbon dioxide is subsequently removed from pyruvic acid, and the resulting acetaldehyde is reduced (hydrogenated) to ethyl alcohol.

\[
\begin{align*}
\text{O} &= \text{C} \quad \text{OH} \\
2 &= \text{C} \quad \text{O} \\
\text{CH}_{3} &\quad \text{CO}_{2} \\
2 &= \text{H} \quad \text{C} \quad \text{O} \\
\text{CH}_{3} &\quad 2 \text{NAD} \quad 2 \text{NAD}^{+} \\
2 &= \text{H} \quad \text{C} \quad \text{OH} \\
\text{CH}_{3} &\quad \text{Ethyl alcohol} \\
\end{align*}
\]

Part 1 Procedure

Fermentation

1. Label three clean small test tubes A, B, and C. Prepare them as follows (yeast suspensions settle; shake before use).

   Tube A: Fill tube one-third full with yeast suspension. Add water to fill tube completely.
   Tube B: Fill tube one-third full with yeast suspension. Add 0.5 M glucose solution to fill tube completely.
   Tube C: Fill tube one-third full with yeast suspension. Add 0.5 M glucose solution to fill tube two-thirds full. Add 0.1 M sodium fluoride solution to fill tube completely.

2. Obtain three vials and prepare incubation chambers as follows.
   a. Hold filled tube upright.
   b. Place the vial on over the tube.
   c. Hold the tube tightly against the end of the vial.
   d. Quickly invert the incubation chamber as shown. If the procedure is correctly done, only a small air bubble will be present in the upper end of the tube.
   e. Place the vial/test tube set-up in a 100 mL beaker.
3 Measure the length of this air bubble, if present. Record its length in millimeters.
4 Place all three incubation chambers in 37°C incubator.
5 Examine the tubes at 15 min intervals and record the length of any bubbles as they develop and increase in size. Continue observations over a 90 min period. Proceed to Exercise 2 during this period.
6 Subtract the length of the original bubble and record your data in Table 26-1. Then plot the data on a graph. The carbon dioxide gas that evolves is the product of the fermentation reaction, which may be summarized as:

\[
C_6H_{12}O_6 \overset{\text{yeast}}{\rightarrow} 2 \text{C}_2\text{H}_5\text{OH} + 2 \text{CO}_2
\]

Glucose Ethyl alcohol Carbon dioxide

Part 2
Anaerobic Respiration

Conversion of glucose to pyruvic acid is glycolysis. It occurs, with variations, in nearly all organisms, both aerobic and anaerobic. The anaerobic conversion of glucose (and glycogen) to lactic acid occurs in many animal cells.

Yeast are interesting organisms because, depending upon reaction conditions, they can metabolize glucose either anaerobically or aerobically. Yeasts are referred to as facultative anaerobes; that is, they can grow either in the absence of oxygen or in the presence of oxygen. In addition, yeast cells contain the enzyme lactic acid dehydrogenase (used in glycolysis) as well as pyruvic acid decarboxylase and alcohol dehydrogenase (used in fermentation). Animal cells, on the other hand, contain only lactic acid dehydrogenase. Under anaerobic conditions, they accumulate lactic acid instead of ethyl alcohol.

The conversion of pyruvic acid to lactic acid is a reduction process; it follows that the conversion of lactic acid to pyruvic acid is an oxidation process. The enzyme lactic acid dehydrogenase catalyzes both reactions.
In the following exercise we shall investigate the action of this enzyme (in yeast) upon lactate ions. We shall use the dye methylene blue (MB) in place of the natural electron acceptor NAD\(^+\) (the coenzyme) because the color change of the dye will allow us to monitor the extent of enzyme activity.

Summary of Anaerobic Respiration
Both fermentation and glycolysis yield four ATP molecules via substrate-level phosphorylation. But in the process each consumes two ATP molecules. Therefore there is a net gain of only two ATP molecules.

\[
\text{Glucose} + 2 \text{ADP} + 2 \text{Pi} \rightarrow \begin{cases} \text{Ethyl alcohol or CO}_2 & \text{or} \\ \text{Lactic Acid} & \end{cases} + 2 \text{ATP}
\]

Oxygen is not consumed in the overall anaerobic process of breaking down glucose to either ethyl alcohol or lactic acid.

Part 2 Procedure
Glycolysis

1. Label three clean small test tubes A, B, and C, and prepare them as follows.
   - Tube A: Add 5 mL of 0.02% methylene blue solution and 5 mL of yeast suspension.
   - Tube B: Add 5 mL of 0.02% methylene blue solution, 5 mL of yeast suspension that has previously been heated in boiling water for 10 min, and 10 drops of 5% sodium lactate solution.
   - Tube C: Add 5 mL of 0.02% methylene blue solution, 5 mL of yeast suspension, and 10 drops of 5% sodium lactate solution.
2. Mix the contents of each tube well. Then hold each tube at a slight angle and allow 3 or 4 drops of mineral oil to run down the inside of the tube and form a thin film over the mixture. (Mineral oil prevents oxygen from diffusing into the solution.)
3. Place the tubes in a water bath set at 37°C.
4. Examine the tubes at 5 min intervals for 20 min. Note color changes, if any. record your results and graph.
5. What does the graph show? Include the graph analysis in the Results and Discussion portion of your lab report.
DATA SHEET

Table 1  Fermentation

<table>
<thead>
<tr>
<th>Tube</th>
<th>Contents</th>
<th>Length of Bubbles at Time Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>Yeast + Water</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Yeast + Glucose</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Yeast + Glucose + NaF</td>
<td></td>
</tr>
</tbody>
</table>

Table 2  Glycolysis

<table>
<thead>
<tr>
<th>Tube</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>MB + Yeast</td>
</tr>
<tr>
<td>B</td>
<td>MB + preheated Yeast + lactate</td>
</tr>
<tr>
<td>C</td>
<td>MB + yeast + lactate</td>
</tr>
</tbody>
</table>
Post Lab Questions

1. Can we regard fermentation as a complete oxidative process, i.e., one that has released all energy from the chemical bonds of glucose? Explain.

2. What is the effect of sodium fluoride on the fermentation process?

3. Why is lactic acid enzyme called dehydrogenase rather than oxidase?

4. Why is it necessary to exclude oxygen from the contents of the tubes in both procedures?

5. In the glycolysis experiment, why does the color change less in Tube B than tube C?
Esterification of Linoleic and Linolenic Acids from Evening Primrose Oil
Note: The experiment will be completed on week 4. Chromatogram will be distributed on week 5.

Background

Evening Primrose is a biennial plant indigenous to North America. Oil from the plant is cold pressed. Evening primrose oil is composed of fatty acids of oleic acid, palmitic acid, gamma-linolenic acid (GLA) and linoleic acid (LA). Both gamma-linolenic and linoleic acids are essential fatty acids. Evening primrose oil contains 72% linoleic acid and 9% gamma-linolenic acid.

Essential fatty acids (EFA’s) play important roles in our body’s overall health. They are part of every cell, and establish and control the cellular metabolism. EFA’s are essential in four primary body functions: a) provide energy, b) maintain body temperature, c) insulate our nerves and d) cushion and protect body tissues.

Linoleic acid, an omega-6-fatty acid, is not produced by the body and must be obtained through dietary intake. Linoleic acid acts as an energy source and is what the body converts to gamma linolenic acid (GLA). GLA, an omega-6-fatty acid is converted to eicosanoids which are hormone-like substances that regulate platelet aggregation, blood vessel tone, salt and water balance, gastrointestinal function, neurotransmitter function and secretion of insulin. Eicosanoids include the prostaglandin, thromboxanes and leukotrienes. Because EPO is a rich source of gamma-linolenic acid, it is highly valuable for individuals who cannot otherwise form GLA from linoleic acid.

The fatty acids in EPO are easily oxidized when exposed to air. They also have high boiling points making separation by distillation difficult. By esterifying the fatty acids to their methyl esters, the problem of oxidation is alleviated. Carboxylic acids react with alcohol when catalyzed with acid to form esters. The general reaction is:

\[ R_1\text{COOH} + R_2\text{OH} \rightarrow R_1\text{COOR}_2 + \text{H}_2\text{O} \]

In oils, carboxylic acid varies considerably in their fatty acid content. They commonly occur as triglycerides. Triglycerides are triesters of glycerol with, most times, different acid groups attached to the glycerol. If sodium methoxide in methanol is used, the triglycerides are converted to methyl esters cleanly as shown on the next page. The reaction must be done in anhydrous condition. Acetic acid is added to the reaction to acidify the mixture. The methyl esters are extracted into hexane layer. The methyl esters is isolated from the hexane is by distillation.

In this experiment, gamma-linolenic acid, linoleic acid, palmitic acid, oleic acid palmitic acid and stearic acid will be esterified into their corresponding methyl esters and analyzed by gas chromatograph. Other fatty acids may be isolated but will not be identified.

Gas Chromatography

Ideally, separation by vacuum distillation is performed to be able to separate each component. However, this requires a vacuum source that is not available. Instead, separation and quantification of the components will be done by gas chromatography.

Gas chromatography is often used as a tool to separate, identify and quantify components of a mixture. The components are separated through a column that contains an adsorbent that is coated with a liquid. The liquid coated adsorbent is the stationary phase. The sample is injected through a heated injection port. It vaporizes and is carried through the heated column by the carrier gas, commonly helium. The carrier gas is
the mobile phase. The sample is passed through the column and separates. The separation occurs because the components are attracted to mobile and stationary phases differently. As the components separate, they go through the detector at different times. A signal is sent to the detector and peaks are generated as each component goes through the detector.

The distance from injection mark to the peak, multiplied by the chart speed is called retention time. If the chart speed is constant, the distance is normally reported as the retention time. Given there is no change in the flow rate of carrier gas and temperature, the retention time for the given compound will always be the same. The retention times of the components are compared to the retention time of the known standards to identify the components.

The areas of the peaks are proportional to amount of the components. To determine the percent component, the areas of the peaks are measured. Each of the component areas is divided by the total area and multiplied by 100. In the past, the areas of the peaks were calculated by triangulation of the peak, height (at ½ width) x width. For this experiment, the integrator will measure the areas for you. A computer prints out with the areas and % areas will accompany your chromatogram.

In this experiment we will convert the triglycerides present in the EPO to the corresponding methyl esters using the procedure used by Pavia et al. We will identify the presence of GLA and LA and minor fatty acids: palmitic, oleic and stearic acids, in EPO. We will also quantify the fatty acid components of EPO. Gas Chromatography will be used for both identification and quantification of product. A flame ionization detector (FID) with a column specific to fatty acid will be used.

**Materials:**

**Per pair of students**

1 clean dry test tube  
1 cork to fit test tube  
1-50 mL Erlenmeyer flask  
Pasteur pipets and bulbs  
Cotton  
Vial with lids (10 mL capacity)  
Simple distillation apparatus (2-50 mL boiling flasks, 3 way connector, straight tube adapter, thermometer adapter, vacuum adapter, thermometer, 2-water hoses)
Funnel
Filter paper
Ring stand
2 clamps
1 small ring

Per Class
Nitrogen gas
2 sets of water baths set to 40°C with test tube racks. Set one on each side of the lab away from the sink area.

Chemicals:
GC standards (methyl palmitate, methyl oleate, methyl linoleate, gamma methyl linolenate, methyl stearate). Prepare a mixture of 0.2 mL of each of the methyl ester dissolved in 1 mL methylene chloride.
Anhydrous sodium sulfate mixed with 10% (w/w) sodium bicarbonate (10 grams of sodium bicarbonate mixed with 90 grams of anhydrous sodium sulfate).

Set the following chemicals in a dispensing hood.
1. Tetrahydrofuran (THF) dried over molecular sieves (1 week prior to use), stock bottle
2. Sodium methoxide in anhydrous methanol, stock bottle
3. 4 – 2 mL syringes
4. glass syringe (1 mL)
5. Nitrogen gas tank with regulator and a hose hook-up.

Set items 1 to 5 in a hood that is centrally located.

Evening Primrose oil

Set up items in each of the 6 hoods (total of 6 chemical bottles)
6. Glacial Acetic Acid, 25 ml/bottle with droppers
7. Hexane, 200 mL/bottle provide a 25 mL or 10 mL graduated cylinder with each bottle. Label hexane at the bottom of the grad cylinder.

Procedure:
Part A: Esterification of EPO

Important: Maintain an anhydrous condition until the acetic acid is added.

1. Slit open a capsule of evening primrose oil. Place two drops of the oil in a clean and dry small test tube. Label your test tube with your name.
2. Have your instructor add 1 mL of (dry) tetrahydrofuran (THF) and 2 mL of 0.5 M sodium methoxide in anhydrous methanol. This will be done in the dispensing hood.
3. Loosely insert a cork in the test tube containing the reactants mixed above. Swirl gently to mix the reagents.
4. Place the test tube in 40°C bath in the hood. Allow the mixture to react for 10 minutes in the water bath.

Part B: Extraction of methyl ester

Do all of Part B in the hood. Reagents are already in the hoods.

1. Remove the test tube from the water bath and add one drop of glacial acetic acid while swirling gently.
2. Add 5 mL of distilled water and mix with a stirring rod.
3. Add 5 mL of hexane to extract the methyl ester. Stir the mixture for 2 minutes.
4. Let the mixture settle. There should be two layers. If an emulsion forms, it should dissipate on standing.
5. Using a Pasteur pipet, remove the top hexane layer and place it into 50 mL Erlenmeyer flask. Cover the flask with parafilm to prevent contamination.
6. Add another 5 mL of hexane to the aqueous layer to extract the remaining methyl esters. Again remove the upper hexane layer and add it to the first hexane extract.
7. Dry the hexane layer with the anhydrous sodium sulfate/sodium bicarbonate solid mixture to remove any residual water.
8. Fold a filter paper in half and then in quarter. Make a cone and place the coned filter paper in a
ground funnel. Wet the filter paper with hexane (this is a very important step). Filter the hexane
layer into a vial labeled with your name. Turn in the vial to your instructor to be run on the GCMS.

Part C: **Gas Chromatography**
The sample prepare above will be run at Seattle Central Community College (SCCC) unless the new GC is
ready at Bastyr University.
The Gas Chromatograph at SCCC has a mass spectrometer detector, hence GCMS. The sample will be
diluted further in hexane with a 1 uL sample/1 mL hexane. Out of this diluted sample, 1 uL is injected in the
GCMS. HP5MS, a 30 meter, non polar column is used. The components are identified by comparing the
fragment pattern to a library of spectra.
Once the instrumental analysis is complete two chromatograms will be presented, the standard contains
both the retention time (rt) and identified peak that will be referenced to identify the second chromatogram,
the sample.

**Identification of GLA and LA**
1. Identify the peaks based on the retention time of the standards.
2. Determine the percent fatty acid components of your product?

**Post Lab Questions.**
1. How are you able to identify the components of your product?
2. Are you able to identify all the fatty acid components?

Pavia D., Lampman, G, Kriz, G and Engel, R. *Introduction to Organic Laboratory Techniques, A
Small Scale Approach.* Saunders College Publishing. 1998
A Study of Carbohydrates

Objectives:
Carry out several of the more common carbohydrate reactions.
Identify an unknown sugar.

Monosaccharides and Disaccharides
Carbohydrates, also known as sugars or saccharides, are one of the most important classes of compounds dealt with in organic chemistry. These compounds function as sources of energy in living organisms and as structural material for cells. Carbohydrates are complex molecules containing both alcohol and carbonyl functional groups. One of the main sources of carbohydrates in our diet is starch from grains and cereals.

Carbohydrates, such as starch, can also be viewed as polymers of various monosaccharides. A monosaccharide is the simplest type of carbohydrate; it cannot be further hydrolyzed into smaller units. One such example of a monosaccharide is glucose, which is shown below.

![D-(+)
Glucose (hemiacetal form)](image1)

Because glucose has both an alcohol and a carbonyl functional group, it can form a cyclic hemiacetal, as shown in the reaction above. The hemiacetal form is in equilibrium with the open chain form. Because both of these forms exist, one would expect that the chemistry of these sugars would be similar to that of an aldehyde. In the case of glucose, its aldehyde form can be easily oxidized. Carbohydrates that can be oxidized are known as reducing sugars.

A disaccharide is a molecule composed of two monosaccharide units. One such compound is sucrose (structure shown below), also known as table sugar. Sucrose is composed of a fructose unit and a glucose unit. Note that the fructose and glucose units are joined by an acetal link involving both their carbonyl carbons, which means that the aldehyde and ketone forms of these sugars are unavailable. Because sucrose is not in equilibrium with any aldehyde form, it is considered to be a nonreducing sugar.

![Sucrose](image2)

Two other common disaccharides are maltose (composed of two glucose units) and lactose (which contains glucose and galactose).

Polysaccharides are carbohydrates composed of many monosaccharide units linked together. The characteristic linkage of most disaccharides and polysaccharides is an acetal. The formation of an acetal linkage produces water; thus, the addition of water will break acetal linkages (hydrolysis).
The relationships between the various carbohydrates are illustrated by the following hydrolysis reactions. Note that the reactions are acid-catalyzed:

\[
\ce{H+} \quad \ce{H+} \\
\text{polysaccharides + H}_2\text{O} \rightarrow \text{disaccharides + H}_2\text{O} \rightarrow \text{monosaccharides}
\]

starch, cellulose                         sucrose,                            glucose, fructose, 
lactose, maltose                         galactose, xylose

The monomer glucose is used to form two different polymers, starch and cellulose. The difference between these two polymers is how the polymeric linkage is formed. We, as humans, can utilize starch as a source of glucose because we can hydrolyze the linkages between the glucose units in starch (\(\alpha\) linkages). In contrast, we cannot use cellulose as a source of glucose because we do not contain the enzyme (\(\beta\)-glycosidase) to hydrolyze the \(\beta\) linkages found in cellulose.

*Glucose polymer with \(\alpha\) (1-4) glycosidic linkages*

The \(\alpha\) and \(\beta\) indicates the configuration at the anomeric carbon. The \(\alpha\)-anomer has a trans relationship between the hydroxy on the anomeric carbon and the -\(\text{CH}_2\text{OH}\) at C5. In the \(\beta\)-anomer, the relationship is cis.
EXPERIMENTAL PROCEDURE

A. Fermentation

During fermentation, certain carbohydrates are converted into CO₂ gas and ethanol. This transformation is brought about by several enzymes.

Yeast contains enzymes capable of fermenting glucose as well as many other sugars.

PROCEDURE

1. Obtain an unknown sugar solution and record the unknown identification (ID) number on Table 1 of the Data and Report Sheet.
2. Carry out the following procedure and those in Parts B to D with 4% solutions of the sugars fructose, glucose, lactose, sucrose, xylose, and your unknown and 10% solution of honey.
3. Label five test tubes with the identity of the sugar solutions and add 3 mL of the appropriate sugar solution to each tube. Label another test tube, honey and another, unknown. Each student will have their own different unknown solution to analyse.
4. Add 3 mL of yeast suspension to each tube. Shake the yeast bottle before using. Cover the test tube with a plastic top.
5. Invert once to mix and place the tubes in a 250-mL beaker. Place the test tubes in a 45º C
incubator.
6. Allow the tubes to remain undisturbed for 1 hour. Gas (CO₂) will form, bubble off and create a foam on the top of some of the tubes within an hour.
7. While this test is proceeding, go on to other tests.
8. Record in Table 1 which samples produced CO₂ and which did not.

B. Benedict's Test

Benedict's reagent is a mild, alkaline oxidizing agent that will oxidize all monosaccharides, whether they are aldoses or ketoses, and some disaccharides. Sugars are classified as reducing or nonreducing, depending on whether or not they react with the Benedict's reagent. A color change from blue to orange, red, or dark brown indicates a reaction has taken place.

\[
\text{reducing sugar} + \text{Cu}^{2+} \xrightarrow{\text{heat}} \text{oxidized sugar} + \text{Cu}_2\text{O}
\]

**PROCEDURE**

1. Label nine test tubes and place 20 drops of the appropriate (listed on Table 1 of the Data sheet) solutions in each tube.
2. Add 20 drops of Benedict's solution to each tube and agitate to mix the contents.
3. Place all the tubes in a boiling-water bath at the same time.
4. Heat them for 2 minutes after the water starts boiling again.
5. Note and record in Table 1 the color of the agitated contents of each tube after the 2-minute boiling is complete.

C. Barford

Barfoed's reagent is a slightly acidic solution containing Cu²⁺ ions, but it is a weaker oxidizing agent than the alkaline Benedict's solution. Barfoed's reagent will oxidize monosaccharides (and produce the red-colored precipitate Cu₂O) but will not oxidize disaccharides. Thus, Barfoed's reagent serves to distinguish between monosaccharides and disaccharides.

**Procedure**

1. Label nine test tubes and place 20 drops of the appropriate solution in each tube (listed in Table 1).
2. Add 20 drops of Barford's reagent to each tube and agitate to mix the contents.
3. Place all the tubes in a boiling-water bath at the same time.
4. Heat them for 2 minutes after the water starts boiling again.

At the end of 2 minutes, note and record in Table 1 the appearance of any red precipitate.

D. Seliwanoff's Test

Hexoses (sugars containing six carbon atoms) are dehydrated and form hydroxymethylfurfural when heated with HCl. Ketohexoses (such as fructose) and disaccharides (such as sucrose) that contain a ketohexose yield larger amounts of this product and react faster than aldohexoses.

These differences are the basis of the Seliwanoff test for ketoses in which resorcinol is used to form a red product with the generated hydroxymethylfurfural. Other sugars produce gray, yellow, or faintly pink colors.
Dehydration of Aldohexose and Ketohexose to Hydroxymethylfurfural

CAUTION: Seliwanoff's reagent is 6 M in hydrochloric acid, and will vigorously attack tissue. Wear gloves. If contact occurs, wash the contacted area with cool water.
PROCEDURE
1. Label nine test tubes with the solutions listed on Table 1.
2. Place 2 mL of Seliwanoff's reagent in each tube.
3. Add 1 drop of each solution to the appropriate tube and mix well.
4. Place all the samples in a boiling-water bath at the same time.
5. Heat them for 3 minutes after the boiling starts again.
6. Note and record in Table 1 the resulting color of each solution.

A to D. Summary of Sample Behavior
1. Use the positive results summarized in Table 2 and the test results for each sample recorded in Tables 1 through 4 to decide which samples gave positive results for each test.
2. Record these results in Table 3 by placing an X in the blanks to indicate which samples gave positive results.

A to D. Identification of Unknowns
1. Your unknown contained a single sugar. Use the results in Table 3 to identify the sugar.
4. Honey contains two sugars; one is glucose. Identify and record the name of the other one.
TABLE 1
DATA TABLE

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Fermentation</th>
<th>Benedict's Test</th>
<th>Barfoed’s Test</th>
<th>Seliwanoff’s Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(CO₂ Gas)</td>
<td>(Color of solution and color and degree of ppt)</td>
<td>(Red Precipitate)</td>
<td>(Color)</td>
</tr>
<tr>
<td>Water (negative control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Honey</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown #</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown #</td>
<td></td>
<td></td>
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<td></td>
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</table>

TABLE 2
POSITIVE RESULTS

<table>
<thead>
<tr>
<th>TEST</th>
<th>SUGARS THAT GAVE POSITIVE RESULTS</th>
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</thead>
<tbody>
<tr>
<td>Fermentation</td>
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</tr>
<tr>
<td>Benedict’s Test</td>
<td></td>
</tr>
<tr>
<td>Barfoed’s Test</td>
<td></td>
</tr>
<tr>
<td>Seliwanoff’s Test</td>
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</tbody>
</table>
TABLE 3
SUMMARY OF SAMPLE BEHAVIOR

<table>
<thead>
<tr>
<th>SUGAR SOLUTION</th>
<th>FERMENTATION</th>
<th>BENEDICTS</th>
<th>BARFOED’S</th>
<th>SELIWANOFF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Fructose</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lactose</td>
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<td></td>
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<tr>
<td>Sucrose</td>
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<tr>
<td>Xylose</td>
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<tr>
<td>Glucose</td>
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<td></td>
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<tr>
<td>Honey</td>
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<td></td>
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<tr>
<td>Unknown #</td>
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<td>Unknown #</td>
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</tr>
</tbody>
</table>

Post lab Questions:
1. Find the structures for the following carbohydrates (sugars) in a reference work or textbook and decide whether they are reducing or non-reducing carbohydrates (sugars): sorbose, mannose, ribose, maltose, raffinose and cellulose.