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UNIVERSITY COLLEGE
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Faculty of Science

Laboratory Manual

ADVANCED BIOCHEMISTRY

Master of Science in Biotechnology

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LINCOLN UNIVERSITY COLLEGE
FACULTY OF SCIENCE (DEPARTMENT OF BIOTECHNOLOGY)
LABORATORY SAFETY RULES

The following rules must be obeyed by all students in the science laboratory of the faculty. Wilful or repeated in advertent non-compliance may result in dismissal or suspension from the laboratories

- **No entry without permission:**
  - Outsiders are not allowed to enter the laboratory without permission.
  - No student is allowed to enter the laboratory unless permission has been given by a laboratory assistant or a lecturer.

- **At work in the laboratory:**
  - No experiment may be attempted without the knowledge and permission of a lecturer.
  - Students must wear shoes in the laboratory. Students wearing slippers or sandals are not allowed to work in the laboratory.
  - Lab coat must be worn at all times during practical work in the laboratory.
  - Do not mouth pipette chemicals.
  - Do not eat or smoke in the laboratory.
  - Do not taste any chemicals, including dilute solutions. If any acid or alkali accidentally enters your eyes or mouth, wash immediately with plenty of tap water. Inform your lecturer, and seek medical attention if necessary.
  - Paper should be used to light up the Bunsen burners.
  - Used match sticks, filter papers, and other solid waste must never be thrown into the sinks. They must be thrown into the dustbins provided. Lighted match sticks and smoldering materials must be extinguished with tap water before thrown in to the dustbins.
  - Any equipment broken or damaged must be reported to the laboratory assistant.

- **Before leaving the laboratory:**
  - All the equipment and benches must be cleaned at the end of each practical session.
  - Wash hands and arms with soap and water before leaving the laboratory.
  - No student is allowed to take away any chemicals, equipment or other property of the laboratory.

INTRODUCTION

1. The Scientific Method

- Making observations
- Generating hypotheses
- Making predictions
- Designing and carrying out experiments
- Constructing scientific models

2. Practical Exercises

To get the most out of the practical exercises, you need to follow carefully the instructions given. These instructions have been designed to provide you with the experience in the following skills:

- Following instructors
- Handling apparatus
- Having due regard for safely
- Making accurate observations
- Recording results in an appropriate form
- Presenting quantitative results
- Drawing conclusions

3. Following Instructions

Instructions are provided in the order in which you need to carry them out. We would advise that before carrying out the instructions, you read through the entire exercise. This will help you to remember what you have learned.

Each practical exercise in the book begins with a few lines describing its purpose in most cases the following headings are also used:

- Procedure**-**numbered steps that need to be carried out.
- For consideration **-**some questions to help you think carefully about the results you have obtained.
- Materials**-**a list of the apparatus, chemicals and biological materials you need.

4. Handling apparatus

Biologists need to able to use many different types of apparatus, for example, photometers (to measure water uptake by plants), respirometers (to measure oxygen uptake or carbon dioxide production), Petri dishes (for plating out bacteria and other microorganisms) and the light microscope (to magnify specimens). Many of the practical exercises are designed to help you derive the maximum benefit from a piece of apparatus.

5. Having Due Regard for Safety

Surveys have been shown that science laboratories are among the safest places to be. Nevertheless, this is no cause for complacency.

- Always move slowly and carefully in a laboratory.
- Never put your fingers in your mouth or eyes after using chemicals or touching biological specimens until you have washed your hands thoroughly with soap and warm water, and dried them.
- Make sure glass objects (e.g, thermometers, beakers) cannot roll off tables or be knocked onto the floor.
- Wear safely goggles whenever there is a risk of damage to the eyes.

<u>Situations of risk include:</u>

- Heating anything with a Bunsen burner (even heating water has its dangers')
- Handling many liquids, particularly those identified as corrosive, irritant, toxic or harmful

- Handling corrosive or irritant solids
- Some dissection work
- Allow Bunsen burners, tripods, gauzez and beakers to cool down before handling them.
- Never allow your own body fluids (especially blood and saliva) to come into contact with someone else, or theirs into contact with you.
- Keep long hair tied back and do not wear dangly earrings.
- Do not allow electrical equipment to come into contact with water.
- If you are unsure how to carry out a scientific procedure, ask.
- Make sure you understand why you are going to do something before you do it.
- Wear a lab coat when using chemicals or handling any biological specimens.
- Follow exactly agreed procedures with regard to cuts, burns, electric shocks and other accidents (e.g. with chemicals).
- Follow exactly all specific safely instructions given in this book or provided by your teacher for particular practical exercises (e.g. use of gloves, disinfection)

With practice, these procedures should become second nature to you. They will enable you to carry out practical work in safety.

6. **Making Accurate Observations**

In most cases the practical exercise will make it clear what you need to observe, e.g. the time taken for a certain volume of gas to be evolved or the width of a sample cells. Ensure that you know how to use any necessary equipment before starting practical. Think carefully about the precision with which you will make your observations.

7. Recording Results in an Appropriate Form

Results can be recorded in various ways. Often it is helpful to record raw data in a table. Most data will be in the form of numbers, i.e. they will be quantitative data (also known as numerical data). However, some data, e.g. flower colour, will be qualitative data.

One form in which some biological findings can be recorded is a drawing. You don't need to be professional artist to make worthwhile biological drawings. If you follow the following guidelines, a drawing can be of considerable biological value:

- Ensure that your completed drawing will cover at least a third of A4 page.
- Plan your drawing so that the various parts are is proportion and will not be drawn too small. Small marks to indicate the length and breadth of the drawing are a great help in planning and a faint outline can be rapidly drawn to show the relative positions of the parts.
- The final drawing should be made with clean, firm lines using a sharp HB pencil and, if needed, a good quality eraser (not a fluid).If important details are too small to be shown in proportion, they can be put in an enlarged drawing at the side of the main drawing.
- Avoid shading and the use of colour unless you are an excellent artist and they really help, for example when drawing soil profiles.
- When drawing structures seen with the naked eye or hand lens, use two lines to delineate such things as blood vessels and petioles. This will help you to indicate the relative widths of such structures.
- When drawing low power plan drawings from the light microscope, do not attempt to draw individual cells-just different tissues.
- When drawing plant cells at high power under the light microscope, use two lines to indicate the width of cell walls, but a single line to indicate a membrane.
- Always put a scale on each drawing.

8. <u>Presenting Quantitative Results</u>

Presentation of data is all about using graphs or other visual means to make it easier to see what your results tell you. The following four ways of presenting data are the most frequently used in biology: line graphs, bar charts, histograms and scatter graphs (Figure 1).

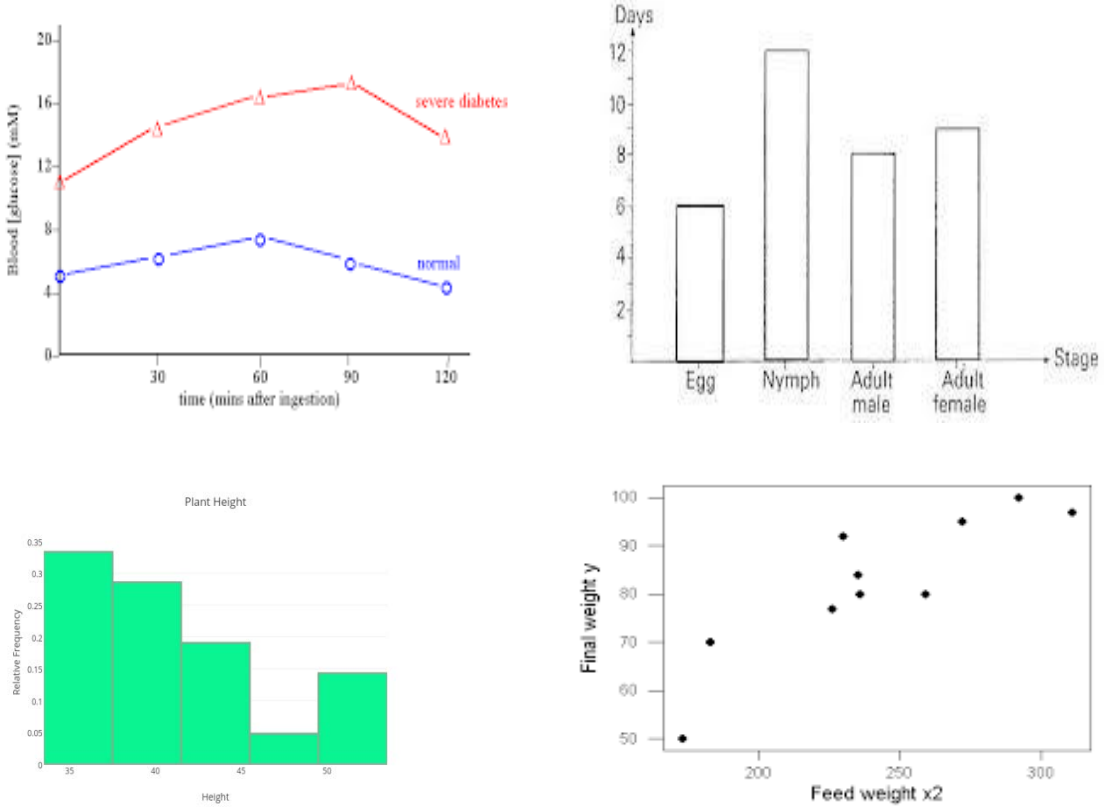


Figure 1: Line graphs, bar charts, histograms and scatter graphs

9. Drawing Conclusions

Finally, you will need to draw conclusions. If your practical exercise has involved the testing of a hypothesis, for example that the enzyme pepsin works better at low pH than in neutral or alkaline conditions, your conclusion should indicate whether the hypothesis has been refuted (i.e. shown not to be the case) or supported. Of course, even if your hypothesis has been supported, it doesn't mean that it has been confirmed with 100% certainty- in other words it isn't proved. Science proceeds more by showing that certain ideas are wrong than by showing that others are right (think about that!). Your conclusion might therefore include further ways of testing the original hypothesis, or might raise new possibilities to be investigated.

Often you will only be able to arrive at your conclusions after statistically analysing your data.

10. Writing a Scientific Lab Report

Title

- Communicate the subject investigated in the paper.

Introduction

- State the hypothesis.
- Give well-defined reasons for making the hypothesis.
- Explain the biological basis of the experiment.
- Cite sources to substantiate background information.

- Explain how the method used will produce information relevant to your hypothesis.
- State a prediction based on your hypothesis. (If the hypotheis is supported, then the results will be.)

Materials and Methods
- Use the appropriate style.
- Give enough detail so the reader could duplicate your experiment
- State the control treatment, replication and standardized variables that were used.

Results
- Summarize the data (do not include raw data).
- Present the data in an appropriate format (table or graph).
- Present tables and figures neatly so they are easily read.
- Label the axes of each graph completely.
- Give units of measurement where appropriate.
- Write a descriptive caption for each table and figure.
- Include a short paragraph pointing out important results but do not interpret the data.

Discussion
- State whether the hypothesis was supported or proven false by the results, or else state that the results were inconclusive.
- Cite specific results that support your conclusions.
- Give the reasoning for your conclusions.
- Demonstrate that you understand the biological meaning of your results.
- Compare the results, with your predictions and explain any unexpected results.
- Compare the results to other research or information available to you.
- Discuss any weaknesses in your experimental design or problems with the execution of the experiment.
- Discuss how you might extend or improve your experiment.

Conclusion
- Restate your conclusion.
- Restate important results.

Literature Cited
- Use the proper citation form in the text.
- Use proper citation form in the Literature Cited section.
- Refer in the text to any source listed in this section.

Acknowledgement
- State any appropriate acknowledgement that you think is necessary.

ADVANCED BIOCHEMISTRY

Table of Content

Practical 1
Title: Determination of competitive and non-competitive inhibitors

Objectives:
After completing the practical, you will be able:

1. Determine competitive and non-competitive inhibitor

Introduction:

For an enzyme inhibitor, two properties are of interest. The first is the *type* of inhibitor (competitive or mixed), and the second is the K*i*, the *dissociation constant* of the inhibitor for the enzyme. In many explanations of enzyme kinetics, inhibition type is considered to have three categories: competitive, uncompetitive, and non-competitive. Kinetically, these are all borderline cases. Using only a kinetic definition, "competitive inhibitor" means that the inhibitor only binds to the free enzyme, and prevents binding of substrate, "uncompetitive inhibitor" means that the inhibitor only binds to the ES complex (and inactivates it), and "non-competitive inhibitor" means that the inhibitor binds to (and inactivates) the enzyme with the same affinity regardless of the presence of substrate. The kinetic definition makes no claims about the actual location of the inhibitor binding site.

Kinetic experiments can be a method for examining the mechanism and site of inhibitor binding. In many (although not all) cases, either the inhibitor binds to the active site and is a competitive inhibitor, or it binds elsewhere and is a mixed inhibitor. The best method for determining the inhibitor type is to perform K*m* determinations at a variety of inhibitor concentrations.

In most cases, the type of inhibitor is determined by measuring velocity for several different substrate concentrations in the presence of several different inhibitor concentrations. These data allow the comparison of the apparent K*m* and V*max* in the absence of inhibitor to the values obtained in the presence of several inhibitor concentrations. In the other experiment (below) you will collect such data, which should both allow you to assess the inhibitor type and allow you to calculate the K*i* for the inhibitor.

Materials:

Lactate Stock Solution

120 mM lithium lactate

10 mM Tris-HCl (pH 8.6)

NAD+ Stock Solution

12 mM NAD+

10 mM Tris-HCl (pH 8.6)

Bicarbonate Stock Solution

18 mM NaHCO3

0.5 M NaCl

Pyruvate Stock Solution

60 mM Pyruvate

10 mM Tris-HCl (pH 8.6)

NADH Stock solution

0.5 mM NADH

10 mM Tris-HCl (pH 8.6)

Tris buffer for Enzyme Dilution

10 mM Tris-HCl (pH 8.6)

0.5 mM 2-Mercaptoethanol

Putative Inhibitor Stock Solution

300 mM oxalate

10 mM Tris-HCl (pH 8.6)

Putative Inhibitor Stock Solution

300 mM malonate

10 mM Tris-HCl (pH 8.6)

Procedure:

1. Different compounds may inhibit enzymes. Test each of pyruvate, oxalate, and malonate for their ability to inhibit lactate dehydrogenase (LDH) using lactate (with NAD) as the substrate.
2. If time allows, try testing the compounds for their ability to inhibit LDH using pyruvate (with NADH) as the substrate.
3. Perform an I_{50} determination for each of the potentially inhibitory molecules. When performing the I_{50} determination, it is usually best to use a constant substrate concentration near the K*m*, and to vary the concentration of inhibitor over a fairly wide range.
4. In performing the enzyme assays, add an additional 10 µl of either buffer or inhibitor to each cuvette (this maintains the same volume in all of the cuvettes).

Experimental procedures: LDH inhibitor type determination

1. Make a series of lactate dilutions, as you did for determining the K*m* for lactate. Make up enough of each dilution so that you can run several measurements.
2. Collect the data for a velocity *versus* substrate concentration plot for each of at least three separate inhibitor concentrations (note: one of these “inhibitor concentrations” should be “zero”).
3. The inhibitor concentrations you should use should be low enough to allow you to readily detect activity but high enough so that you will clearly observe inhibition. (Reasonable concentrations are those that should yield about 30 to 40% and about 50 to 60% inhibition.)

Result

Questions:

1. What were the I*50* values from your data?
2. Is the value you obtained using pyruvate similar to the value you obtained using lactate?
3. Is there anything obviously different about the inhibition with pyruvate as substrate?
4. Based on your data, what are potentially useful concentrations of the inhibitor to use in K*m* determination experiments?
5. Why do you want to take a time point at 0.25 minutes?
6. Why are you adding lactate to the reaction mixture for the second chemical modification experiment?
7. What is the function of the "stop solution"? How does it work?
8. What is the composition of the reaction mixture? How much of each reagent will you need to add? (In other words, do the dilution calculations you need, so that you will be ready to perform the reaction before coming to class.)
9. Why might arginine modification alter the activity of LDH?
10. Plot the results of the chemical modification experiment shown in the table below. What is the qualitative effect of the lactate? What might account for this?

Practical 2
Title: Thin layer chromatographic separation of sugars and membrane lipid

Objective:
After completing the practical, you will be able:

1. To learn the separation technique of thin layer chromatography

Introduction:

Thin layer chromatography (TLC) and high-performance thin layer chromatography (HPTLC) – now also called planar chromatography are, like all chromatographic techniques, based on a multistage distribution process. This process involves: a suitable adsorbent (the stationary phase), solvents or solvent mixtures (the mobile phase or eluent), and the sample molecules. For thin layer chromatography the adsorbent is coated as a thin layer onto a suitable support (e.g. glass plate, polyester or aluminum sheet). On this layer the substance mixture is separated by elution with a suitable solvent. The principle of TLC is known for more than 100 years now. The real break-through of TLC as an analytical method, however, came about 35 years ago as a consequence of the pioneering work of Egon Stahl. After some time of stagnation thin layer chromatography has gained increasing importance as an analytical separation technique, which is probably due the effects of instrumentalization and automatization. At the same time the applicability of thin layer chromatography was enhanced by the development of new adsorbents and supports.

Materials:
For each student:

- N-(1-naphthyl) ethylenediamine dihydrochloride
- Normal and high sucrose soya beans
- Measuring cup
- Kitchen strainer
- One 1-liter glass container with no metal or plastic parts
- Two pairs standard laboratory gloves and goggles
- One 5 cm x 10 cm Whatman K5 thin-layer chromatography plate Carbohydrate standards
- Three 10-microliter (µl) micropipettes One pencil (Do not substitute a pen.)
- One ruler marked in millimetres (mm) and cm
- One piece of plastic wrap (Saran wrap or similar) large enough to enclose the chromatography plate
- Two glass beakers, mixing bowls, or other containers each large enough to contain 1/2 cup of soybeans plus enough water to keep them covered overnight
- Three or four paper towels
- Three microscopic plates
- Two 1.5 ml microcentrifuge tubes Masking tape and marker pen

Group of students can share:

- A blender
- Two wide-mouth (at least 5 cm wide) glass jars with lids. Wide-mouth mason jars such as those used for canning garden produce will work.
- A hairdryer that has low/high heat settings

Procedures:

Day 1

1. Depending on the availability of blenders, your teacher may wish to have you work in pairs or in groups of two or more pairs. Clean the bowls, measuring cups, beakers, strainers, and blender containers with hot soapy water before you begin, unless your teacher has already completed this step.
2. Measure one-half cup (75 g) of normal soybeans into a container and add enough tap water (about two cups) to keep the soybeans covered as they soak for 18-24 hours. The soybeans should stay covered with water for this entire time, so designated students or the teacher should check the containers periodically. Before leaving for the day, add a sufficient amount of water to cover the soybeans overnight. You cannot add too much water. Use the masking tape and marker to label the container "Normal."
3. Repeat step two with the high sucrose soybeans. Label the container "High Sucrose."
4. Each group or pair of students should prepare a thin layer chromatography plate for the next day's activity. Turn the plate white-side-up. Use the ruler to make a very light line 15 mm from one side of the plate, as shown in figure 1. It is important to use a pencil to draw lightly on the plate. Alcohol dissolves ink which would migrate up the plate, staining it.
5. Your teacher may want you to make your own standard plate for pure sucrose, raffinose, and stachyose, as well as testing the two kinds of soybeans. If so, use a pencil to make three dots along the length of the horizontal line 1.5 cm apart. Be sure that you do not make any dots closer than 1 cm to the edge of the plate. See Figure 1.

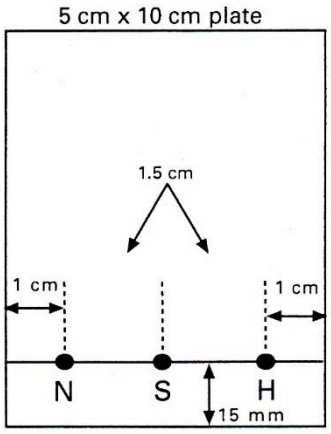


Figure 1

If the standard plate for pure sucrose, raffinose, and stachyose was made by the teacher, you need to make only two dots on the horizontal line.

6. Label the left dot by very lightly writing "N" for normal, label the middle dot by very lightly writing "S" for sugar standards, and label the right dot by very lightly writing "H" for high sucrose. Write the labels under each dot.

Day 2

1. Drain the water from the high sucrose and normal soybeans.
2. Measure the normal soybeans into a measuring cup. Put the soybeans and an equal amount of distilled water in a blender and blend on high for one minute.
3. While the mixture is blending, place a double layer of coffee filters in a kitchen strainer. Filter the mixture, now a whitish liquid, through the double layer of coffee filters into a glass beaker or container.

Note: At this point, your teacher may wish to centrifuge the liquid (filtrate) to obtain better results. If desired, centrifuge the filtrate with either a clinical or microcentrifuge for 10-20 minutes. Continue to prepare the dilution in step 4 from the top layer of the filtrate.

4. Prepare a 1/5 dilution by transferring a drop of the mixture to a 1.5 ml microcentrifuge tube. Add 4 drops of distilled water to make the 1/5 dilution.
5. Transfer 2 drops of the dilution liquid to a clear microscopic slide.
6. Use the marking pen to label the slide "Normal."
7. Repeat steps 2 through 5 with the high sucrose beans. Label the second microscopic slide "High Sucrose."
8. One student in each pair should place one end of a 10 µl micropipette into the liquid on the slide labelled "Normal." The micropipette should fill through capillary action. Place the end of the micropipette on the

TLC plate labelled “N” until a drop of liquid about 2.5 mm wide transfers to the plate. The smaller the dot, the better. See figures 2 and 3.

Figure 2

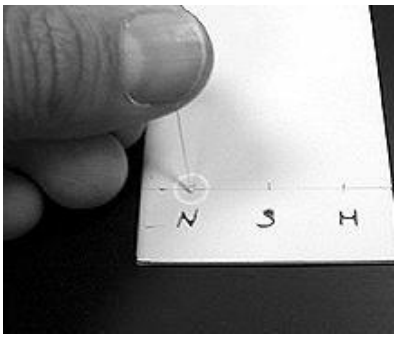


Figure 3

9. Using a new 10 µl micropipette, repeat step 8 with the standard mixture. This time, the drop should be placed on the pencil dot labelled “S.”
10. Using another new 10 µl micropipette, repeat step 8 with the high sucrose soybeans. This time, the drop should be placed on the pencil dot labelled “H.”
11. When all drops are on the plate and have been dried, put on lab gloves. Place the plate in the solvent jar so that it is standing vertically, leaning against the side of the jar with the pencil line at the bottom as shown in figure 4. Cap the jar tightly. The level of solvent should be about halfway between the bottom of the plate and the horizontal line.

Figure 4

12. The solvent will rise up the plate by capillary action, turning the plate a grey, damp colour. The solvent will raise to the top of the plate in about 1.5 to 2 hours. At this point, your teacher may wish to continue this experiment on the following day. If this is the case, wearing lab gloves you (or the teacher) should carefully remove the plate and place it on several thicknesses of paper towels to dry. Leaning the plate upright is the best way to dry it. If the plate is dried horizontally, the coated side should face up. The dry plates should be covered with plastic wrap if left overnight to prevent dust contamination. Be sure the TLC

plates are dry before wrapping them with plastic wrap. Re-cap the solvent jar tightly. A second option is to leave the plate in the jar overnight and then dry it following the directions just described.

Day 3

1. Your instructor should pour dipping reagent into a glass jar to a minimum depth of 15 cm or equal to the height of the TLC plate. A mason jar works well.
2. Wearing lab gloves, you or your teacher should dip the TLC plate into the reagent to immerse it. The plate should be immersed completely for only a second as shown in figure 5.

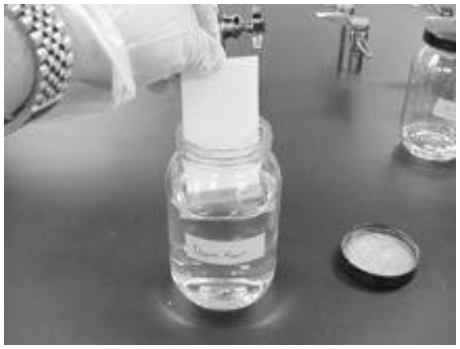
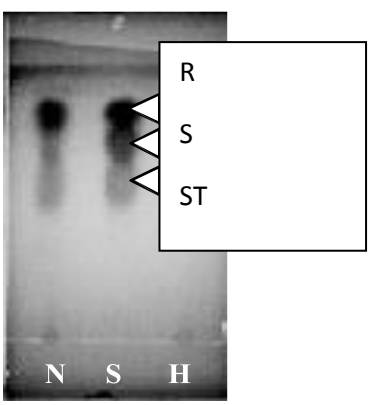
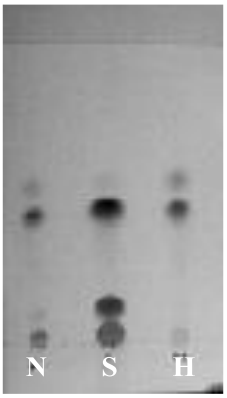


Figure 5

3. Place the plates on several thicknesses of paper towels to air-dry or use the hairdryer on high setting to dry. Leaning the plates upright is the best way to dry them. If dried horizontally, the coated side of the plates should face up. The plates are dry when the white color returns. Continue to dry/heat the plates until the dark spots appear. Be careful handling a hot plate.
4. Varying sizes and densities of black spots will appear in a lane that stretches above each labeled pencil dot on the plate. Using figure 6 as a guide, locate the spots that correspond to sucrose, raffinose, and stachyose in the "S" lane. Compare the spots that appear in the lanes above the "N" for normal soybeans and "H" for high sucrose soybeans to the positions of the spots for pure sucrose, raffinose, and stachyose that appeared in the "S" lane. Use a pencil to mark the spots with an "S" for sucrose, "R" for raffinose, and "ST" for stachyose.





70% isopropyl alcohol

No centrifuge

1/5 filtrate concentration

91% isopropyl alcohol

No centrifuge

1/5 filtrate concentration

Figure 6

5. Compare the size and blackness of the sucrose, raffinose, and stachyose spots that appear in the normal soybean lane with those that appear in the high sucrose soybean lane.

Questions:

1. What are the three carbohydrates that you are trying to identify in this lab activity?
2. How do these carbohydrates differ molecularly?
3. Explain why the three carbohydrates found in soybeans move up the plate at different rates.
4. Why is it important to test soybeans for the presence of the carbohydrates?
5. Why could it be important socially and commercially to have a soybean high in sucrose?

Practical 3
Title: Determination of protein concentration

Objective:
After completing the practical, you will be able:

1. Determine the presence of protein and its concentration in a sample

Introduction:

Bradford Protein Assay

In biochemistry, it is frequently necessary to know the total protein concentration in your solution. A number of methods have been developed for measuring total protein concentration. The method you will use is called the Bradford Assay, after the name of the scientist who developed the method. The method was first described in a paper written more than 30 years ago.

The Bradford reagent binds proteins; when it does so, its extinction coefficient at 595 nm increases. Because the magnitude of the extinction coefficient change is somewhat dependent on the conditions, it is necessary to calibrate the change in absorbance induced by different amounts of protein. This calibration procedure is called "generating a standard curve".

Ideally, the absorbance readings obtained in your standard curve should be linearly proportional to the protein concentration (which means that there are limits as to how much protein you can use; too much protein will give absorbance readings too high to be meaningful). Time constraints mean that you will probably be able to run each sample only once. In an actual experiment, you would run replicates for both the standard curve and the unknown samples, so as to be able to assess the uncertainty in your data.

Materials

- Bradford Reagent
- 1 mg/ml Bovine serum albumin (BSA)
- Cuvettes

Procedure:

1. **Standard Curve**: For this experiment, you will be using a stock solution of 1 mg/ml bovine serum albumin (BSA) as your standard protein. Note that 1 mg/ml = 1000 µg/ml. You will want to make up several dilutions of the BSA stock, and then use a constant volume from each protein dilution in the actual assay tubes.
2. **Make 25 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml, 150 µg/ml, and 200 µg/ml solutions of BSA.** Since you need 100 µl of the protein solution, it is probably best to make convenient dilutions with final volumes of 150 to 400 µl.

3. **Decide how to make up these dilutions *before* you come to class.** Mix 800 µl of H2O, 200 µl of the Bradford Coomassie Blue solution and 100 µl of the BSA standard dilution together, allow them to equilibrate for a few minutes, and measure the absorbance at 595 nm. In addition to BSA protein solutions, run one Bradford sample without protein (*i.e*. which contains the same assay mixture, using buffer instead of protein).

4. Plot of the absorbance at 595 nm *versus* protein concentration should be drawn. You will also need to dilute your LDH purification protein solutions. You will prefer to use small volumes of your precious protein solutions (you worked hard to prepare those protein solutions, and you will need them again for later experiments).

5. You will find that most, if not all of the samples you have will be too concentrated to run undiluted in the assay. If you dilute the initial sample 1:10 (*i.e*. 30 µl sample + 270 µl of H2O), and then prepare several 1:3 serial dilutions from this sample, eventually you will obtain samples that fall within the standard curve.

6. Run assays to determine the protein concentration on the following samples: Crude homogenate Desalted ammonium sulfate fraction Peak fraction(s) from the Cibacron Blue column Once you have measured the absorbance values for the standard curve and for your unknown protein samples, you will need to use the standard curve to determine the protein concentrations for your unknowns.

7. The best method is to plot the values obtained for the standard curve, determine the slope of the best-fit line, and then use the equation of the line to give you the protein concentrations for your unknown samples.

Questions:

1. Does the Bradford Assay allow you to calculate the LDH concentration? Why?
2. What wavelength do you use for the Bradford assay? Why is this different from the wavelength you used for the LDH assay?
3. You have 0.5 ml of a stock solution of 1 mg/ml BSA. You need at least 200 µl of the solutions listed below. How do you make them up?
   25 µg/ml, 50 µg/ml 75 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml
4. You set up a Bradford assay. Your protein sample gives an absorbance of about 2.5. What do you need to do?
5. You perform a Bradford assay. You obtain the absorbance values listed below from the BSA samples; your protein sample yields an absorbance of 1.3; what is the protein concentration of your sample?

BSA (µg/ml)	Absorbance
0	0.5
25	0.65
50	0.8
75	0.95
100	1.1
150	1.4
200	1.75

Practical 4
Title: Separation and identification of serum protein by PAGE

Objective:
After completing the practical, you will be able:

1. To learn method of PAGE to identify serum protein

Introduction:

Once a protein purification procedure has been completed, the resulting protein must be characterized for both purity and physical properties. The first technique is SDS polyacrylamide gel electrophoresis. SDS PAGE allows assessment of purity of the preparation, estimation of approximate quantity of the protein, and measurement of the size of the protein. Electrophoresis is a process in which molecules are exposed to an electric field and separated on the basis of their differential mobilities in that field. The differential mobility is a result of different charge on different molecules, and the result of different resistance to movement through the medium. For molecules with similar shapes, the mobility is proportional to the charge-to-mass ratio of the molecule. For molecules of similar shapes and similar charge-to-mass ratios, the motion through the medium will be proportional to the size of the molecule, because friction increases as a function of size.

The velocity of a charged molecule is given by:

$$v=qE/f$$

where *q* is the charge on the molecule, *E* is the electrical potential gradient, and *f* is the frictional coefficient of the medium for the molecule.

For similarly shaped molecules, *f* is proportional to size of the molecule. For molecules in which charge increases in proportion to size, larger molecules move more slowly than small ones, because *f* increases faster than charge. Gel electrophoresis uses a matrix of large uncharged molecules to provide the required friction. The matrix also serves to inhibit diffusion, and therefore to prevent degradation of the separation that is achieved. The separation of proteins usually involves the use of polyacrylamide as the matrix.

Polyacrylamide is formed by polymerization of acrylamide monomers in the presence of *N, N´*-methylenebis (acrylamide). The bis (acrylamide) contains two double bonds, which allow the compound to act as a cross-linker between polyacrylamide chains. The presence of the cross-linking agent results in formation of a gel matrix rather than a simple linear polymer.

In the synthesis of polyacrylamide, the polymerization occurs via a free radical chain reaction mechanism. The formation of the free radicals is initiated by the unstable compound ammonium persulfate. The sulfate radicals formed then react with *N,N,N´,N´*-tetramethyl ethylenediamine (TEMED), forming TEMED radicals, which then react with acrylamide molecules to begin the actual polymerization reaction. Varying the amount of acrylamide monomer and bis-acrylamide cross-linker present controls the formation of the matrix. The use of larger amounts of these components results in a denser matrix. Denser matrices are used for separating smaller proteins; larger proteins may find the pores in a dense matrix too small to enter, and may therefore not enter the gel at all.

Materials:

Chemicals required:

12% Resolving gel 1.5 ml 1.5 M Tris-HCl, pH 8.8 1.8 ml **40% acrylamide** 0.06 ml 10% SDS 2.94 ml water	**12% Resolving gel** 1.5 ml 1.5 M Tris-HCl, pH 8.8 2.4 ml **30% acrylamide** 0.06 ml 10% SDS 2.04 ml water

- Add **last** to initiate reaction:
- 30 µl 10 % ammonium persulfate and 5 µl TEMED
- Pour the gel and layer 1-butanol on top of the polymerizing solution. After polymerization is complete, rinse off the top of the gel to remove the butanol.

Stacking gel 0.875 ml 1.0 M Tris-HCl, pH 6.8 0.437 ml **40% acrylamide** 0.035 ml 10% SDS 2.153 ml water	**Stacking gel** 0.875 ml 1.0 M Tris-HCl, pH 6.8 0.583 ml **30% acrylamide** 0.035 ml 10% SDS 2.07 water

- Add **last** to initiate reaction:

30 µl 10 % ammonium persulfate and 5 µl TEMED

Pour the gel and insert the comb to create the wells.

- **5x Sample Buffer**

60 mM Tris-HCl, pH 6.8

25% glycerol

2% SDS

14.4 mM β-mercaptoethanol

1% bromophenol blue

- **Electrophoresis tank buffer**

25 mM Tris

192 mM glycine, pH 8.8

0.1% SDS

Coomassie Staining Solution (10% Acetic acid, 25% Methanol, 0.05% Coomassie R- 250 or Bio-Safe Coomassie staining solution)

- **Hardware required**:

Electrophoresis apparatus

Gel loading tips

Weigh boats for gel staining

100°C water bath

Microcentrifuge tubes

Procedures:

1. Set up the gel apparatus.
- Mark location of wells before adding tank buffer
- Remove bubbles from bottom of gel

2. Prepare your samples. You will want to run all protein samples that contain LDH activity. This should include:
- Crude homogenate
- Ammonium Sulfate Pellet/Desalting Column Elution
- Peak Cibacron Blue Elution Fraction(s)

3. The gel apparatuses you will be using have 10 wells, so you can plan on running 3 lanes for each of your samples. Each sample should contain 20 µl of diluted protein and 5 µl 5x sample buffer.
4. **Loading the same volume of each sample tends to allow the gel to run more evenly**. Prepare **2 µg, 10 µg, and 50 µg aliquots** for the crude homogenate and desalted ammonium sulfate samples; prepare **2 µg and 10 µg aliquots** for the peak fraction(s). (Note: do not prepare the 50 µg sample for your peak fraction(s); this would result in an overloaded lane.)
5. To calculate how much of each sample to use you must refer back to the protein concentration determined by the Bradford assay in Experiment 3.

6. If your protein concentration is too low to allow preparation of the more concentrated aliquots, make the most concentrated aliquots you can.

7. Add 5x sample buffer to each sample. The sample buffer should be added at 1/5 the final volume. Your samples contain 20 µl so you should add **5 µl sample buffer** (5µl is 1/5 of 25 µl). If your samples do not contain 20 µl of diluted protein, you must determine how much sample buffer to add.
Calculate how you are going to prepare all of your samples *before* coming to class!

8. Heat all samples at 100°C for 2 minutes. Spin down the protein solution for 5 seconds, so that all of the liquid is at the bottom of the microfuge tube.
9. Apply samples to wells. Record how the samples were loaded!
10. Attach the electrodes to gel apparatus, and turn on the power supply. Run the gel at constant current (15 mA per gel) until the tracking dye reaches the end of the gel.
11. **Be careful – the high current during gel electrophoresis is dangerous**!

12. Turn off the power supply and remove the electrodes. Remove the gel sandwich. Carefully separate the plates. Gently remove the gel from the plate and cut one lower corner at lane #1 to distinguish the two sides. Place it in a plastic tray containing stain solution..
13. Do not handle the gel with your bare hands: **acrylamide is a cumulative neurotoxic agent and is absorbed through the skin!** Residual unpolymerized acrylamide will be present in the gel. The gel will be stained overnight and transferred to the destaining solution before the next lab period. The results should be visible in the next lab period.

Results:

Minimum inhibitory concentration (MIC): _____

Questions:

1. Why is SDS added to the sample and the gel?
2. What are the two layers of a gel used in SDS-PAGE? What is the function of each layer?
3. What information can you obtain from SDS-PAGE?
4. Why are you running the SDS-PAGE today?
5. In SDS-PAGE, do the proteins migrate toward the anode (positive electrode) or cathode (negative electrode)? Why?

Practical 5
Title: Identification and quantification of the activity of amylase

Objective:
After completing the practical, you will be able:

1. To learn method of quantification of amylase

Introduction:

Starch is broken into monomer units by the enzyme amylase produced by the organism. However starch reacts with iodine and develops blue coloured complex. Moreover, intensity of the colour developed is directly proportional to the concentration of starch present in the sample.

Materials:

Growth Medium

- Yeast extract - 0.3g
- Malt extract - 0.3g
- Peptone - 0.5g
- Soluble starch - 1.0g
- Distilled water - 1000ml
- pH = 6.0

Reagents

- Iodine solution - 0.01N
- Starch solution - 0.1%

Procedures:

1. Prepare growth medium, dispense 200 ml medium in flask and autoclave it.
2. Prepare pure culture of *Aspergillus niger and prepare* fresh culture in tubes.
3. Transfer 10ml of inoculum in sterilized growth medium and incubate at 30ºC for 24 to 48 hours.
4. Filter the filterate through sterile Whatman filter paper no.42. Collect supernatant and measure amylase activity of filterate by starch iodine method.
5. Take different aliquots of starch solution ranging from 0-2ml and make initial volume to 16ml by adding distilled water. Blank will lack starch.
6. Add 4ml of 0.01N iodine solution. Measure O.D after 10min of incubation at 578nm.
7. Draw graph between O.D and starch concentration.

To estimate the activity of α- amylase

Principle: - α -amylase E.C. No. 3.2.1.1 i.e α endo α 1,4 glucan hydrolase. It occurs widely in bacteria and fungi. All α amylase are endo active enzymes which specially cleaves α 1,4 glycosidic linkages in amylose , amylopectin and glycogen yielding sugar in α configuration. They are unable to hydrolyse α 1-6 branch points in amylopectin but are able to bypass this branch point. Two types of microbial α amylase are existing i.e. saccharifying α amylase and liquefying α amylase. They are distinguished by the fact that saccharifying α amylase produce an increased quantity of reducing sugar about twice of liquefying α amylase.

Properties	**Alpha amylase**	**Beta amylase**	**Amyloglucosidase**
Molecular wt.	50,000	55,000 – 1,60,000	50,000 – 1,12,000
Optimum pH	3.5 -7	5.5 -7.5	4.0 -6.0
Optimum temperature	35° -90° C	37° -55°C	40° – 70°C
Metal ion req.	Ca++	-	--
Specificity	Alpha 1,4 linkages can bypass alpha 1,6 linkages	Alpha 1,4 linkages cannot bypass alpha 1,6 linkages	Alpha 1,4 and alpha 1,6 linkages
Product	Maltodextrin	Maltose and beta unit dextrin	Beta glucose, amylase and amylopectin
Substrate	Amylase, glycogen, amylopectin	Amylase, glycogen, amylopectin	glycogen, amylopectin,dextrin maltose.
Nature	Endosplitting	Exosplitting	Exosplitting

Assay: - α amylase are generally quantified by beta amylation of reducing group formed due to hydrolysis of soluble starch. The simplest method is dinitrosalicyclic acid method. Starch iodine colorimetric method is also used.

β amylase: - β amylase i.e. 1-4 β 1-4 glucanmalto hydrolase E.C. No. 3.2.1.2. It is a saccharifying amylase i.e. widely distributed in plants and also in microorganisms. β amylase are exosplitting enzymes which attacks amylase, amylopectin, glycogen at non reducing terminal resulting in formation of maltose in β configuration, β amylases is specific for, α 1-4 linkages and is unable to bypass α 1,6 branch point.

Assay: - Since the main product for β amylase hydrolysis are the disaccharides β maltose units, β unit dextran, the reducing sugar assay described for alpha amylase may be employed. Amyloglucosidase E.C. No. 3.2.1.3 also called glucoamylase. Amyloglucosidase are exosplitting amylopectinamylopecic enzyme which attack amylase, amylopectin and glycogen are specifically hydrolysed α 1, 4 and α 1, 6 linkages from non-reducing end.

However, α 1,6 glucosidic linkages are cleaved by less readily than 1,4 glucosidic linkages. The product of reacton is glucose.

Assay: - The major product of amylase action is glucose. The spectrometric assay for glucose may be employed.

Procedure: - Take 0.5 ml of α amylase enzyme in a test tube and 1 ml of buffer and 0.5 ml of starch. The glucose production in this tube is to be determined. Incubate these tubes for 10 min at 37 C then add 1 ml of DNSA reagent in it. Boil test tube for 10-20 min in water bath. A control tube is also performed in which DNSA is added before addition of the starch so, as to deactivate the enzyme. Arrange the test tubes as per protocol. After addition boil these test tubes for 10 – 15 min. Optical density of these tubes are to be read at 520 - 540 nm in colorimeter.

Result

OBSERVATION:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
OD(450nm)										

Result: - The activity of α amylase was found to be_____ µg/ml from the graph.

Practical 6
Title: Quantification of alkaline phosphatase

Objective:
After completing the practical, you will be able:

1. To learn method of quantification of alkaline phosphatase

Introduction:

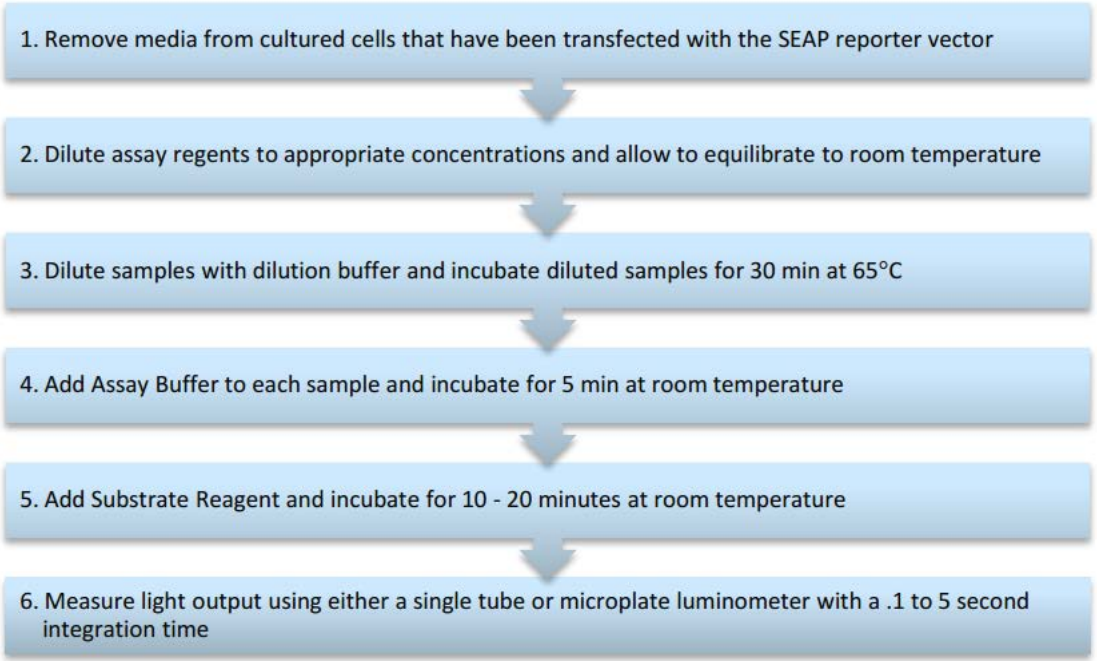
Alkaline phosphatase (ALP) is an enzyme found in several tissues throughout the body. The ALP in blood samples of healthy adults comes mainly from the liver, with most of the rest coming from bones (skeleton). Elevated levels of ALP in the blood are most commonly caused by liver disease, bile duct obstruction, gallbladder disease, or bone disorders. This test measures the level of ALP in the blood.

In the liver, ALP is found on the edges of cells that join to form bile ducts, tiny tubes that drain bile from the liver to the bowels, where it is needed to help digest fat in the diet. ALP in bone is produced by special cells called osteoblasts that are involved in the formation of bone. Each of the various tissue types produces distinct forms of ALP called isoenzymes.

ALP blood levels can be greatly increased, for example, in cases where one or more bile ducts are blocked. This can occur as a result of inflammation of the gallbladder (cholecystitis) or gallstones. Smaller increases of blood ALP are seen in liver cancer and cirrhosis, with use of drugs toxic to the liver, and in hepatitis.

Procedures:

Regardless of the assay kit used, the SEAP assay is fairly simple to run and consists of the following steps:



Once Substrate Reagent has been added to the samples, the light output reaches a stable level after 10 minutes and is stable for up to 1 hour.

1. The dilution buffer contains inhibitors that target non-placental alkaline phosphatase from both serum and endogenous alkaline phosphatase.
2. The combination of inhibitors and heat inactivation step eliminates non-placental alkaline phosphatase activity.
3. However, certain cell lines, such as HeLa and others derived from cervical cancers, may express placental alkaline phosphatase which may produce high assay background and are not recommended for use with SEAP systems.

Result

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