**BIO112 - LAB 1 THE METRIC SYSTEM**

**The goals of this lab are to:**

1. learn the standard units of measure in the metric system for length, mass, volume, and temperature
2. learn how these units can be scaled to create smaller or larger units of measure, e.g. meters to millimeters or meters to kilometers
3. gain a sense of the approximate size of the metric units and apply them in appropriate real world situations
4. learn how to convert between metric units
5. learn how to calculate area of and volume
6. learn how to measure liquid volumes with the appropriate glassware
7. learn to use an electronic balance
8. learn the relationship between water volume and water mass
9. know all the terms from this Lab Handout that are in **bold face** type.

**Materials:**

1. meter stick and metric ruler
2. graduated cylinders
3. flasks
4. beakers
5. transfer pipettes & dropper bottles
6. pipettes and pi-pump
7. burette
8. thermometers in ice and boiling water

**Procedure:**

1. Introduction
2. Work as a group to read the handout and complete the procedures
3. Completer all of the practice problems for doing the metric conversions. You must be able to do them easily. Answers will be posted on Dr. Rohrer's website.

**Readings:**

Sadava, 8th ed., p. 70

## INTRODUCTION

All science is based on observation. Observations can be direct or indirect. Observations can refer to any of the senses. Science only studies things that are observable.

Observations can be grouped into two categories.
Types of data:

1. qualitative data - observations fall into discrete categories
   e.g., male and female, one merely places the things in each category
2. quantitative data – the variable that is being measured varies continuously, e.g., height, weight, a measurement system is needed

## METRIC SYSTEM

The sciences use a measurement system named the metric system. The metric system employs standard units for measuring length, mass, volume, electricity, and light. These standard units are based on physical constants so that they are available to anyone who has access to simple lab equipment.

| | **STANDARD UNITS** | **ABBREVIATION** |
|---|---|---|
| Length | meter | m |
| Mass | gram | g |
| Volume | liter | l |
| Temperature | degrees Celsius | $^{o}C$ |

A standard unit may be either too large or too small to easily make a particular measurement. For example, a meter would be appropriate for measuring the length of a room, but it would be awkward for measuring the length of a screw or nail. The metric system handles this problem by "scaling" the standard units to sizes that are appropriate to a particular task. The units are "scaled" by powers of ten. The name of the "scaled" units is made by placing a prefix in front of the unit name that indicates how much bigger or smaller it is. For example, the prefix milli- means one thousandth. Thus, a millimeter is $1/1000^{th}$ meter. The millimeter would be the unit appropriate for measuring the diameter of a coin. The most commonly used prefixes are listed below.

| **Prefixes** | **Abbreviation** | **How big is this unit compared to the Standard Unit?** | | **How many of this unit per Standard Unit?** |
|---|---|---|---|---|
| **kilo-** | k | 1,000. | $= 10^{3}$ | 0.001 |
| **-----** | -- | 1 | $= 10^{0}$ | 1. |
| deci- | d | 1/10 | $= 10^{-1}$ | 10. |
| centi- | c | 1/100 | $= 10^{-2}$ | 100. |
| **milli-** | m | 1/1000 | $= 10^{-3}$ | 1,000. |
| **micro-** | μ | 1/1,000,000 | $= 10^{-6}$ | 1,000,000. |
| **nano-** | n | 1/1,000,000,000 | $= 10^{-9}$ | 1,000,000,000. |

One of the advantages of the metric system is that conversions between the various units are easy since they only differ by powers of ten. All that you have to do is move the decimal point to make the conversion.

Examine the chart above to see how many places you move the decimal point.

-If you are converting from a small unit to a bigger unit, you move the decimal place to the left. For example, if you have a string that is 50 millimeters long, you can convert this to meters by moving the decimal point 3 places to the left. It is 0.050 meters long.

-If you are converting from a large unit to a smaller unit, you move the decimal place to the right. For example, if you want to express the length of a 50 millimeter string in micrometers, you would move the decimal point 3 places to the right. It is 50,000 μm long.

This lab will only introduce the units of the metric system that we commonly use. The following list shows how the units relate to each other. You should become familiar with them and recognize which units are appropriate for common measurements. You should also be able to make conversions between the units.

**Units of length.** The meter is the standard unit of length.

kilometer (km) = 1000 m
meter (m) = 1/1000 km, 100 cm, 1000 mm, 1,000,000 μm, 1,000,000,000 nm.
centimeter (cm) = 1/100 m or 10 mm
millimeter (mm) = 1/10 cm or 1000 μm
micrometer (μm) = 1/1000 mm or 1000 nm
nanometer (nm) = 1/1000 μm or 10 Angstrom
angstrom (Å)= 1/10 nm The **Angstrom** (= $10^{-10}$ m) is no longer used in biology

What units of length would be appropriate for measuring

-your desk _____ -cell parts _____ -your jewelry _____
-body parts _____ -a building _____ -distances for travel _____

**Units of volume.** The liter is the standard unit of liquid volume.

liter (l) = 1000 ml.
milliliter (ml) = 1/1000 l

What units of volume would be appropriate for measuring

-gasoline sales _____ -drinking water _____ -daily urine production _____

**Units of mass (weight).** The gram is the standard unit of mass.

kilogram (kg) = 1000 g
gram (g) = 1000 mg
milligram (mg) = 1/1000 g

What units of mass would be appropriate for measuring

-human body weight _____ -weight of a coin _____ -medicine _____

**Temperature.** Degrees Celsius is the standard unit.
The Celsius scale divides the temperature range between the freezing point of water and the boiling point of water into 100 divisions = degrees.

**Derived Measures** are calculated from standard measurements. Examples include area and volume.

Area

The area of a rectangle = length x width

Example: A room is 5 meters by 4 meters. Determine the area of the room

Area = 5m x 4m = $20m^2$

*Note:* Make sure that the Units are the same *before* you calculate the area.

Volume

The area of a solid = height x area

Example: A box is 5 cm by 40 mm by 0.1 m. Determine the volume of the box.

1. Convert units to cm:
   40mm x 1cm/10mm = 4 cm
   0.1m x 100cm/1m = 10 cm
2. calculate Volume = 5cm x 4cm x 10cm = 200 $cm^3$

**The Measurement of Liquid Volume**

In this lab you will frequently need to measure specific volumes of liquid reagents. Many types of glassware can be used to measure liquid volume. These various types of glassware vary as to their accuracy. Which type you choose to use depends on the degree of accuracy required by a particular experiment. Some of the most common types of glassware used to measure liquid volume are described below. Examples of each will be on the back lab bench.

Low accuracy:

1. **dropper** - by counting drops, small volumes can be delivered. Twenty drops are approximately equal to 1ml. However, each dropper is different and should be calibrated using a small graduated cylinder to determine how many drops actually equal 1ml.
2. **dropper bottles** - at times, liquid reagents will be on your lab tables in dropper bottles (small bottles with a dropper as part of the cap). Always use the dropper to dispense these reagents. Never pour or pipette from these bottles.
3. **graduated flasks or beakers** - these containers are mainly used to hold a volume of liquid. They should only be used to measure an approximate volume.

Medium accuracy:

1. **graduated cylinder** - this is the type of glassware which we will usually use to measure liquid volume. Cylinders come in a range of sizes, e.g. 10, 25, 50, 100, 200, 1000 ml. You should use the size which is close to the volume that you are measuring. Make your reading at the bottom of the meniscus = the curved water surface

High accuracy:

1. **pipette** (pipet) – generally used for volumes less than 10 mls
   a. blowout pipette - use your pipetting bulb to blow all the liquid from the pipette
   b. most pipettes have ”TD” written on them. “TD” means “to deliver” and you do not blow these out. A small amount of liquid remains in the pipette, but the pipette has already delivered the correct volume of liquid.
   c. **volumetric pipette**- the greatest accuracy with pipettes.
2. **burette (buret)** - useful for larger volumes

**STUDENT WORK**

Learn to recognize the types of lab glassware and their uses. Examples of the glassware are located on the back lab bench.

A. CALIBRATE THE DROPPER - take a dropper and the 10ml graduated cylinder
   a. Count the number of drops of water needed to fill the graduated cylinder to the 1ml line.
   b. Repeat the process two more times.
   c. What is the average number of drops which equal 1ml? _____

B. LEARN TO USE THE ELECTRONIC BALANCE.
   1. Use the "Mode" button to set the balance so that it gives you a reading in grams.
   2. Place a glass beaker on the balance. Record the weight. _____ grams.
   3. Push the "Rezero" button. What happened? The balance will now subtract this amount of weight from each subsequent weighing, i.e., the weight of the container is subtracted from the total weight. Thus, you only get the weight of the contents. The weight of the container that is subtracted is called the **tare** weight.
   4. Place a small object into the beaker and determine its weight. _____ grams

C. DETERMINE THE RELATIONSHIP BETWEEN WATER VOLUME AND MASS.
   There are small glass vials on the table.
   1. Use a centimeter ruler to measure the vial's internal diameter.
   2. Weigh the empty vial. Push the Rezero button so that the vial's weight is subtracted.
   3. Fill the vial ¾ full with water.
   4. Use the balance to find the weight of the water in grams. _____ g
   5. Use a centimeter ruler to measure the height of the water in the vial.
   6. Calculate the volume of the water in the vial.
      The volume of a cylinder = $\pi r^2 h$ (r=radius = half of diameter)
      V = (3.14)(r in cm)(r in cm)(h in cm) = _____ $cm^3$ or _____ cc (=cubic centimeters)
      Note: the unit $cm^3$ is spoken as "cubic centimeters" and it is abbreviated as "cc"
   7. Pour the water from the vial into the 100ml graduated cylinder. Measure the volume of water in milliliters. _____ ml

   ⇒ Compare the volume of the vial in cc to the volume of the vial in ml? What is the relationship between the cc and the ml (is it approximately 1:1 or 10:1 or 1:10 or something else?). _____

   ⇒ Compare the weight of water in grams to the volume of the vial in ml. What do you think the definition of a gram would be? _____

   Hint: By definition, 1 liter = volume of 1 kg of water at $4^0$C

D. LEARN TO USE A PIPETTE
   Always use a pi-pump or other pipetting device for pulling liquid into the pipette and for ejecting the liquid from the pipette. There are many types of devices.
   1. Weigh the empty vial. Push the Re-zero button so that the vial's weight is subtracted.
   2. Use the pi-pump to pipette exactly 10 ml of deionized water into the vial.
   3. Measure the weight of the deionized water. Record the weight _____
   4. Repeat the procedure using 10 ml of Salt water (10% NaCl). Record the weight _____

   ⇒ Is there a difference in weight between 10ml of deionized water and 10ml of salt water? _____

E. CALIBRATE A THERMOMETER

Scientists commonly have to calibrate their measuring devices. The device can measure some feature such as length or light intensity, but the readout from the device does not use standard units. Calibration is the process of relating the readings to standard units. For example, you can measure the length of the lab bench using your shoe as the measuring device, but you would have to compare the length of your shoe to a meter stick in order to convert the length measured in "shoes" into standard units.

Let's calibrate a thermometer so that you can see one way that this can be done. You can easily calibrate a thermometer because the Celsius scale is based upon dividing the temperature range between the boiling point of water and the freezing point of water into 100 equal divisions. Suppose that you are given a thermometer with evenly spaced marks, but numbered from -10 to 130. You can calibrate it by doing the following. Place the un-calibrated thermometer into ice water and record the value on the thermometer. Let's suppose that the value is 3. You know, however, that this reading corresponds to $0^0$C. Next move the un-calibrated thermometer into boiling water and record the value. Let's suppose that you get a value of 108. You know that this reading corresponds to $100^0$C. Next you have to find the relationship between the values on your un-calibrated thermometer and the standard thermometer. You can do this using algebra or a graph. We will use a graph. Draw a graph with the X axis marked into 100 divisions and the Y axis divided into 110 divisions. The X axis represents the standard thermometer and the Y axis represents the readings from <u>your</u> thermometer. Draw horizontal lines from the points on the Y axis that represent the freezing and boiling points. Draw vertical lines from $0^0$C and $100^0$C on the X axis to intersect the horizontal lines. Draw a straight line through the intersecting points. This line shows the relationship between values on your thermometer and the "true" temperature. All readings from your thermometer can then be converted into standard units by using the graph.

See the thermometer display on the side bench.

**SAMPLE PROBLEMS**

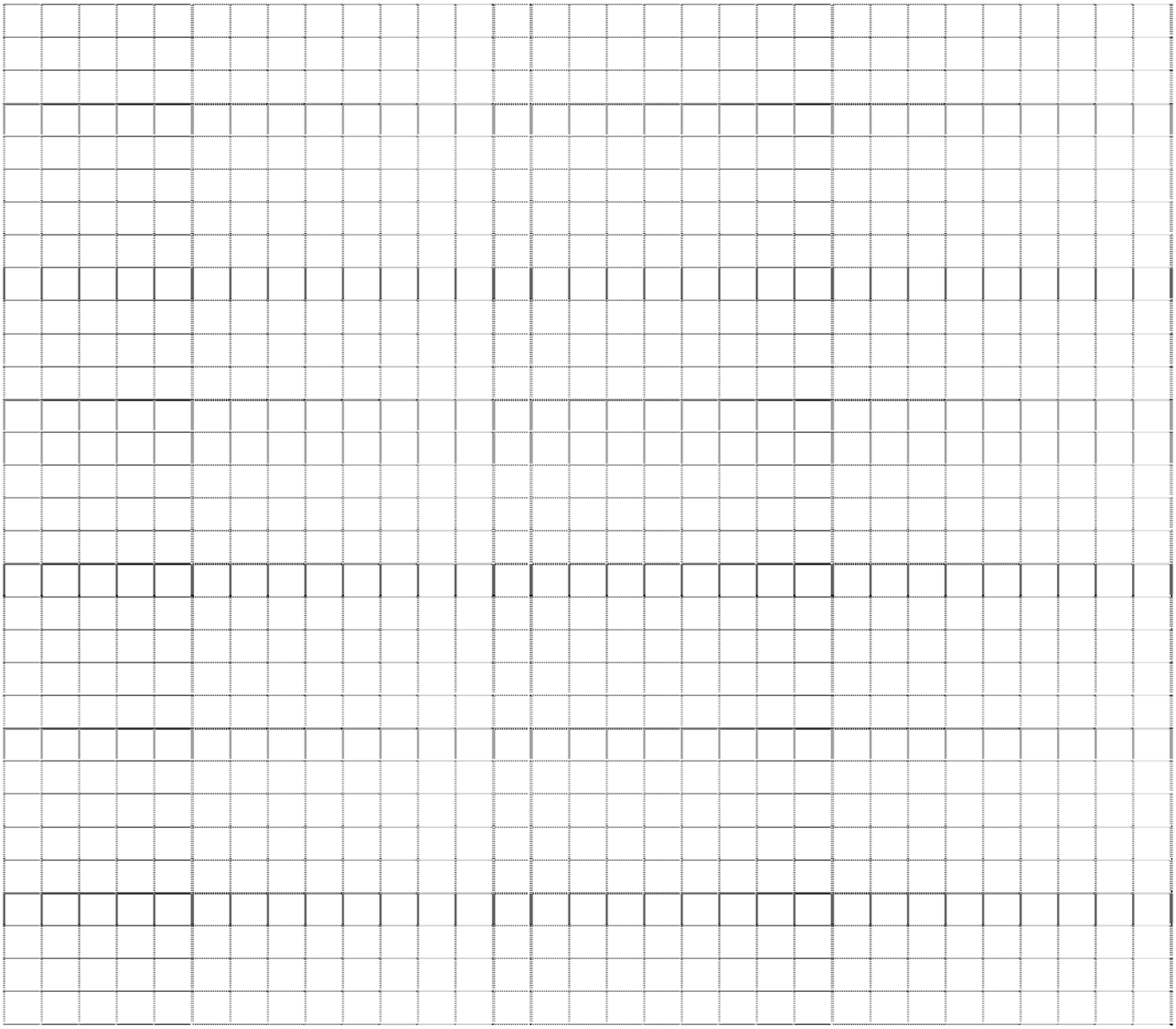
Make the following metric system conversions:

1. Convert 2.3 m to _____ cm.
2. Convert 69 mm to _____ cm.
3. Convert 8.1 cm to _____ mm.
4. Convert 2.1 mm to _____ μm.
5. Convert 3.4 μm to _____ nm
6. Convert 9 nm to _____ Å.
7. Convert 43 nm to _____ μm.
8. Convert 264 μm to _____ mm.
9. Convert 53 ml to _____ l.
10. Convert 1.8 l to _____ ml.
11. Convert 744 g to _____ kg.
12. Convert 1.8 kg to _____ g
13. Convert 50 g to _____ mg.
14. Convert 890 mg to _____ g
15. Assume that a cell is a cube that is 20 μm across.
    a. How many cells would have to be lined up side-by-side to span a distance of 1 mm? _____ cells
    b. What is the cell's volume? _____ $\mu m^3$
    c. Tough question: How many cells of this size would be found in 1 cubic centimeter of tissue? _____ cells

18. Growth of a cell results in a change in the ratio of surface area to volume. This limits the potential size of a cell. Complete the following table to demonstrate the changing surface to volume ratio. Assume that the cell shape is a cube. How many sides does the cell have?

| Cell Length (μm) | Surface Area ($\mu m^2$) | Cell Volume ($\mu m^3$) | Surface Area to Cell Volume Ratio =Surface Area/Cell Volume |
|---|---|---|---|
| 5 | | | |
| 10 | | | |
| 20 | | | |
| 40 | | | |
| 80 | | | |

Graph the Surface Area to Cell Volume Ratio (y-axis) as a function of Cell Length (x-axis)



**BIO112 - LAB 2 MICROSCOPY**

**The goals of this lab are:**

1. to learn the proper steps in the operation of a compound light microscope
2. to become comfortable with the operation of the compound microscope
3. to learn how to measure objects with the microscope
4. to learn how to calibrate and use an ocular micrometer
5. to learn the parts of the microscope & terminology used in microscopy
6. to learn the steps in the preparation of fixed slides and how to make a wet mount
7. to learn how to use a dissecting microscope
8. to become acquainted with other types of light microscopy
9. to become acquainted with transmission and scanning electron microscopy
10. to know all the terms in this handout that are in **bold face** type

**Materials:**

1. Prepared slides: Letter e; crossed threads; Tilia; squamous epithelium; frog blood
2. Samples for wet mounts: pond water; cultures of ciliates
3. Insects for viewing with dissecting scopes
4. Ocular micrometers and stage micrometers
5. Electron microscopy film

**Procedure:**

1. We will work together as a class to learn how to use the microscope and to gain an understanding of the following:
   a. Magnification
   b. Resolution
   c. Field of View
   d. Depth of Field (Focus)
   e. Working Distance
   f. Optical Sections
   g. measurement of field diameter & objects
   h. calibration of the ocular micrometer
2. You will practice using the fixed slides
3. You will make wet mounts.
4. You will practice using the dissecting microscopes.
5. A microscope with both Dark Field and Phase Contrast capabilities will be set up so that you can see specimens using these methods.
6. You will watch a movie on electron microscopy.
7. Be sure to study the Microscopy notes. We won't discuss all of this in lab. You don't need to worry about memorizing details of the material in the section "OTHER TYPES OF LIGHT MICROSCOPY".

**Readings: Sadava 8th ed,, pp. 70-72**

**INTRODUCTION**

The microscope we use in this room is a light microscope. It uses a light bulb to illuminate the microscope slide. It is also called a compound microscope because two lens systems are used to produce the image that your eye sees.

The compound microscope is one of the principal tools of the biological laboratory. As a biology student you must learn proper use of the microscope. Proper use of the instrument demands practice. A little extra time now will pay off later when you are asked to do much more difficult microscopy.

A microscope is really only a sophisticated arrangement of magnifying lenses, constructed to see small objects. The compound microscope consists of a light source, three glass lens systems, plus the human eye. The lenses focus light. The important parts of the microscope are:

1. **light source**
2. **condensing lens system** to collect and focus light from the source onto the specimen
3. **objective lens system** to form and magnify the image of the specimen
4. **ocular lens** to enlarge the image made by the objective lens and to project this image onto the retina of the eye or to photographic film. The ocular produces no new detail. The detail you see is produced by the objective lens.

Use the wall chart to learn these parts of a compound microscope and their function:

1. **ocular lens**
2. **objective lenses**
3. **rotating nosepiece**
4. **base**
5. **arm**
6. **tube**
7. **stage**
8. **condenser**
9. **iris diaphragm**
10. **coarse focus knob**
11. **fine focus knob**

**Resolution** (or **resolving power**) is the ability to see two objects that are close together as two separate objects rather than one blurred object. The human eye, unaided by optical devices, can resolve about 0.2 mm. By means of the light microscope, objects as small as 0.2 micrometers or 200 nanometers can be seen and resolved. This represents a 1000X improvement in resolution beyond that of the naked eye. The compound microscope is limited in its ability to produce "useful" magnification. At high magnification, the image is greatly enlarged but details are blurry ("empty magnification").

The resolving power of an optical system is limited by the wavelength of the light used to illuminate the specimen and the numerical aperture of the objective lens. The shorter the wavelength of light that is used, the better the resolution will be. The **numerical aperture** (N.A.) of an objective lens is calculated from certain physical properties of the lens and the angles at which light enters and leaves the lens. The N.A. is engraved on the side of the objective lens. N.A. can be used to mathematically calculate the resolution of each objective lens. The larger the N.A., the greater the resolving power. Thus, the oil immersion lens (see table below) has the greatest resolving power.

| Common Name | Magnification | Working Distance | Numerical Aperture |
|---|---|---|---|
| scanner (scanning lens) | 2.5-4X | 25-55 mm | about 0.10 |
| low power | 10X | 5-10 mm | about 0.25 |
| high power | 40-50X | 0.15-0.80 mm | 0.65-1.00 |
| oil immersion | 90-100X | 0.15-0.15 mm | about 1.25 |

## RULES FOR HANDLING THE MICROSCOPE

**A compound microscope is a delicate and expensive precision instrument. Treat it with care!**

1. Always carry the microscope upright with two hands: one hand on the arm and one hand under the base.
2. Only use lens paper to clean the lenses. Any other type of paper may scratch the glass lenses, produce lint, or transfer oil from your fingers to the lens. Never touch the lenses with your fingers. You can distinguish between dirt on the ocular and dirt on the objective by rotating the ocular while looking through the microscope. If the dirt is on the ocular, the dirt will rotate with the lens.

3. Never touch the lens to water. Always use a **cover slip** when making a wet mount. Cover slips are very thin squares or circles made from glass or plastic.

4. When you are finished with the microscope, put the 4X objective in place and raise the stage as high as it will go. Wrap the electrical cord loosely around your hand and drape the coil over the tube of the microscope. Do not wrap the cord around the base because this will damage the condenser lens, diaphragm, and the cord. Put the dust cover back on. Return the scope to cabinet.

## PROCEDURE FOR LOOKING AT A SLIDE

Always use this procedure when you look at a new microscope slide:

1. Begin with the 4X objective lens in place.
2. Put the slide in the holder on the stage. Use the micro-positioner to center the slide over the opening in the stage.
3. Use the coarse focus knob to move the stage as close to the lens as it will go (a brake will prevent the slide from actually hitting the lens)
4. Use the coarse focus knob to focus on the object.
   The lens will be moving away from the stage so there is no danger of hitting the slide.
5. Adjust the light using the iris diaphragm and condenser lens. Move the slide so that the specific object that you want to see is centered.
6. Slowly turn the 10X objective lens into place.
7. Carefully focus using the fine focus knob.*
8. Adjust the light using the iris diaphragm and condenser lens. Move the slide so that the specific object that you want to see is centered.
9. Slowly turn the 40X objective lens into place**.
10. Use the fine focus knob to focus*. Never use the coarse focus knob with the 40X objective.
11. Adjust the light using the iris diaphragm and condenser lens. Move the slide so that the specific object that you want to see is centered.
12. Do NOT use the 100X objective.

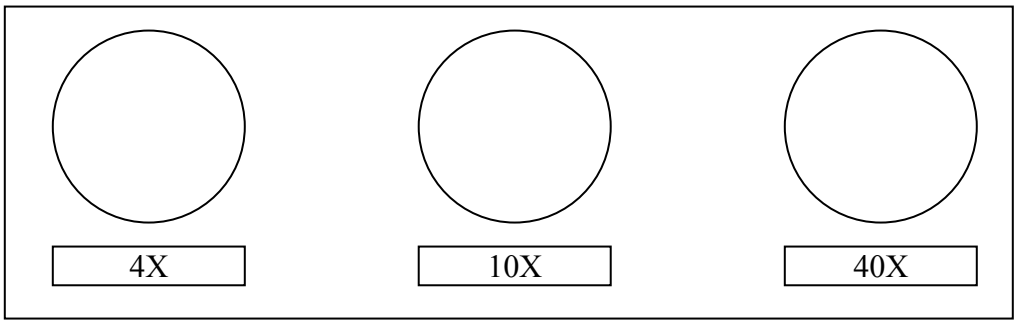
*Note: This microscope is **parfocal**. Parfocal means that when the image is in focus with one objective lens, it should be in focus (or close to it) at the next higher magnification. You should only need the fine focus knob to bring it into perfect focus.

**Note: We sometimes use or make slides that have a thick specimen mounted on them. This may prevent you from using a higher power objective. You should watch from the side as you move the 40X objective into place to avoid hitting the lens on the specimen. This may damage the lens.

Generally, you will need the 10X objective when viewing a tissue and the 40X objective when viewing individual cells. As you become more experienced, you will learn which magnification is most appropriate for the tissue that you are examining.

**STUDENT MICROSCOPE WORK:**

1. Take the letter "e" slide from the slide folder.
2. Hold the slide upright (so that you can read the normal "e" that is beneath the coverslip) and mount it on the stage so that the "e" is upright when viewed with the naked eye.
3. Focus on the "e" with the 4X objective. Raise the condenser lens to its highest position. Adjust the lighting with the iris diaphragm.
4. Draw the image that you see in the appropriate space below.
5. Compare the image of the "e" to the "e" on the slide. _____
6. Move the slide to the right. Which way does the image move? _____
7. Move the slide to the left. Which way does the image move? _____
8. Move the slide away from you. Which way does the image move? _____
9. Move the slide toward you. Which way does the image move? _____
10. Re-center the "e" in the field. Focus. Adjust the light.
11. Turn the 10X objective lens into position.
12. Focus carefully. Adjust the light. Draw the image that you see.
13. Re-center the "e" in the field.
14. Turn the 40X objective lens into position.
15. Focus with the fine focus knob. Adjust the light. Draw the image that you see.




1. How much of the "e" can be seen when using the 4X, 10X, and 40X objectives?

2. How is the image of the "e" different from the actual "e" mounted on the slide? List three ways that the image differs from the object on the slide. Base your answer on your observations.

**Use the following instructions to complete the table below:**

1. **Total magnification** is calculated by multiplying the magnification of the objective lens by the magnification of the ocular. Why?

   A microscope requires two sets of magnifiers. On low power (10X), for example, the objective forms a primary image inside the tube that is 10X larger than the viewed object. The ocular (10X) then magnifies the primary image another 10 times. Thus, the image that finally reaches your eye has been enlarged to 100X the size of the object.

| Objective Magnification | Total Magnification | Diameter of Field (mm) | Working Distance (mm) | Depth of Field (Number of threads in focus) |
|---|---|---|---|---|
| 4X | | | | |
| 10X | | | | |
| 40X | | | | |
| How does this feature change as you go from 4X to 40X? | | | | |

2. The **field** is the circle of light that you see through the ocular. It represents the portion of the microscope slide that you can see at one moment. You can measure the diameter of this field.

⇨ Lay a clear plastic ruler on the stage and measure the **diameter of field** with a total magnification of 40X and 100X. Record the values in the table to the nearest 0.1 mm.
*Tip: When you make this measurement, you must take the thickness of the ruler markings into account. The distance from the left edge of one mark to the left edge of the next mark is 1 mm.*

Knowing the diameter of field makes it possible for you to estimate the size of objects you are viewing. You can also use an **Ocular Micrometer** to make very accurate measurements of the size of objects. This process is described later in this lab.

3. **Working distance** is the distance from the end of the objective lens to the slide when it is in focus. An object must be at this specific distance from the objective lens in order for its image to be in focus.

⇨ Estimate the working distance for each of the objective lenses by standing a ruler vertically on the stage and looking at the ruler from the side. You will not be able to get an accurate measurement, but do your best. Record your results in the table.

4. **Depth of field** refers to the thickness of the specimen that is in focus at any one time. It is a constant value for each of the objectives lens. Each objective lens has a different depth of field.
   a. Obtain the "crossed threads" slide. Using the 4X objective focus at the point where the three threads cross over each other.
      ⇨ How many threads are in focus at any one instant? Record in the table.
   b. Turn the 10X objective into place. Slowly focus up and down.
      ⇨ How many threads are in focus at any one instant? Record in the table.
      Notice that when one point or thread is in focus, the others above and below are blurred.
   c. Increase the magnification by changing to the 40 X objective.
      ⇨ How many threads are in focus at any one instant? Record in the table.
      Can you distinguish one whole thread clearly under high power?
      ⇨ Can you determine the order of the threads on the slide? Focus up and down keeping track of the order in which threads come into focus.
      Notice that if you focus on the bottom thread, the top thread cannot be seen.
   d. Look back at the data that you recorded in the table.
      ⇨ As you go from 4X to 10X to 40X, how does depth of field change?

- ⇨ As you increase magnification, how does this pattern of change compare to what you found for changes in field diameter and working distance?
- ⇨ Based on your observations, at which magnification (4Xor 40X) will the depth of field and the field diameter be largest? Which lens should you use to locate an object on the slide?

**Optical Sections**:
Although the small depth of field at high magnifications makes it difficult to locate objects on the slide, microscopists can use this to their advantage. They can produce "optical sections" of thick slices of tissue. The microscopist can focus on the top of the object and draw a picture of all that is in focus. This is the first "optical section". Then S/he focuses downward below the first section and draws the next region that comes into focus. This is the second "optical section". The process is repeated until the bottom of the object is reached. The optical sections can be used to construct a 3-dimensional model of the object.

**Three tips for seeing an object through the microscope:**

1. You should adjust the amount of light entering your specimen in order to form the best image possible. You will probably need to increase the amount of light at higher magnification. If the image appears "washed out", you will have to reduce the amount of light reaching the specimen.

   Light plays an extremely important role in the operation of a compound microscope. Light is reflected upward through the opening in the stage, passes through the specimen on the slide, and then into the body tube of the scope, ultimately forming an image on the retina of the user's eye. The quality of the light determines the quality of the image so it is important to learn how to adjust the light. The microscope has two mechanisms for this purpose: the iris diaphragm controls the amount of light entering the microscope and the condenser focuses the light.

2. As you learned when studying diameter of field, the diameter of field decreases as you go from scanner to high power. To avoid "losing" the object when you change objective lenses always center the object before changing objective lenses.

3. You have learned that depth of field decreases as you go from low power to high power. As a result, an object that is in focus at low power may not be in focus at high power and you will not be able to see it. To avoid this, you must carefully focus before changing objective lenses to the higher power.

*If you lose the object as you increase magnification, it may be the result of improper lighting; or you did not center it in the field; or you did not focus carefully. How do you find the object again? Change back to a lower power, then adjust the light, re-center the object, and focus carefully. This adjustment should allow you to find it with the higher power lens.*

**The Ocular Micrometer**

An ocular micrometer is a piece of glass that has marks etched into it at uniform intervals. The ocular micrometer is inserted into the tube that holds the ocular lens. When you look through the ocular, you see the etched marks super-imposed on the field of view. The ocular micrometer's image is always the same size regardless of which objective lens is in place. You have seen that the diameter of the field of view changes when you change the objective lens, thus, the space between the marks on the ocular micrometer covers a large distance when using a low power objective lens but a small distance when using a high power objective lens. You must calibrate the ocular micrometer for each objective lens. **Calibration** means that you will determine the relationship between the marks on the ocular and the actual distance on the slide. This calibration procedure involves aligning the marks on the ocular micrometer with a ruler that is placed on the stage. This ruler, called a **Stage Micrometer**, is another piece of glass with etched marks at precise intervals. The distance between these marks is known.

The Stage Micrometer is mounted on a microscope slide. Stage micrometers come in various sizes with marks arranged in a variety of ways. Some marks may be numbered or some may be longer than others. You have to read the label on the slide to determine how it is marked.

> *For our description, assume that the micrometer is 2 mm long and divided into 200 spaces with marks at 10 μm intervals. Your stage micrometer may be different from this example.*

Calibration Procedure

1. Place the stage micrometer on the empty stage of your microscope.
2. Focus on the stage using the 4X objective lens.
3. Move the slide so that the left mark on the ocular micrometer is aligned with the left mark on the stage micrometer. You may have to rotate the ocular in order to do this.
4. Moving your eyes rightward from the two aligned marks, find the first 2 lines that are in alignment. The distance from the left mark to these 2 marks is the same on both micrometers.
5. Now you need to compare the number of intervals on the two micrometers.
   a. Count the number of spaces between the 2 sets of aligned marks on the Ocular Micrometer. Record it here _____
   b. Count the number of spaces between the 2 sets of aligned marks on the Stage Micrometer. Record it here _____
6. You can calculate the distance represented by each space on the Ocular Micrometer. For *this example we said that* each space on the Stage Micrometer = 10 μm *(Note, yours may be different)*. Suppose, that there were 10 spaces on the Stage Micrometer; then that would represent 100 μm (10 spaces x 10 μm/space). If the Ocular Micrometer had only 5 spaces covering the same 100 μm, then each space on the Ocular Micrometer represents 20 μm/space (100 μm / 5 spaces).
7. You can repeat this procedure for the 10X objective.
   Do NOT use the procedure with the 40X objective because the Stage Micrometer slide is too thick. You can calculate the distance represented by each space on your Ocular Micrometer. Hint: The image made with the 40X objective is 4 times bigger than the image made by the 10X objective.

⇨ Record your calibration results here. You will need this for later labs.

**4X Objective:**

**10X Objective:**

**40X Objective:**

**Oil Immersion Lens**

The 100X lens is an oil immersion lens. The working distance of the oil immersion objective is less than one millimeter. This lens is designed to be used with a drop of oil between the lens and the slide. The oil affects the passage of light from the specimen to the lens. When light travels out of glass into air and back into glass its path is bent (refracted). The amount of refraction is reduced when the oil refills the air space. This reduces the loss of light and increases the resolution of the system. The field diameter with oil immersion lens is much smaller than the field diameter with the 40X objective lens; the depth of field with oil immersion is also very small.

**Prepared Slides**

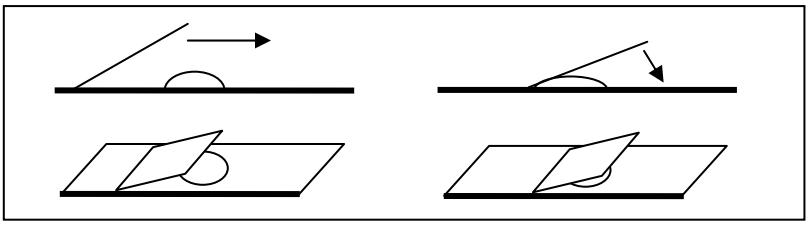
In class you will usually look at slides prepared by a manufacturer. These are the steps needed to produce a prepared slide.

1. **fixing** - cells or tissues are killed and preserved with chemicals such as formaldehyde to prevent self-digestion (**autolysis**) by cellular enzymes and decomposition.
2. **embedding** - the tissue is surrounded by a hard material such as paraffin wax. This makes soft tissue firm enough to be cut.
3. **sectioning** - the tissue is cut into slices that are thin enough to allow light from the microscope to pass through the tissue. These slices are called **sections** and are prepared on a special cutting instrument called a **microtome**. The slides you will see have been sectioned in one of two planes (directions):
   a) **longitudinal section (l.s.)** - cut parallel to the long axis (along the length) of the object
   b) **cross sections (c.s. or x.s.)** - cut at right angles to the longitudinal axis (along the width)of the object.

   Be aware of the plane that sectioned each slide so that you can visualize the real, living material. The type of section is listed on the slide label.
4. **staining** – cell are often gray and transparent. It is difficult to see details of the cell. By staining cells with dyes that color only certain structures (e.g., only the nucleus or the cytoplasm), there will be contrast between different cellular regions. These chemicals are called **stains**. A large number of stains are available. Some color all parts of cells more or less indiscriminately, while others act more specifically on particular structures or chemical compounds within the cell. Some stains, called **vital stains**, can be used with living materials. You will examine cell structures during other labs and will make use of one of these vital stains, e.g. **Janus Green**.

**Wet Mounts**
Wet Mounts are slides made from a fresh specimen. They are temporary slides that you can make yourself. The specimen must be thin so that light can pass through it.

Procedure for making a wet mount:
1. Place your specimen in the center of a slide.
2. Add a drop of water or saline (saline is 0.9% NaCl solution) onto the specimen.
3. Cover the specimen with a small plastic **cover slip** to prevent water from touching the objective lens. Always use a cover slip when liquid is on the slide. Use the following procedure to avoid trapping air bubbles beneath the cover slip. Holding the cover slip at a $30^0$ angle to the slide, touch one edge to the left end of the slide. Drag the cover slip to the right until it contacts the drop of water. The water will flow beneath the cover slip. Then slowly lower the cover slip and release it.



**STEREOSCOPIC MICROSCOPE**
The other microscope you will use in this course is the stereoscopic dissecting microscope.
The microscope has two oculars (binocular), one for each eye. The distance between the oculars can be adjusted to the size of your head. Most dissecting microscopes have a double objective system arranged in such a way as to produce a stereoscopic (three-dimensional) image.

Light passing through an object and then into the microscope is **transmitted** light. In contrast, light going from the illuminator directly to the object and then reflected by the object up into the microscope is **reflected** light. Which form have you already used with the compound microscope?

A stereoscopic microscope can use either transmitted or reflected light. Thus, solid objects through which light cannot pass can be examined with the stereoscopic microscope. The working distance of the stereoscopic microscope is very large so that the specimen can also be very large. The stereoscopic microscope has low magnification. It magnifies approximately the same as the scanner of a compound microscope.

**<u>Student Work:</u>**
⇒ Look at a coin with the stereoscopic microscope. How does the image appear? Is this similar or different from the image seen with the compound microscope?

⇒ Move the coin to the right. Which direction does the image move?
Move the coin away from you. Which direction does the image move?
Are these movements of the image the same as those seen with the compound microscope?

⇒ What are the advantages of the stereoscopic microscope?
What are the disadvantages of the stereoscopic microscope?

## OTHER TYPES OF LIGHT MICROSCOPY

The ordinary **light microscope** is used almost exclusively for examination of fixed and stained materials. In this case, perception of the image is based on differential absorption of light by parts of the specimen binding dye so that variations in intensity or in color give contrast to different parts of the image.

The way that we use the compound microscope is called **brightfield**; the field is a circle of bright light. However, the compound microscope can be modified to provide different views of the specimen. Today, you will see examples of dark-field and phase contrast microscopy with a modified compound microscope.

### Dark-Field Microscopy

This technique involves illuminating a specimen without admitting light directly into the objective. Illumination is achieved by the insertion of a dark-field stop into the condenser mount of the microscope. This produces a hollow cone of light that only allows light from the side to enter the microscope. No direct light passes through the specimen. The specimen appears bright against a dark background.

Dark-field microscopy is used for studying a variety of cells and tissues, for example, unstained bacteria and micro-organisms in aqueous medium, living single-celled organisms in water, fresh blood, unstained epithelial cells, diatoms, and various plant tissue sections.

⇨ Look at the dark-field microscope set up on the side bench in the lab.

- Are the outlines of the unstained cells easier to see under dark-field illumination than with your bright-field microscope?

- Which cellular organelles are clearly visible?

- If you have been studying a fresh cheek epithelium wet mount with dark-field illumination, which organelles/structures appear visible or more distinct with this technique than when viewed under your bright-field microscope?

### Phase Contrast Microscopy

Some parts of cells are thicker than others. Hence, different cellular structures bend (refract) light waves by different amounts. A difference in thickness between different parts of a cell will produce a difference in phase of the light waves passing through. This will cause an average retardation of ¼ wavelength ( $\lambda$ ) of light. The phase contrast microscope transforms the phase differences of the light into variations in brightness within the image. This results in an increased contrast between regions of unstained cells.

The phase contrast microscope is fitted with a special phase condenser and phase objectives. A hollow cone of light passes through the specimen. Light rays entering the object are shifted by an annular diaphragm located beneath the condenser lens. The rays passing through and around the object are shifted again by a phase plate in the objective lens. The result is an increase in the contrast of the object as certain regions appear brighter, while other regions appear darker.

Phase contrast microscopy can be used to study the structure of living cells, cell division, and cell motility. A "halo" is generally visible around cytoplasmic granules, lipid droplets, and the plasma

membrane. This is due to enhanced phase differences in these areas. Internal structures will be bright and clear. Movements inside the cell are clearly visible.

⇨ Observe an unstained fresh wet-mount of cheek epithelium or a drop of protozoan culture with the phase contrast microscope. Your instructor will inform you as to which of these specimens is on demonstration.
  · Describe the appearance of the cells. Which structures can you see?

**Even more types of light microscopy**
Some cell structures show selective absorption of light of specific wavelengths such as ultraviolet; they can be examined with an **ultraviolet microscope**. Living cells, which are highly transparent and absorb virtually no light, can be examined with the **phase contrast microscope** or with an **interference microscope**. Both instruments depend upon differences in refractive index of various cell structures to give contrast to the image produced.

Some cell structures have two planes of light refraction (birefringent) that alters the natural path of polarized light passing through them. They can be viewed with a **polarization microscope**. The presence of certain substances in cells that fluoresce or emit visible light when illuminated with shorter wavelength radiation permits their study with the **fluorescence microscope.** Many of these different kinds of microscopes can be coupled with very sensitive phototubes so that differences in light quality (color) or light quantity (intensity) can be detected and recorded. Such instruments are called **cytophotometers**.

**ELECTRON MICROSCOPY**
Differences in the mass of substances that contain atoms of carbon, oxygen, and nitrogen in different parts of the cell cause scattering of an electron beam and are responsible for image formation in the **electron microscope.**

You will be introduced to the electron microscope during this lab period via a film, Electron Microscopy**.**

There is a physical limit to the magnification that can be produced by a light microscope. A beam of electrons has a much shorter wavelength than visible light and can produce much higher magnification.

Instead of visible light, an electron microscope uses a beam of electrons. Instead of glass lenses, this beam is focused by electromagnets. A beam of electrons would be deflected by collisions with molecules of air so that a vacuum is needed inside the electron microscope. Because of the vacuum only dead material can be studied.

The human eye cannot directly see a beam of electrons. The image produced by the electron microscope must be projected onto photographic film or a television monitor so that it can be viewed.

**The goals of this lab are to:**

1. Introduce you to the basic structure and function of cells.
2. learn the differences between Prokaryotic and Eukaryotic cells
3. learn to recognize the parts of cells that are visible with the light microscope
4. learn the general function of all cell structures that are described in the handout
5. learn to distinguish between plant and animal cells on the basis of their structure as seen with the light microscope.
6. learn about the structure and properties of the cell membranes

**Materials:**

1. Stains/Solutions for making slides: Janus Green, Methylene Blue, Lugol's Iodine (IKI), 10% NaCl
2. Specimens for wet mounts: human cheek cells, *Elodea*, onion (Alium), potato, tomato, beets
3. Reagents for membrane experiment: Lysis buffer (SDS), Protease solution
4. Demo Slides: plant mitochondria, Golgi Apparatus, Mitochondria-turtle liver, Mitochondria-onion

**Procedure:**

1. Short introduction to the cell
2. Watch a film that reviews the parts of a cell and shows many types of cells.
   Learn the general function of the following:
   Plasma Membrane, Nucleus, Nucleoli, Cytoplasm, Cytosol, Smooth & Rough Endoplasmic Reticulum (ER), Ribosomes, Golgi, Mitochondria, Plastids (Chloroplasts, Chromoplasts, Leucoplasts), Vesicles & Vacuoles, Cytoskeleton and Cell Wall
3. You will look at cells taken from inside your cheeks, cells in Onion (*Alium*), and cells in *Elodea* (an aquatic plant) leaves using the compound microscope. You will look at them before and after staining.
   - Draw and describe the cells.
   - You should know which organelles were visible.
   - Stop and compare the plant cells with the animal cells. What structures are common to both plants and animals? What structures are unique to each?
4. Review the structure of membranes. Know which types of molecules are present and how they are arranged. Be able to draw and briefly describe the membrane structure.
5. You will perform experiments on beet root designed to shed light on the chemical properties of the membrane. Know how the experiment works and the expected results.

**Readings:**

Sadava, 8th ed. Chpt 4

This chapter describes the structure of membranes, cell organelles, and show electron micrographs. There is more detail in these descriptions than you need right now. You will get the details in lecture. For the time being, just get to know the basic functions of the organelles, their general appearance, and gain some sense of how they contribute to the cell's survival.

**EXAM 1** will be given in the first hour of next week's lab. The test will include material from the first three labs: Metric System, Microscopy, and Cell Structure & Function. We will do the Lab 4 experiments after the exam.

**INTRODUCTION**

One of the central ideas of modern biology is the **cell theory.** According to this theory, all living things are composed of one or more cells; the cell is the simplest and smallest thing that can be said to be alive (i.e. it possesses all the characteristics of life); all cells arise from pre-existing cells. Put another way, the cell theory establishes the cell as the fundamental unit of life and the building block of all organisms. Because cells can only come from other cells they must have common ancestors.

Only a small number of cellular structures are visible with the light microscope. An electron microscope is needed to see most of these cellular components. In this lab, you will look at those cell parts that are visible with the light microscope.

There are two basic types of cells. **Procaryotic cells** are only found in bacteria (Kingdom Monera). The cells of the other four kingdoms are called **eucaryotic cells**. Procaryotic cells are approximately 1000X smaller in volume than eucaryotic cells and lack many cell structures, including the nucleus, which are found in eucaryotic cells. We will study only eucaryotic cells in this course.

All cells are enclosed by the **plasma membrane**. The plasma membrane surrounds all the parts of the cell and serves as the boundary of the cell. It plays an active role in the entry and exit of material from the cell.

Plant cells are surrounded by a non-living **cell wall**. The cell wall is located outside of the plasma membrane and is not a part of the living cell. The cell wall is mainly made of cellulose that is secreted from the cell's interior and builds up as a protective covering on the outside of the living cell's plasma membrane. Because the plasma membrane is so close to the cell wall, it cannot be seen with the compound microscope. Animal cells do not have a cell wall.

A large spherical structure can be seen in most eucaryotic cells. This is the **nucleus**. The nucleus is the control center of the cell. Within the nucleus, although not visible with the light microscope, are a specific number of long molecules of DNA. These DNA molecules store information that is used to control the cell's metabolism. This is the genetic information that will be studied later in the semester. Often one or two darker staining areas may be seen within the nucleus. These are **nucleoli** (singular = nucleolus). The nucleoli produce ribosomes, structures where all cellular proteins are produced.

The rest of the cell is called the **cytoplasm**. The cytoplasm can be a liquid or a gelatinous material. The liquid cytoplasm is called the **cytosol**. The cytosol is a watery liquid with many dissolved and suspended substances. The rest of the cytoplasm is composed of structures called **organelles**. There are numerous kinds of organelles. Each has a specific structure and function. Few organelles are visible with the light microscope. In some plant cells **chloroplasts**, the site of photosynthesis, are visible. Their green color is due to chlorophyll, a pigment that captures solar energy.

Most cellular organelles are too small to be seen by the light microscope. Most can only be seen with the electron microscope. Your textbook shows photomicrographs of these organelles.
Know the basic function of these organelles:

**Ribosomes** - protein synthesis
**Endoplasmic Reticulum (ER)** - a network of membranes where lipids and proteins are made
**Golgi apparatus** - packaging of proteins
**Mitochondria** - aerobic cellular respiration i.e. for releasing energy from sugar
**Lysosomes** - contain digestive enzymes for breaking down old cell parts

**Student Work:**

1. Examine a typical animal cell:
   Lightly scrape the inside lining of your cheek with a toothpick. This will scrape off some of the epithelial cells of your cheek lining. Make a wet mount of these cells (see the Microscope Lab for instructions) and examine them with the microscope. After you have seen the cells in their natural state, gently lift the cover slip and add a small drop of **methylene blue**. Methylene blue is a stain that will improve the contrast within the cells. Examine the cells again. Note the nucleus, cytoplasm, and plasma membrane. Draw the cell. Estimate the diameter of your cells by comparing them to the field diameter (you measured the field diameter in the Microscope Lab).
   **DISPOSE OF THIS SLIDE, TOOTHPICK, CELLS, COVERSLIP, ETC IN THE BIOHAZARD CONTAINER.**

2. Examine a typical plant cell
   Make a wet mount of an *Elodea* leaf. Note the cell wall, chloroplasts, and central vacuole of each cell. Draw one of the leaf cells. Estimate the length of the cells.

   After seeing the normal leaf, put a drop of 10% NaCl at the left edge of the cover slip. Then hold a piece of paper towel to the right edge of the cover slip. This will remove the water from beneath the cover slip and draw the 10% NaCl under the cover slip. Water will diffuse from the leaf cells and the cells will shrink in size. The cell will shrink, but the cell wall will remain the same size. This will pull the plasma membrane of the plant cell away from the cell wall and allow you to see that a plant cell also has a plasma membrane.

   See the demonstration slide of *Elodea* leaf XS. How many cell layers are found in the leaf?

3. Break a wedge of onion, peel off a thin strip, and make a wet mount. Note the nucleus, cytoplasm, and cell wall of each cell. Why can't the plasma membrane (which is present) be seen? Why are no chloroplasts present? Draw a cell. Estimate the length of the cells.

   After examining the slide, lift the cover slip and add a small drop of the vital stain **Janus green**. The cell's mitochondria will be stained a light blue-green color. The mitochondria look like tiny rods inside the cell.

4. The **plastid** is another organelle that is visible with the light microscope. Plastids are found in plant cells but not in animal cells. There are three main kinds of plastids.
   a. **Chloroplasts -** Chloroplasts are green because they contain green chlorophyll. You have already seen chloroplasts in the wet mount of an *Elodea* leaf. Make a new wet mount if you have forgotten their appearance. What is the function of chloroplasts?

   b. **Chromoplasts -** chromoplasts do not contain chlorophyll. Chromoplasts contain crystals of **carotenoid** pigments (such as beta-carotene) which are responsible for the color of carrots, tomatoes, and flowers. Chromoplasts give color to flowers and fruit to attract animals that pollinate the flowers or disperse the seeds.

      Using a scalpel, carefully and gently peel off a thin layer of the skin of a tomato. Try not to get any tomato pulp along with the skin. Make a wet mount of the tomato skin.

      Examine a thin area of the skin. Look for circular orange chromoplasts. A few of the chromoplasts may have ruptured membranes and may appear to be "spilling their contents". Do you see any crystals of pigment in the chromoplasts?

c. **Leucoplasts** - leucoplasts are colorless because they contain no pigments. They store starch. Starch is the polysaccharide that plants use to store extra sugar.

Use a scalpel to cut a thin slice of potato (an underground stem for starch storage). Make a wet mount of the thin piece. Focus on the edge of the slice.

The leucoplasts will resemble "pearly" ovals. Each starch grain consists of concentric layers of starch and may resemble an onion. By cutting the potato, you have cut the plasma membrane of many cells. The leucoplasts will have spilled out of the ruptured cells and can be seen separately.

Lift the cover slip and add a small drop of iodine. Using the compound microscope, look at the slide again. Iodine reacts with starch and gives a characteristic color. The leucoplasts, stained by dyed starch, will now be easier to see. What is the color?

5. Can we learn about cell structure chemistry?
All cellular membranes are similar in structure. They are made from a double layer (bilayer) of phospholipids that has proteins embedded in it.

The experiment that follows will demonstrate the structure of cellular membranes by treating plant cells in various ways that damage the membrane.

a. Cut four rectangles from a red beet. Each should be closely the same size: approximately 1 mm x 3 mm x 30 mm. Be sure that the rectangles are thin (0.5cm). Place them in a beaker and wash them for several minutes in cold, running water to remove any beet pigment which was released from cut cells. The red pigment is **anthocyanin**. It is water soluble and is located inside the central vacuole of the intact plant cell.
b. Place four (3) test tubes in a rack. Label them #1 through #3 and prepare them as follows:
   Test Tube #1 - 5 mls cold water = the **control**
   Test Tube #2 - 5mls water. Place this test tube in a boiling water bath for 2 minutes so that the water comes to a boil.
   Test Tube #3 - 5 mls of water. Add 5 drops of the Protease solution. The protease damages protein.
   Test Tube #4 - 5 mls SDS (Sodium Dodecyl Sulfate) solution. SDS is an anionic detergent. Detergents dissolve lipid.
c. Place one rinsed rectangle of beet in each of the test tubes and let them stand for 10-15 minutes. Keep Test Tube #2 in the boiling water bath during this period.
d. Arrange the tubes in order from most red color to least red color.

   ? Why did you get this variation in red color?
   ? What does this tell you about the contribution of phospholipids and proteins to membrane structure?
   ? What was the function of Test Tube #1? What did this test tube tell you?

## BIO112 - LAB 4 SEPARATION OF CELL COMPONENTS

**The goals of this lab are to:**

1. understand why cell fractionation is used by cell biologists
2. understand the difference between differential centrifugation and density gradient centrifugation
3. understand the procedure that we used for cell fractionation
4. be able to explain which organelles were found in each fraction and the basis for this
5. be able to predict how a particles that differ in density will separate in a hypothetical fractionation process
6. know the stains by which we identified leucoplasts & nuclei
7. know the method by which we were able to identify the presence of mitochondria
8. know the conditions required to preserve enzymatic activity in the tissue sample
9. know all the terms in this handout that appear in boldface print

**Materials:**

1. Food blender
2. table top centrifuge
3. electronic balance
4. $37^0$C water bath
5. microscope
6. peas that have been soaked in water for 24 hours
7. Buffered sucrose solution, pH 7, at 4 $^0$C
8. Stains: Aceto-orcein & Iodine
9. Tetrazolium
10. centrifuge tubes
11. cheese cloth for filtration

**Procedure:**

You will take exam 1 during the first hour.

You will work in groups of 3 or 4 to homogenize peas. You will then fractionate the tissues by means of filtration followed by differential centrifugation. You will examine each fraction with the light microscope and determine which cell structures are present. Stains will be used to identify leucoplasts and nuclei. The presence of mitochondria will be revealed by the action of mitochondrial enzymes on tetrazolium.

**Readings:**

Sadava, p. 75

**The Separation of Cell Components: Homogenization and Cell Fractionation**
Many techniques have been developed for studying cells components. One method is to break apart the cells and then isolate the cell parts for study by microscope or biochemistry. The process of breaking the cells into pieces is called **homogenization**. The process of isolating the cell parts from each other is called **Cell fractionation.** Most commonly, a **centrifuge** is used to separate the various components of the cell according to their size and density.

There are different methods to homogenize cells. The tissue may be ground to a pulp in food blender or it may be ground with a mortar and pestle. In either case, the tissue is mechanically torn apart and the cell membranes are ruptured releasing the cell organelles and bits of plasma membrane. The tissue is usually homogenized in an aqueous (water) medium. The homogenizing medium may be a salt solution, but it is usually a sucrose solution because salts tend to harm organelles in various ways, particularly in the high solute concentrations required for adequate separations.

**Differential Centrifugation Method**
This method of centrifugation separates cell organelles and macromolecules of a cell homogenate by subjecting the homogenate to a series of centrifugations of increasing centrifugal force. The homogenate is first spun at low speeds for a short time. A **pellet** is formed at the bottom of the centrifugation tube and a **supernatant** (the solution floating on top of the pellet) is left above. The supernatant is then poured into a new tube and spun for a longer time at a higher speed to form a second pellet and leave a second supernatant. This can be repeated a 3$^{rd}$, 4$^{th}$, etc. time.

Progressively higher speeds and prolonged times are required to sediment the smaller, lighter cell components. In general, the smaller the subcellular component, the greater the centrifugal force required to sediment it. Thus, large particles sediment out first, then medium-sized particles settle out, then the smaller-sized components are deposited at the bottom of the centrifuge tube.

**Density Gradient Method**
A finer degree of separation can be achieved through the use of **density gradients** in an **ultracentrifuge** which rotates at speeds up to 80,000 rpm and produces forces up to 500,000 times gravity. The centrifuge tube is filled with a solution of sucrose or some other soluble material. The concentration of the sucrose is made to vary from the bottom of the tube to the top. It is very concentrated at the bottom and less concentrated at the top. This is the density gradient. The cell homogenate is carefully layered on top and centrifuged at a high speed. The larger particles move down through the gradient at faster rates than the smaller particles; and the particles come to rest in an area of the density gradient equivalent to their own density. The rate at which each component sediments depends on its size and shape. This rate, which is roughly related to the molecular weight of the particle, is expressed as the **sedimentation coefficient** or **S** value. Larger S values indicate relatively larger particles. This technique makes it possible to separate very small particles such as viruses and macromolecules (proteins, DNA, RNA). Very high centrifugation speeds are needed to separate such small particles.

**Procedure for the Isolation of Cellular Components**
Cell components (nuclei, chloroplasts, mitochondria, etc.) differ in size, shape, density, and chemical composition. The cell components can be isolated from homogenized cells as described above and studied independently. In fact, under carefully controlled conditions, they will perform some of their normal functions outside of the cell for a limited period of time. The purpose of this laboratory exercise is to isolate several cellular components then identify them through microscopic observation, staining, and chemical reaction.

Scientists and biology students in more advanced courses use large floor-model refrigerated centrifuges or ultracentrifuges which operate at high speeds and in a vacuum while maintaining the sample at 4°C. The vacuum reduces friction, thereby preventing the rotor from heating and changing the temperature of the sample. The temperature must be kept low to prevent damage to the cells and cell parts. In this experiment you will use a lower speed table-top model centrifuge, but you will still be able to separate fractions of large and medium-sized organelles. You will use pea seeds as your source of cells. The pea seed is made of a variety of cell types so their appearance will vary. You will use two stains to help you identify cell organelles in the cell fractions: (1) **Iodine** combines with starch to form a black precipitate, and (2) **Aceto-Orcein** stains nucleic acids (DNA and RNA) red. You will also use a chemical reaction to detect the presence of mitochondria in some fractions. In this reaction the enzymes in mitochondria cause the color of **Tetrazolium** to change from clear to red.

Work together in groups of three (3) or four (4) and divide the work to be done. Work independently when viewing the microscope slides.

1. Obtain a beaker containing soaked pea seeds (dry weight of seeds = 10g.) Pour out the water that covers the seeds and add 100 ml. of cold sucrose-buffer solution. Pour the contents of the beaker into the blender and homogenize the seeds at high speed for 45 seconds. (Keeping the solution cold prevents excessive damage to the cell components released during the homogenization.)

   Pour the homogenate into a beaker and rinse out the blender vessel immediately!
   The homogenate contains pieces of the seed coat, clumps of intact cells, unbroken cells, and fragments of cell walls as well as the cell components.

2. Pour the homogenate through a pad of cheesecloth (4 layers) stretched across the top of a 250ml beaker. Fold the corners of the pad and squeeze the liquid into the beaker. This liquid is the filtrate.
   - Save the filtrate for Step 3.
   - Save the residue in the cheesecloth for microscopic examination. (Step 4)

   Wash all glassware **thoroughly** after use because you are working with a concentrated sugar solution. Use wet paper towels to wipe up any solution that you may have spilled.

3. Stir the filtrate in the beaker to re-suspend the solid material. Pour some of the filtrate into 2 large plastic centrifuge tubes. **DO NOT FILL THE TUBES MORE THAN ¾ FULL.**

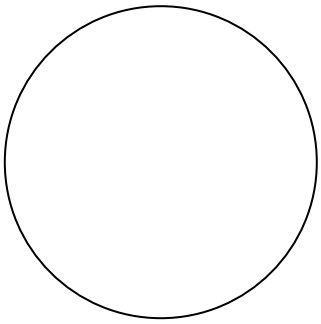
   Each tube must be **FILLED EQUALLY** to balance the centrifuge. Place each tube directly opposite its partner in the centrifuge. Always run 2 or 4 tubes (even if you have to fill one tube with water) to keep the centrifuge balanced.

   Close the top of the centrifuge. Set the speed _____ and time _____ indicated by your instructor. The setting should give a force of 200 x gravity.

4. Examine the residue while the centrifuge is running. Spread a tiny amount of the residue from the cheesecloth onto two slides. Add a drop of iodine to one microscope slide and a drop of aceto-orcein stain to the other slide. Apply a coverslip to each slide and observe under both low and high magnifications with your microscope.

Try to identify any fragments you find.

- ? Do you see any cell wall fragments? (They look like bricks in a wall).
- ? Which structures have turned a blue-black color in the iodine stain?
- ? Have any structures been stained with aceto-orcein?
- ? Can you tentatively identify any other structures on your slide? If so, name them.
- ? Compare their relative sizes. Are they large, medium, or small structures?



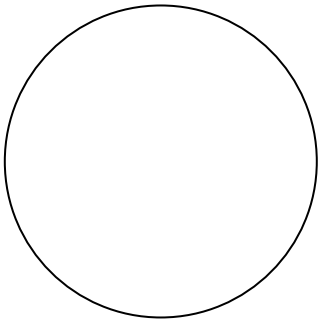
Use the space to sketch your slide.

5. Remove the centrifugation tubes.
   Carefully pour the supernatant (Supernatant #1) into 2 clean centrifuge tubes.
   Keep the pellet (Pellet #1) for step 6.
   You will now centrifuge Supernatant #1 with greater force (1300x gravity).
   Be certain to balance the centrifuge! Close the centrifuge cover.
   Set the centrifuge to the speed _____ and time ____ indicated by the instructor (1300X gravity).

6. Examine Pellet #1 while you wait for the centrifuge to finish running. Remove a small drop of the pellet with a long-tipped pipette and put a small amount on two microscope slides. Add a drop of iodine to one slide and a drop of aceto-orcein to the other slide. Apply a coverslip to each slide and observe them under the microscope.

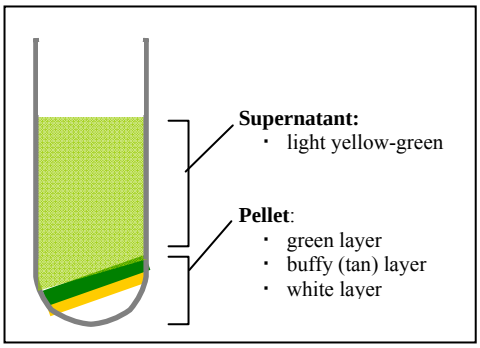
   Which organelles do you see?

   - ? Do you see any nuclei?
   - ? Are any starch grains present? If so, are there many or only a few?
   - ? If starch grains are present, measure several and record their size. How do they compare in size to any starch grains you may have observed on the slides of the cheesecloth residue?

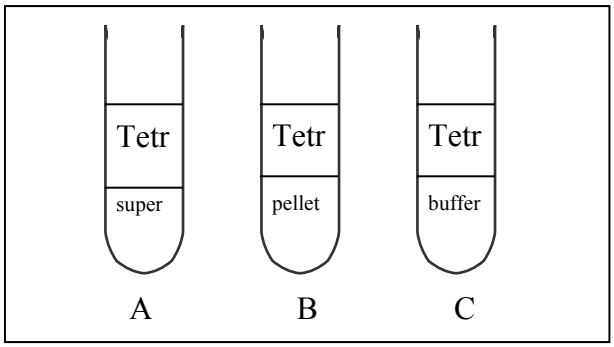


Use the space to sketch your slide and record the sizes.

7. After the second centrifugation, your centrifuge tubes should appear as shown in the sketch.




   a. Set aside one of the tubes from the second centrifugation to use in #8 below. Be careful not to disturb the layers in the tube.
   b. Last week you saw the small size of mitochondria. They are very hard to see with a light microscope. Are they in the pellet after the second spin or are they in the supernatant of the second spin? To find out we will use a chemical indicator (Tetrazolium) which will change color in the presence of mitochondria.
      1. Label three small test tubes "A", "B", and "C"
      2. Pipette enough supernatant to fill test tube "A" one third (1/3) full.
      3. Gently pour off the rest of the supernatant into a waste beaker. Add a small amount (about 3 mls) of sucrose buffer solution to the centrifuge tube and stir in order to re-suspend the pellet. Pour some of this suspension into test tube "B" so that it is 1/3 full.
      4. Pour sucrose buffer solution into test tube "C" so that it is 1/3 full.
      5. Carefully add a layer of **tetrazolium** solution to each tube (A, B, & C) so that each test tube is 2/3 filled. The tetrazolium should form a separate clear layer on top of the material already present in the test tube. **BE CAREFUL NOT TO SHAKE THE TUBES!**
      6. Place the three test tubes in the test tube rack in the warm (35-40$^{o}$ C) water bath on the side table. A red color will appear in the sample containing mitochondria. Red indicates the presence of mitochondria with active respiratory enzymes (dehydrogenases).




   ? Which tube(s) develop a red color?

   ? Would you expect to find mitochondrial activity in tube C? Why? What is the purpose of tube C?

8. Visually examine the centrifuge tube that you set aside after the second centrifugation (7a above). Be careful not to disturb the layers in the tube.

   a. Examine the supernatant (Supernatant #2) with the microscope. Use a clean pipette to gently remove some of the supernatant liquid from the tube. Place a small drop of it on two slides. Add a drop of iodine to one slide and a drop of aceto-orcein to the other. Place a coverslip on each slide. Examine them under low and high magnification.
      - ? What can you see? Which organelles are present?
      - ? Are leucoplasts still present? Why?

   b. Visually examine the pellet in the centrifuge tube (Pellet #2). There should be 3 layers with different colors.
      - ? Which layer of the pellet has a greater volume? Green, buffy, or white?
      - ? You've already seen that the white layer is composed of starch grains in leucoplasts. Why would you expect to find such an unusually large concentration of starch in pea seed cells?
      - ? Plant seeds are one of the most basic food supplies for humans (corn, rice, etc.). Based on your observations, can you explain why this is true?

   c. Examine the upper layers (green & buffy) of the pellet (Pellet #2) with the microscope. Carefully pipette out some material from the upper layers (green & buffy) of the pellet. Place a drop of this material on each of two slides. Add a drop of iodine to one slide and a drop of aceto-orcein to the other slide. Place a coverslip on each slide. Examine under your microscope.
      - ? Which organelles are present? What color are they?
      - ? Do you see any leucoplasts in the iodine wet mount? How do they compare in size to those you saw on the slide after spin #1 (step 6 above)?

   d. The buffy-colored sediment consists of cell nuclei. These structures are usually damaged by the experimental treatment. Thus, it is difficult to find them intact on your slide, even with aceto-orcein.

**SUMMARY**

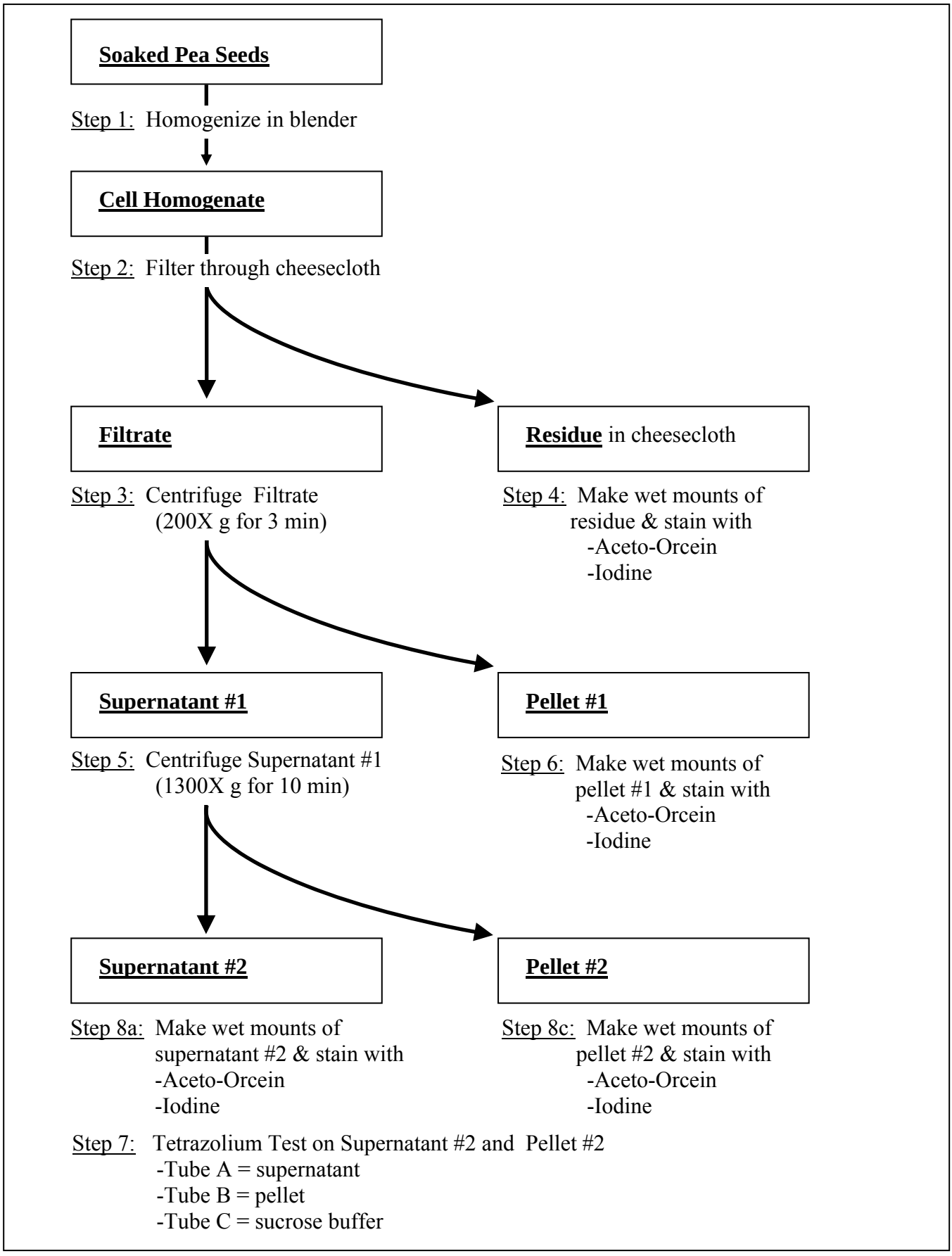
? Why did you centrifuge the homogenate twice? Why not use one centrifugation at 1300X gravity or greater force?

? List the organelles that you identified in each fraction that you collected.
- Residue
- Pellet #1
- Pellet #2
- Supernatant#2

? Rank in order, according to density and size, the cell components you obtained in this experiment. Start with the largest and most dense component.

? Describe how you identified each cell component.

## FLOW CHART: SEPARATION OF CELL COMPONENTS FROM PEA SEED CELLS



## BIO112 - LAB 5 THE STUDY OF CARBOHYDRATES & LIPIDS

**Objectives:**
By the end of this lab you will
1. be able to describe the structure of the major groups of carbohydrates: monosaccharides, disaccharides, and polysaccharides
2. know examples of each class of carbohydrate
3. know various functions of carbohydrates in the cell
4. know the chemical basis for the term "reducing sugar"
5. know how to perform a Benedict's Test for reducing sugars and interpret the results
6. know how to perform a Barfoed's Test for monosaccharides and interpret the results
7. know how to perform the Iodine Test for starch and interpret the results
8. you should be able to determine the identity of an "unknown" sample using these tests and appropriate "knowns"
9. know the chemical structure of the lipids known as triglycerides and waxes
10. know the major functions of the triglycerides and waxes
11. understand how the chemical structure of triglycerides and waxes causes them to be Hydrophobic
12. know how to perform the Grease Spot Test for lipids and how to interpret the results

**Materials:**
1. Reagents: Iodine, Benedict's Solution, Barfoed's Solution
2. brown paper for the grease spot test
3. Paraffin block
4. mortar and pestle for grinding
5. Materials for testing:
   a. Student Desks –
      - Carbohydrate Knowns = 1% solutions of fructose, glucose, maltose, sucrose, starch
      - Oil
   b. Side Bench –
      - Olive oil, potato, table salt, butter, milk or cream, apple, peanuts, Miracle Whip (salad dressing) and 2% solutions to use in the grease spot test
   c. Instructor's Desk
      - Carbohydrate Unknowns labeled "A". "B". "C" and "D". These are 1% solutions of fructose, glucose, maltose, sucrose.
6. Demonstration Slides: Tissue stained for lipids: Liver stained with Sudan IV and osmic acid

**Procedure:**
You will learn how to use several simple tests (Benedict's, Barfoed's, and iodine tests) to identify reducing sugars, monosaccharides, and polysaccharides. You will also learn about stains and a simple test (the grease spot test) to identify lipids.

Each group of four will test run the Benedict's, Barfoed's, and iodine tests on known carbohydrate samples. Next the tests will be used to identify 2 unknown carbohydrates. Each group will also look at a variety of food items using the Benedict's, Iodine, and grease spot test to determine whether they contain reducing sugars, polysaccharides, or lipids.

You will also see demonstration slides that have been stained for lipids.

**Readings:**
Sadava: pp 49-56

# Carbohydrates

**Chemical Structure of Carbohydrates**

Carbohydrates are molecules that have the following general formula $(CH_20)_n$ One carbon atom has a carbonyl group (C=O) and the rest have a hydroxyl (–OH). The smallest carbohydrates are the monosaccharides which have between 3 and 7 carbons. Examples of monosaccharides with 6 carbons include glucose, fructose, and galactose. Examples of monosaccharides with 5 carbons include ribose, and deoxyribose. Larger carbohydrates are synthesized by joining 2 or more monosaccharides together through dehydration reactions. Disaccharides are carbohydrates made from two monosaccharides. Examples include maltose, sucrose, and lactose. Mono- and disaccharides are commonly referred to as sugars. Oligosaccharides consist of short chains of monosaccharides. Polysaccharides consist of long chains of monosaccharides, usually glucose. Examples include starch, glycogen, and cellulose.

**Tests for Carbohydrates**

We will describe two tests used to characterize mono- and disaccharides and a third test for identifying polysaccharides. Each test produces a characteristic color change when a particular reagent reacts with a particular type of carbohydrate.

**Benedict's Test for Reducing Sugars**

A reducing sugar is one that has a free, or potentially free, aldehyde group or a ketone group. Examples include glucose, fructose, and maltose. Sucrose is not a reducing sugar. Benedict's Reagent is an *alkaline* solution that contains $Cu^{+2}SO_4^{-2}$. Its color is blue. When Benedict's Reagent is heated in the presence of a reducing sugar (either mono- or disaccharide), the solution turns red as the copper ions are reduced to form $Cu^{+}_2O$. A positive Benedict's Test (solution turns red) indicates the presence of reducing sugars; a negative Benedict's Test (solution remains blue) indicates that no reducing sugars are present.

$Cu^{+2}SO_4^{-2}$ + Reducing Sugar ⟶ (heat 3 minutes, *alkaline pH*) $Cu^{+}_2O$ + Oxidized Sugar

Benedict's Solution (BLUE) → (RED)

***Benedict's Test Procedure:*** Mix 8 drops of Benedict's Reagent and 8 drops of the sample in a test tube. Heat for 3 minutes in a boiling water bath. Remove from the bath and examine for color change. Any change from blue to yellow, to orange, to red is positive. Red indicates larger amounts of reducing sugar.

**Barfoed's Test for Monosaccharides**

Barfoed's Reagent is an *acidic* solution of $Cu^{+2}SO_4^{-2}$. Its color is blue. When Barfoed's Reagent is heated for *up to* 1½ minutes in the presence of a monosaccharide, the solution turns red as the copper ions are reduced to form $Cu^{+}_2O$. A positive Barfoed's Test (solution turns red) indicates the presence of a monosaccharide. A negative result (solution remains blue) indicates that no monosaccharides are present.

Obtaining good results with the Barfoed's Test can be problematic since the acidic conditions can cause hydrolysis of disaccharides so that monosaccharides form. You can avoid this by not heating too long. You should also run the test on a known disaccharide at the same time as your unknown, then you will know if you have heated for too long.

$Cu^{+2}SO_4^{-2}$ + Monosaccharides ⟶ (heat 1+1/2 minutes, *pH 4.5*) $Cu^{+}_2O$ + Oxidized Sugar

Barfoed's Solution (BLUE) → (RED)

***Barfoed's Test Procedure:*** Mix 16 drops of Barfoed's Reagent and 8 drops of the sample in a test tube. Heat for exactly 2 minutes in a boiling water bath. Remove from the bath and examine for color change. Any change from blue to yellow, or to orange, or to red is positive. Red indicates larger amounts of reducing sugar.

**Iodine Test for Polysaccharides**
Iodine has a dark amber color. Starch binds to iodine and produces a blue-purple color. A positive Iodine test (color changes to blue-purple) indicates the presence of starch. A negative Iodine test (no color change) indicates that no starch is present.

***Iodine Test Procedure:*** Mix 1 drop of Iodine solution (IKI) and 3 drops of the sample together in a spot plate. Look for the color change.

## Student Work

Perform a Benedict's Test, Barfoed's Test, and an Iodine Test on all of the Known substances (1-6) listed in the chart and record your results.

Next perform the tests on the Unknowns (7-10). The Unknowns are the same solutions as the Knowns, but their identity is hidden from you. Identify the Unknowns by comparing these results with your Knowns.

**STUDY OF CARBOHYDRATES**

**Indicate whether test is positive and the relative amount (+, ++, +++) or negative (-).**

| Carbohydrates | Test | | |
|---|---|---|---|
| | Benedict's | Barfoed's | Iodine |
| 1. Water* | | | |
| 2. 1%Starch Solution* | | | |
| 3. 1% Glucose Solution* | | | |
| 4. 1% Fructose Solution* | | | |
| 5. 1% Maltose Solution* | | | |
| 6. 1% Sucrose Solution* | | | |
| 7. Carbohydrate Unknown A** | | | |
| 8. Carbohydrate Unknown B** | | | |
| 9. Carbohydrate Unknown C** | | | |
| 10. Carbohydrate Unknown D** | | | |

* Located on the student benches ** Located on the Instructor's bench.

Why did you run the tests on Water?

Identify each Unknown:
A =

B =

C =

D =

# Lipids

## Chemical Structure of Triglycerides and Waxes

The lipids include four types of molecules that share one property: they are not water soluble. They are not water soluble because the molecules are composed of hydrocarbon chains that have very few oxygen atoms. As a result, the molecules are non-polar and will not mix with water (they are **hydrophobic**). In contrast, sugars, which have many more oxygen atoms are polar and therefore mix with water (they are **hydrophilic**). Lipids are soluble in non-polar organic solvents such as acetone. The four types of lipid are triglycerides, phospholipids, steroids, and waxes.

Triglycerides are also known as fats and oils. Triglyceride molecules are formed when dehydration reactions join 1 molecule of glycerol to 3 fatty acids. Fats are triglycerides that are solids at room temperature. Oils are liquids at room temperature. Fats and oils are important energy storage molecules. They contain approximately double the amount of energy per gram as protein or carbohydrate. They also serve as insulation in animals.

Phospholipids are a major structural component of cell membranes. Each is made from 1 glycerol joined by dehydration reactions to 2 fatty acid chains and 1 phosphate-containing group.

Steroids include cholesterol and a variety of hormones. All the steroid molecules include a molecular structure composed of 4 rings of carbon that are fused together. Each steroid has unique molecular groups attached to this 4-ringed structure.

Waxes are composed of a long fatty acid chain linked to a long chain alcohol. The hydrocarbon chain in both the fatty acid and the alcohol is saturated and longer than those found in the triglycerides. As a result, waxes are very non-polar. Waxes are found coating many surfaces in plants and animals. They help slow down water loss across these surfaces.

## Tests for Triglycerides

### Grease Spot Test

We will use a simple test for lipids. We will place a spot of the sample on a piece of paper and see if it causes the paper to turn translucent. Lipids will leave a translucent "grease" spot on the paper. Aqueous solutions will cause the paper to become translucent, but when they dry the paper will be opaque.

> ***Grease Spot Test Procedure:*** Place the sample on a piece of paper. If it is a liquid, let it dry completely. If it is a solid, smear it onto the paper then let it dry. Hold the paper up to the light and see if it is translucent. A transparent spot on the paper indicates that oil was present. Do the grease spot test on the substances listed in the table on the next page.

**Sudan IV and Sudan Black are tissue stains for lipids.**
Adipose Tissue is composed of cells (adipocytes) that store droplets of lipid. Adipose tissue stained with **Sudan IV** will be red-orange. Adipose tissue stained with **Sudan Black** will be black.

# Student Work

1. Observe the prepared slides that have been stained for lipids on the microscopes at the Demonstration Table.
2. Do the following experiments which illustrate the Hydrophobic Nature of Lipids

**Interaction between Water and Oil:**
*Procedure:*

1. Pour 2 ml of colored water into a CLEAN test tube and 2 ml of plain water into another CLEAN test tube.
2. Gently layer 15 drops of oil onto the surface of the water in each test tube. This can be accomplished by allowing the oil drops to flow gently along the inside of the test tubes.
   - Does the oil mix with either the colored water _____ or with the plain water?_____
3. Stopper the test tubes and shake them well.
   - Does the oil mix with the water in either tube? _____
   - Describe what happened? How does this illustrate the hydrophobic nature of oil?

**Interaction between Water and Wax:**
***Procedure:*** Obtain a block of paraffin wax. Use an eye dropper to place 1 small drop of plain water, 1 small drop of colored water, and 1 small drop of olive oil onto the block of paraffin wax.

- Explain why the water "beads up".
- Did the food coloring in the colored water have any visible effect on the hydrophobic property of the lipid?
- Look closely at the shape of the 3 drops. Compare the shape of the oil drop to the shape of the water drop.

## STUDY OF CARBOHYDRATES & LIPIDS

Perform a Benedict's Test, an Iodine Test, and a Lipid Spot Test on all of the substances listed in the chart. These materials are located on the side bench. Record your results. Where testing solids (e.g. potato) are to be tested for carbohydrate, take a small portion of the food, mince with a paring knife, and then grind with the mortar and pestle. Add 1 or 2 ml of distilled water and grind again. Use the resulting mixture for testing. When testing solids for lipids, simply smash a small amount on the paper, then remove the excess.

**Indicate whether each test is positive & relative mounts (+, ++, +++) or negative (-).**

| Sample | Test | | |
|---|---|---|---|
| | Benedict's | Iodine | Grease Spot |
| 1. Water | | | |
| 2. 1% Starch Solution** | - TRANSFER | YOUR - | |
| 3. 1% Glucose Solution | - DATA | FROM - | |
| 4. 1% Fructose Solution | - THE | EARLIER - | |
| 5. 1% Maltose Solution | - | EXPERIMENTS ! | |
| 6. 1% Sucrose Solution | | | |
| 7. Olive Oil | | | |
| 8. Potato | | | |
| 9. Table Salt (NaCl) | | | |
| 10. Butter | | | |
| 11. Milk | | | |
| 12. Apple | | | |
| 13. Miracle Whip | | | |
| 14. Peanut | | | |

## BIO112 - LAB 6 PROTEINS

**Objectives:**
By the end of this lab you will
1. be able to describe the structure of proteins
2. know various functions of proteins in the cell
3. know some methods for separating proteins by exploiting differences in solubility, charge, and size
4. know what Chromatography is and some of the various types
5. know how to perform Paper Chromatography and interpret the results
6. know how to calculate the $R_f$
7. know the chemical basis for the Ninhydrin, Pauly, & Biuret tests for protein
8. know how to perform these tests and interpret the results
9. know how to create a series of protein standards which vary in the amount of protein
10. know how to operate the Spec 20
11. know how to construct a Standard Curve and use it to determine the amount of protein in a sample

**Materials:**
1. Paper Chromatography: all materials are on the side bench
   - 4 flasks with amino acids: Methionine, Alanine, Tyrosine, Proline, and
   - 1 flask labeled "Amino Acid Mixture" contains a mixture of these 4 amino acids
   - Hair dryer, ruler, and applicator sticks
   - Chromatography tank filled to 1 cm depth with chromatography solution
2. Qualitative Tests for Protein:
   - Reagents are at the student bench: Sodium Acetate (powder in a jar), Ninhydrin Reagent, Sulfanilic Acid, 5% Sodium Nitrite, 20% Sodium Carbonate
   - Samples for testing are on the side bench: 0.1% egg albumin, histidine, tyrosine, glycine
3. Quantitative Determination of Protein: all materials on the side bench
   - Burets filled with Biuret's Reagent
   - Burets filled with 0.5 M KCL solution
   - Burets filled with Serum Albumin (2.5 mg/ml)
   - Vortex Mixer
4. Spec 20

**Procedures:**
You will learn about strategies and methods used to separate proteins from a mixture. You will learn to do paper chromatography, test for the presence of protein and certain amino acids in samples, and measure the quantity of protein in a sample.

Each group of four will carry out all of the procedures. Each group should start with the chemical tests for qualitative and quantitative tests for protein since these will take the most time. The "spotting" of amino acid samples on the chromatography paper can be done during incubation periods in the chemical tests.

The results of the paper chromatography experiment will be analyzed next week.

**Readings:**
Sadava: pp 37-45

**PROTEINS**

The proteins account for roughly half the dry biomass of most tissues. They include a very large number of molecules. For example, human cells are able to make roughly 30,000 different proteins. Proteins play a variety of roles in the cell. Some form stable structures (e.g. fibers in hair, bones), while others are capable of movements that allow them to function as intracellular machines (pumps, motors, enzymes).

Proteins are made of one or more peptides. A **peptide** is a polymer made of 2 or more amino acids that are linked together by **peptide bonds**. The peptide bond is formed by a dehydration reaction between the amine group of one amino acid and carboxylic acid of another amino acid. Peptides vary in length from 2 to over one thousand amino acids. The sequence of amino acids in a peptide is called its primary structure. The peptide can twist and fold back upon itself to give it a 3-dimensional shape (secondary and tertiary structure) which is unique to each peptide. The interaction of the primary structure with the surrounding environment determines this shape. The shape of a protein is what determines the type of function that it will have in the cell.

**EXTRACTION OF PROTEINS FROM A MIXTURE**

Cell biologists are commonly interested in looking at the activity of a single protein. There are a variety of methods that can be used to isolate one protein from a mixture of many proteins. The methods take advantage of differences in solubility, size, and charge of the different proteins in the mixture. Usually, several methods must be used in sequence to isolate a particular protein. **Dialysis** takes advantage of differences in molecule size. **Electrophoresis** separates proteins on the basis of size and charge. **Chromatography** uses differences in the rate at which molecules migrate across some medium. The mixture is dissolved in a liquid or gas (the "mobile phase"), then the mixture is applied to a solid material such as paper (the "stationary phase"). The speed at which each molecule migrates depends upon its size, solubility, and interaction with the solid. Chromatography takes many forms including thin layer, paper, column, high pressure liquid, and gas chromatography.

You will carry out two procedures to demonstrate these methods:

1. **Extraction by Differential Solubilities**

   Egg white includes 2 proteins: albumins and globulins. Each is soluble in saline solutions, but only albumins are soluble in distilled water. We will take advantage of their solubility differences in order to separate these two proteins from each other.

   ***Procedure:*** Separate the white from the yolk of an egg. Discard the yolk. Put the egg white into a 400 ml beaker and slowly add 200 mls of distilled water. Stir slowly. A white stringy precipitate of the globulins will form. You can separate the solid globulin from the soluble albumin by centrifuging at low speed for 10 minutes. Discard the extracted proteins.

2. **Separation of Amino Acids by Paper Chromatography**

   We will do the procedure that is given on the following page to separate and identify amino acids in a mixture. We carry out the separation this week. We will identify the amino acids in the mixture and calculate their $R_f$ values the following week.

## SEPARATION OF A MIXTURE OF AMINO ACIDS BY PAPER CHROMATOGRAPHY

Chromatography is a technique used to **separate a mixture of chemicals**. We will use a sheet of paper to separate a mixture of amino acids. Each chemical moves through the mesh of fibers (of which the paper is made) at different speeds based upon their individual size, shape, electrical charge, solubility, etc. We will also include samples of the amino acids ("standards") on the chromatography paper so that we can identify the components of the mixture.

We only have two chromatography chambers so that each class will only do one sheet of paper.

1. The first group that does the procedure will draw a faint line in pencil (the solvent would dissolve ink) parallel to the bottom of the chromatography paper. The line will run the length of the paper. The line should be 4 cm from the bottom of the paper.

2. Each group will draw small dots at 2 cm intervals along the pencil line. Label each dot with these letters: M, A, T, P, X as shown below. Write a name to identify your lab group. You will apply your samples at these spots on the paper.


3. The samples are in flasks on the side bench. Four flasks contain a known amino acid (**M**ethionine or **A**lanine or **T**yrosine or **P**roline). These are your "standards". The fifth flask contains a mi**X**ture of all of these amino acids.
   - Use the wooden stick in each flask to put a small drop of sample on the appropriate dot. Then let the sample air-dry or use the hair dryer.
   - Repeat this process of applying the samples to each dot 5 times. Keep adding drops and drying until you have a large amount of sample on each "X".
     - ⇨ Your goal is to keep the diameter of the spot with each amino acid sample small, but put lots of amino acid on the paper.

The instructor will stand the paper in the solvent (alcohol & acetic acid) at the bottom of the chamber. As the solvent is absorbed and moves up the paper it will dissolve the samples and carry them up the paper at different speeds.

The experiment must be stopped before the solvent reaches the top of the paper. This requires 1-2 hours. At that time the paper will be removed from the tank and allowed to air dry.

When the paper is dry, it will be sprayed with **ninhydrin.** Ninhydrin is a general stain for amino acids. The amino acids will become visible as purple spots (proline will be a yellow spot).

The papers will be available in the next lab period so that you can identify the components of the mixture.

**Procedure for next week.**

1. Calculate the **$R_f$ value** of each spot. The $R_f$ value is an index of the distance moved by each substance in that particular solvent. It is used for comparing results from 2 chromatograms.

   $R_f$ = distance the sample moved / distance the solvent moved

2. Identify each of the amino acids in the mixture. How can you identify the unknowns?

Questions:

- Why do you need to stop the solvent before it reaches the top of the paper?

- Why use the $R_f$ instead of the actual distance moved when comparing two chromatograms?

**CHEMICAL TESTS FOR PROTEIN**

1. **Qualitative Tests to Detect Protein**

Qualitative tests simply indicate the presence or absence of a particular substance. You will perform the following two tests on the substances indicated in the tables below. Reagents are at your desks. Samples of amino acids & proteins are on the side bench.

a. **Ninhydrin Test for Protein**:

Ninhydrin reacts with the amine group at the end of a peptide chain. It cleaves off the amine, forming ammonia. The ammonia and ninhydrin react to form a violet purple precipitate. The test indicates the presence or absence of peptides. Use the following procedure to test the samples that are listed in the table below.

Procedure:

1. Add 3 ml of sample to a test tube
2. add a scoopful of Sodium Acetate (entire end of the spatula)
3. Add 8 drops of Ninhydrin Reagent
4. Boil the contents of the test tube for 3 minutes.
5. Results
   + test = purple or violet color
   – test = no purple color  If there is no color change, let the test tube cool to room temperature and check again before recording a negative result

| Sample | Color | Conclusion |
|---|---|---|
| Distilled Water | | |
| 0.1% albumin | | |
| | | |

b. **Pauly Test for the amino acids Tyrosine and/or Histidine:**

Sulfanilic acid reacts with Tyrosine and Histidine under alkaline conditions to produce a color change. Use the following procedure to test the samples that are listed in the table below.

Procedure:

1. Add 2 ml of sample to a test tube
2. Add 1 ml of Sulfanilic Acid
3. Add 1 ml of 5% Sodium Nitrite
4. Mix and let stand for 30 minutes
5. Add 3 ml of 20% Sodium Carbonate. Mix.
6. Results
   + test = red color
   - test = not red

| Sample | Color | Conclusion |
|---|---|---|
| Distilled Water | | |
| Histidine | | |
| Tyrosine | | |
| Glycine | | |
| Unknown | | |

2. **Quantitative Chemical Determination of Protein**

The **Biruet Reaction** occurs when a peptide bond reacts with a solution of copper sulfate to produce a complex with a purple-blue color. The amount of color that is produced is directly proportional to the number of peptide bonds present. Since the number of peptide bonds per gram of protein is approximately the same in all proteins, we can also use the amount of color produced by the biuret reaction as a measure of either the total amount of protein in a sample (or its concentration). In order to measure the total amount of protein in a sample we will need (1) a device to measure the amount of color, and (2) the relationship between the amount of color and the amount of protein (or its concentration).

**Colorimeters & Spectrophotometers**

Colorimeters & spectrophotometers are devices that can measure the amount of light of a particular wavelength (i.e. color) that is absorbed as it passes through a sample. You will use a spectrophotometer (Spec 20) to measure the amount of light that is absorbed by the purple biuret reaction product. This product absorbs light with a wavelength of 540 nm.

**The Standard Curve**

Following treatment of a sample with Biuret's Reagent, the $Absorbance_{540}$ is proportional to the amount of protein in the sample. The relationship between Absorbance and the amount of protein (or its concentration) is linear as long as the amount of protein is within certain limits. You might think that you could turn to a reference book and find what absorbance value corresponds to a particular amount of protein, but this is not the case. These measurements are subject to variation due to the spectrophotometer, the reagents, and the tube that holds the sample. For this reason, it is necessary to determine the specific relationship between the absorbance and the amount of protein (or its concentration) every time that you want to measure the amount of protein present in a sample.

In order to determine the relationship between Absorbance and the amount of protein in a sample, you have to construct a Standard Curve. This is done by setting up a series of tubes that differ in the amount of protein present. These tubes that contain known amounts of protein are called "Standards". The absorbance by each tube is measured with the spectrophotometer and then plotted on graph paper. Since the relationship between absorbance and the amount of protein is known to be linear, a "best fit" straight line can be drawn to estimate what the absorbance would be for amounts of protein that are in-between the known values. This Standard Curve can then be used to determine the amount of protein in a sample. The absorbance of the unknown sample must be measured using the same reagents, vial, and spectrophotometer that was used in constructing the Standard Curve. The Standard Curve has value only for that particular experiment; it must be created new each time you need to measure the amount of protein in a sample.

**Procedure:**

You will create a Standard Curve and use it to determine the amount of protein in two Unknown samples. Follow the instructions below and use the table on the next page to guide your preparation of the Standards and the Unknowns for the Biuret's Test. After taking the readings from the spectrophotometer, you will draw a Standard Curve and use it to determine the Total (amount of) Protein in the Unknowns.

1. **Set up the protein "Standards"**, i.e. a series of tubes with different known amounts of Protein. You will do this by diluting a stock solution of Serum Albumin (2.5 mg/ml) with 0.5 M KCl. The amount of Serum Albumin will be different in each tube, but the total volume in each tube will be 2.5 ml (See columns B and C in the table). The total amount of Protein in each tube is shown in Column E of the table. Make certain that you understand how this is calculated!

2. **Set up the Unknowns.** Set up two small test tubes and add 2.5 mls of Protein Unknowns A and B. You will <u>not</u> dilute these with KCl. Your goal is to determine the quantity of protein in each sample.

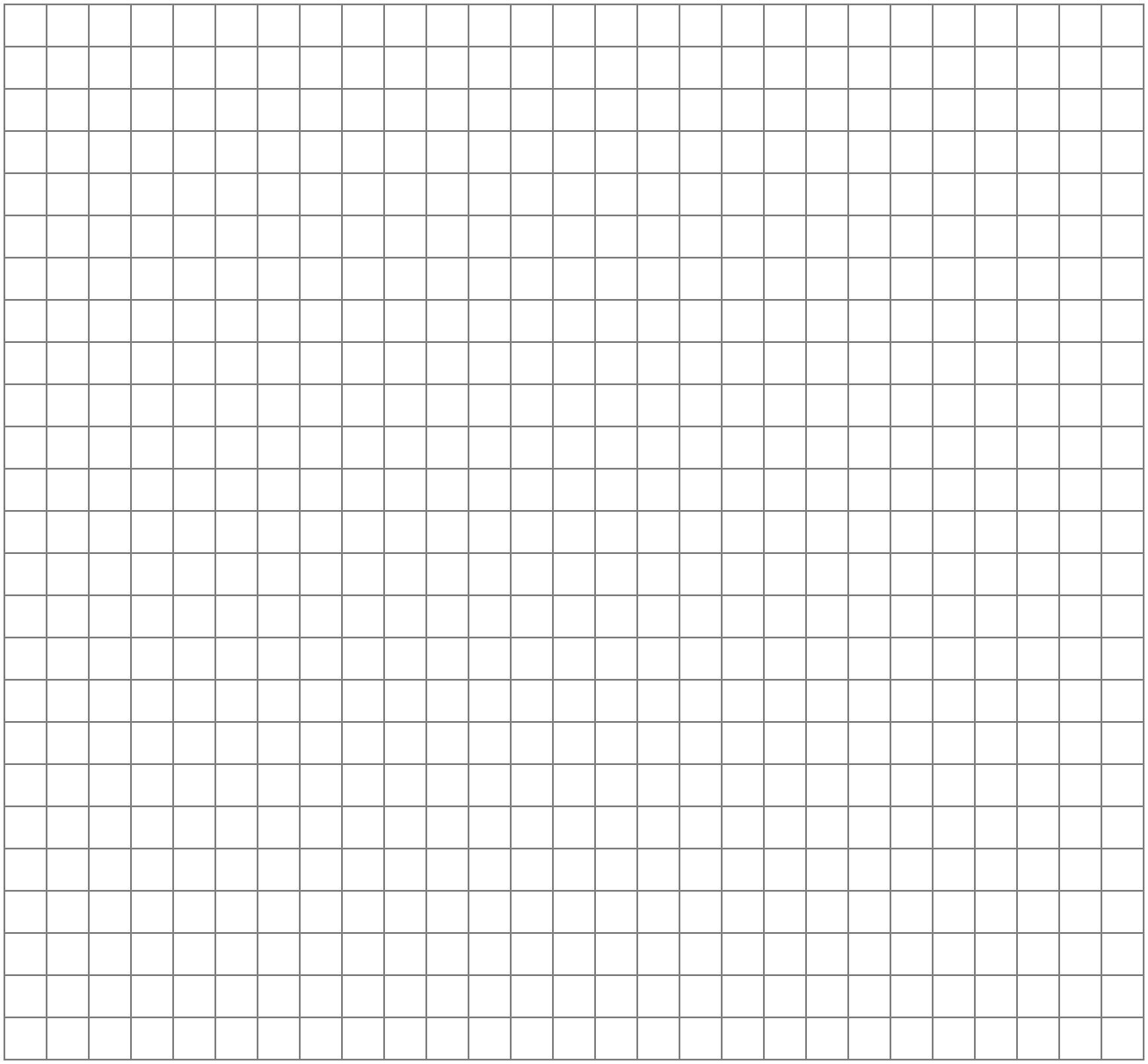
3. After setting up the 8 test tubes with the Standards and Unknowns, add 1.5 ml of Biuret Reagent to each tube and <u>mix</u> with the Vortex mixer.

Look!

4. Let the tubes stand for **20-60 minutes** so the biuret reaction can take place.

Look!

5. Turn on the Spec 20 and allow at least 15 minutes for it to warm up.
6. Follow the directions for using the Spec 20. Be sure to set the wavelength to 540 nm.
7. Tube 1 is the "BLANK". It contains no protein and is used to calibrate the Spec 20.
8. Measure the Absorbance for tubes 2-6 and your Unknowns. Record the values in the table.
9. Use the absorbance data for tubes 2-6 to construct the Standard Curve on the graph paper. Follow the instructions below.
10. Find the Total Protein in your Unknowns by comparing the absorbance to your standard curve. Follow the instructions below. Write the amount of protein for Unknowns A and B in the column E.



| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| | Tube | Volume of Serum Albumin (2.5 mg/ml) (mls) | Volume of 0.5M KCl (ml) | Volume of Biuret Reagent (ml) | [1] Total Protein in tube (mg) | [2] Spectrophotometer Readings | |
| | | | | | | $A_{540}$ | %T (optional) |
| Protein Standards for Constructing the Standard Curve | 1 "Blank" | 0.0 | 2.5 | 1.5 | 0 | 0.0 | 100 |
| | 2 | 0.5 | 2.0 | 1.5 | 1.25 | | |
| | 3 | 1.0 | 1.5 | 1.5 | 2.5 | | |
| | 4 | 1.5 | 1.0 | 1.5 | 3.75 | | |
| | 5 | 2.0 | 0.5 | 1.5 | 5.0 | | |
| | 6 | 2.5 | 0.0 | 1.5 | 6.25 | | |

| | | Volume of Unknown Protein (ml) | Volume of 0.5M KCl (ml) | Volume of Biuret Reagent (ml) | Total Protein in tube (mg) | Spectrophotometer Readings | |
|---|---|---|---|---|---|---|---|
| | | | | | | $A_{540}$ | %T (optional) |
| Samples with Unknown Amounts of Protein | A | 2.5 | 0.0 | 1.5 | ? | | |
| | B | 2.5 | 0.0 | 1.5 | ? | | |
| | | | | | | | |

Use the Standard Curve & your Spec 20 readings to determine the Total Protein for these two samples.

[1] Total Protein (mg) = (Vol. Serum Albumin) X (concentration of Serum Albumin)
For example, in test tube #4 there is 1.5 ml of serum albumin which has a concentration of 2.5 mg/ml.
The total amount of protein in tube #4 = (1.5 ml) X (2.5 mg/ml) = 3.75 mg

[2] The spectrophotometer does not measure absorbance directly; it measures the amount of light that is transmitted. Transmittance, T, is defined as: $T = I/I_o$, where $I_o$ = the intensity of light that enters the sample and I = the intensity of the light that passes through the sample. T can also be expressed as %T (multiply 100 x T).

The relationship between protein concentration and %T is not linear (it is logarithmic) which makes plotting the Standard Curve more difficult since you have to plot it on semi-log paper. An alternative, is to convert %T to Absorbance. Absorbance (aka Optical Density) is defined as follows: $A = \log_{10} {}^{I}/_{T} = \log {}^{Io}/_{I} = -\log T$. According to the **Lambert-Beer Law**, the concentration of solute is directly proportional to Absorbance. This means that if you plot Absorbance vs concentration, the Standard Curve will be a straight line and you only need to plot a few points.

Our Spec 20 converts %T to Absorbance for you and displays it on the digital readout screen. Some models do not report Absorbance, but you can make the conversion yourself by finding the –log of T or by using a conversion chart.

## SPECTRONIC 20 D+

Illustrated instructions are printed on the top right front of the machine.

Mark the top of all the test tubes with a red marking pencil. Always put the test tubes into the machine in the same direction each time (use the red mark you made). Wipe the tube with a paper towel before placing it into the sample holder. Dirt, fingerprints, air bubbles in the glass, etc. can affect the readings of the machine.

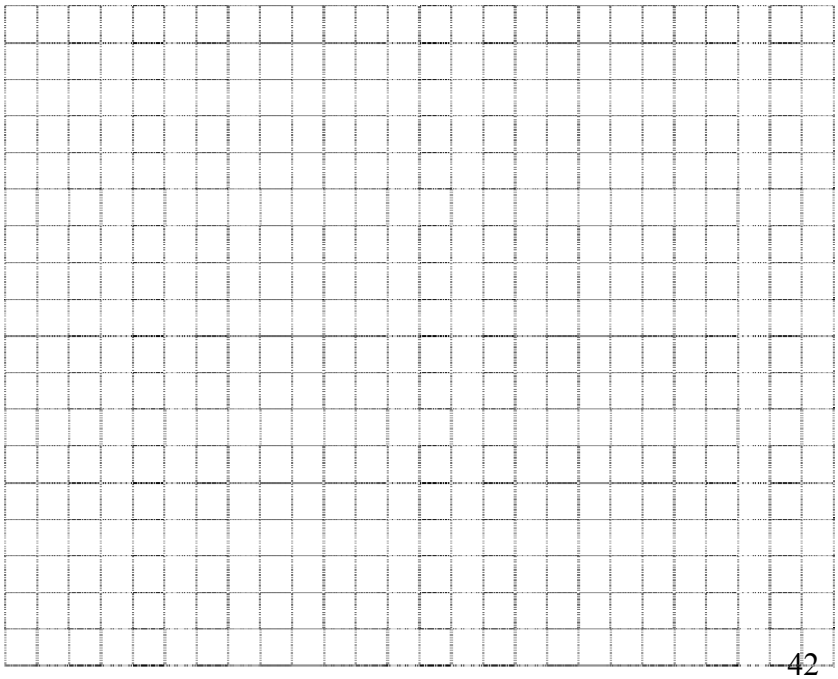
1. Turn the machine on (left front knob). Let the machine warm up for 15 minutes.
2. Be sure that the filter position (lever at lower left) is on the black (340-599 nm).
3. Set the wavelength (top right knob).
   Use 540nm for the biuret reaction.
4. Set the mode button to %Transmittance.
5. Set the empty machine (with the lid closed) to 0% T (left front knob). After you set it to 0, you should **not touch this knob again**. When the chamber is empty, no light reaches the detector and there should be 0% transmittance.
6. Insert the "BLANK" (**= test tube #1** = the tube that has no protein). Close the lid.
7. Set the machine to 100% T (right front knob). The amount of color in the "BLANK" will be subtracted from all of the other test tubes.
8. Set the mode button to Absorbance.
9. Read the Absorbance of each of your test tubes with the Spec-20.

### Procedure for drawing the Standard Curve. You will plot Absorbance vs. Total Protein (mg).

1. Set up the axes for the graph. Make your graph as big as possible.
   - Plot "Total Protein (mg)" on the X axis. The values should cover the range of values for your Standards (Column E in the data table above).
   - Plot Absorbance$_{540}$ on the Y axis. The values should cover the range of values obtained from the Standards (Column F in the data table above).
2. Plot the points for each pair of "Total Protein" and Absorbance values. These values are the X,Y coordinates for each point on your graph.
   - Locate the point on the X axis that corresponds to the Total Protein and draw a vertical line.
   - Locate the point on the Y axis that corresponds to the Absorbance reading and draw a horizontal line.
   - Place a dot at the intersection of these two lines.
3. Draw the "best fit" line through the points that you plotted above. The points should all lie on the line. This is the Standard Curve. It shows the relationship between Total Protein and Absorbance.

Sample Data

| Tube | Total Protein (mg) | Absorbance |
|---|---|---|
| 1 | 0 | 0 |
| 2 | 1.25 | 0.074 |
| 3 | 2.50 | 0.152 |
| 4 | 3.75 | 0.218 |
| 5 | 5.00 | 0.281 |
| 6 | 6.25 | 0.328 |



**Procedure for using the Standard Curve to determine the Total Protein in a sample.**
You will have measured the absorbance of your Unknown(s) at the same time that you created the Standard Curve.

1. Find the value on the Y axis of the Standard Curve that matches Absorbance value that you measured. You recorded this in the table.
2. Draw a horizontal line from this point to the Standard Curve.
3. Draw a vertical line from the intersection of the lines in step 2 down to the X axis.
4. Read the value at the X axis. This is the Total (amount of) Protein in the tube. Enter this value in the data table (column E).