Chlorophyll and Photosynthesis

Lab IV-1

EQUIPMENT AND MATERIALS

You'll need the following items to complete this lab session. (The standard kit for this book, available from *www.thehomescientist.com*, includes the items listed in the first group.)

MATERIALS FROM KIT

- Goggles
- Beaker, 250 mL
- Bromothymol blue
- Centrifuge tubes, 15 mL
- Centrifuge tubes, 50 mL
- Chromatography paper
- Forceps
- Gram's iodine stain
- Hydrochloric acid
- Pipettes
- Ruler (mm scale)
- Slides (flat) and coverslips
- Test tube clamp
- Test tube rack
- Thermometer

MATERIALS YOU PROVIDE

- Gloves
- Coin with milled edge (optional)
- Cotton balls
- Elodea (water weed; see text)
- Isopropanol, 70%
- Leaves, various (see text)
- Light source (see text)
- Meter stick or measuring tape
- Microscope (with reflection illumination)
- Microwave oven
- Saucer
- Scissors
- Soda straw
- Toothpicks (plastic)
- UV light source (optional)
- Watch or clock with second hand
- Water, distilled

BACKGROUND

In this lab session we'll investigate *photosynthesis*, the conversion of solar energy to stored energy in the form of saccharides, which is the basis of nearly all life on Earth. (Only nearly all, because a few deep-sea organisms use energy directly from thermal vents on the sea bottom.) Photosynthesis depends largely on *chlorophylls*, a closely related group of organic chemical compounds that contain a central magnesium atom.

In its most common form, photosynthesis is a chemical reaction that converts carbon dioxide and water, in the presence of light as an energy source and chlorophyll as a catalyst, to glucose (or another saccharide) and oxygen.

$6\ CO_2 + 6\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2$

The products of this reaction, saccharides and oxygen, are the basis of all animal life. Without the saccharides produced by plants, we'd starve; without the oxygen, we'd suffocate. Note the balance. We animals consume the saccharides and inhale the oxygen produced by plants and exhale the carbon dioxide that plants in turn use to produce more saccharides and oxygen.

Plants, algae, and cyanobacteria (formerly called blue-green algae) are *autotrophs* or self-feeders. They use photosynthesis to produce food—saccharides and other organic carbon compounds—from carbon dioxide and water. Autotrophs comprise about 5% of all species, and are the producers in the food chain. The other 95% of species—animals and most other bacteria and fungi—are *heterotrophs*, which are the consumers in the food chain.

Some species of sea slugs, notably *Elysia chlorotica*, use chlorophyll from plants they eat to perform photosynthesis for themselves; no other animal has been found to have this ability.

PROCEDURE IV-1-1: OBSERVING CARBON DIOXIDE UPTAKE

In this procedure, we'll observe the uptake of carbon dioxide (carbon fixation) and the release of oxygen by plants during photosynthesis. We'll do this by establishing various environments in which the three key elements—sunlight, carbon dioxide, and chlorophyll—are present or absent in different combinations.

During photosynthesis, carbon dioxide is consumed and oxygen is produced. We'll use a simple test for the presence of carbon dioxide. When carbon dioxide is dissolved in water, it produces a slightly acidic solution. In slightly acidic solutions (< pH 6.0) the pH indicator bromothymol blue is yellow. In slightly basic solutions (> pH 7.6) it is blue. Between pH 6.0 and pH 7.6, it transitions through various greenish shades.

We'll produce a saturated solution of carbon dioxide by the simple expedient of exhaling through a soda straw into water that has been tinted with bromothymol blue. As the carbon dioxide from our breath dissolves in the water, the indicator turns yellow. If a chemical reaction occurs in that solution that consumes carbon dioxide, we expect the solution color to shift toward blue (or at least greenish) as the carbon dioxide is removed and the pH of the solution increases.

We have no simple chemical test for the presence of oxygen, so we'll depend on the fact that oxygen gas is much less soluble in water than carbon dioxide gas. If in fact a reaction occurs that consumes carbon dioxide and produces oxygen, we would expect that oxygen to be visible as tiny bubbles in the reaction vessel. In this procedure, we'll observe the uptake of carbon dioxide by elodea (a water plant) and determine which combination of three factors—the presence of carbon dioxide, the presence of light, and the presence of chlorophyll—are necessary for photosynthesis to occur.

We used Elodea (water weed) as our plant. You can purchase Elodea (also called Anacharis) at a pet store or aquarium shop, or you can simply gather it from a lake, pond, or in shallow water near the banks of a stream.

1. If you have not already done so, put on your goggles, gloves, and protective clothing.

2. Label six 15 mL centrifuge tubes as follows:

   A. Light – Elodea – CO_2

   B. Light – Elodea

   C. Light – CO_2

   D. Dark – Elodea – CO_2

   E. Dark – Elodea

   F. Dark – CO_2

3. Place 7 cm to 9 cm lengths of elodea sprig into each of tubes A, B, D, and E.

4. Transfer about 175 mL of distilled water to the 250 mL beaker. Add sufficient bromothymol blue indicator solution to the water to tint it a distinct blue or blue-green color and stir to mix the solution.

5. Fill tubes B and E nearly full of the blue solution you produced in step 4.

If the solution in the tubes is not distinctly colored, return the contents of the tubes to the beaker and add more bromothymol blue. It's necessary that the color of the solution in the tubes be intense enough that any color changes are readily visible.

6. Add one drop of hydrochloric acid to each of tubes B and E, and swirl them to mix the contents. If the solution does not turn yellow, continue adding hydrochloric acid dropwise, with swirling, just until the solution turns yellow. Cap the tubes. Note the appearance of the tubes—color, presence or absence of bubbles, and so on—and record your observations in your lab notebook. Place tube B on a windowsill or other location where it is exposed to direct sunlight. Place tube E in a drawer or other dark location.

The tubes will stand on their caps if you invert them. Just make sure the cap is tight.

7. Use the soda straw to blow bubbles (gently) into the solution in the beaker until it assumes a distinctly yellow color. (Obviously, don't suck any of the solution into your mouth.)

8. Fill tubes A, C, D, and F nearly full with the yellow solution you produced in step 7. Cap the tubes. Note the appearance of the tubes and record your observations in your lab notebook. Place tubes A and C on where they are exposed to direct sunlight. Place tubes D and F in a dark location. Retain the excess solution in the beaker for use in the next procedure.

9. Observe each of the six tubes every few minutes and record your observations in your lab notebook.

PROCEDURE IV-1-2: DETERMINING THE EFFECT OF LIGHT INTENSITY ON PHOTOSYNTHESIS

In the preceding procedure, we established that photosynthesis requires the presence of chlorophyll, carbon dioxide, and light. In this procedure, we'll determine the effect of light intensity on the rate of photosynthesis. We'll measure photosynthesis rate by determining the amount of time needed for water saturated with carbon dioxide and tinted by bromothymol blue to change color when exposed to light at differing intensities, indicating that photosynthesis has consumed the carbon dioxide present.

To do that, we'll need an intense light source, ideally one that is full-spectrum and as close as possible to a point source. We used a 600W quartz-halogen work light, which among common light sources comes closest to meeting those criteria. If you don't have a quartz-halogen light, substitute any bright light source.

The amount of time necessary for a change to occur depends on the intensity of the light acting on the chlorophyll in the Elodea, which in turn depends on both the actual intensity of the light source and its distance from each tube. With our 600W quartz-halogen light, visible changes occurred very quickly; with a less-intense light source, it may require several minutes or longer for changes to become evident.

1. Label five 15 mL centrifuge tubes: two as "0.25 m," and one each as "0.5 m," "1.0 m," and "2.0 m."

2. Cut four sprigs of Elodea. It's important to make the sprigs as similar as possible in terms of leaf area.

3. Place each sprig in one of the centrifuge tubes, leaving one of the 0.25 meter tubes without a spring. Fill all five tubes nearly to the brim with the yellow solution from the beaker, and cap the tubes.

4. Set up your light source, but leave it off for now. Use the meter stick to place each tube at the corresponding distance from the light, inverted and sitting on its cap. Position each tube so that it will be fully exposed to the light once you switch it on.

The intensity of a point light source decreases with the square of the distance. For example, light intensity at 2 meters is one-quarter that at one meter, and one-sixteenth that at 0.5 meter. If the rate of photosynthesis varies directly with light intensity, we would expect the nearest tube to change color quickly, with tubes at greater distances taking correspondingly longer to change.

5. Note the time to the second and turn on the light.

6. Watch the 0.25 meter tube with the sprig carefully, and note the elapsed time when you first notice a change in the color of the liquid in the tube. Do the same for the 0.5 meter tube, the 1.0 meter tube, and finally the 2.0 meter tube.

7. Record all of your observations in your lab notebook.

PROCEDURE IV-1-3: CHROMATOGRAPHY OF PLANT PIGMENTS AND LOCATING CELLULAR ENERGY RESERVES

Although chlorophylls are the primary pigment in most vegetation, there are others. Yellow and orange carotenoids are present in leaves year-round, but during the spring and summer their colors are ordinarily masked by the intense green of chlorophyll. Red and purple anthocyanins are not present in spring and summer foliage, but are actually produced during the autumn color change.

In this procedure, we'll extract chlorophyll and other plant pigments from green leaves and use paper chromatography to separate those pigments. In doing so, we'll discover how many pigments are present in our leaf specimens. (The number and types of pigments varies from one plant to another and with the time of year, so your results may differ from ours.)

Some of the pigments are relatively soluble in alcohol but nearly insoluble in water, and vice versa. To extract as many pigments as possible from our specimens, we'll use 70% isopropanol (drugstore rubbing alcohol), which we expect to be a reasonably good solvent for all of the pigments present.

We'll also examine a decolorized leaf microscopically. Plants store the glucose produced by photosynthesis in the form of starches. These cellular energy reserves are ordinarily colorless and therefore difficult or impossible to discriminate visually. We'll therefore stain a decolorized leaf with Gram's iodine solution, which reacts with colorless starches to form an intensely blue-black complex that is readily visible if present.

You can use any type(s) of green leaves available. If you're performing this procedure at a time of year when the leaves have changed color or fallen, you can use evergreen needles or supermarket produce such as spinach. Ideally, you should obtain deciduous leaves during the spring or summer while they're green, and then obtain specimens from the same trees during the autumn leaf color season.

We obtained spring/summer and autumn leaf specimens from two species of maple. For both species, the spring/summer leaves are the intense green of chlorophyll. In autumn, the red maple leaves turn red, due to the presence of anthocyanins, which are absent during the spring and summer. The golden maple leaves in autumn are a brilliant yellow color, due to the presence of carotenoid pigments, which are present in the leaves year-round, but with their yellow color screened by chlorophyll during the spring and summer. We expected anthocyanins to be visible in chromatograms of red maple leaves only in autumn specimens, while we expected carotenoids to be visible in chromatograms of golden maple leaves from any time of year.

1. If you have not already done so, put on your goggles, gloves, and protective clothing.

2. Prepare each of your leaf specimens by cutting the leaves into small pieces, the smaller the better. Rough squares about 5 mm on a side are adequate, but even smaller pieces allow more of the plant pigments to be extracted quickly. Cut enough leaf pieces to fill a 15 mL centrifuge tube to at least the 3 mL or 4 mL line loosely packed.

3. Transfer the pieces of each specimen to a labeled 15 mL centrifuge tube and add enough 70% isopropanol to the tube to cover the leaf fragments completely. A small excess of alcohol is fine, but we want the resulting extract to be as concentrated as possible, so don't use a large excess.

For quick-and-dirty chromatograms, you can skip this extraction process by transferring plant pigments directly to the chromatography paper. To do so, press a leaf against the paper and rub the edge of a milled coin (such as a US quarter) against the leaf to produce a line of plant pigment on the paper. Develop the chromatogram as usual. The results may not be as good as those using the alcoholic extract, but the process is fast enough to allow you to run many more chromatograms if your time is limited.

4. Cap the tube and allow the leaf pieces to soak in the isopropanol for at least several days, inverting the tubes occasionally. It does no harm to allow the extraction process to continue for weeks or even months. For example, we obtained summer leaves and allowed them to continue soaking in the alcohol until autumn leaves were available.

If you haven't prepared the extracts ahead of time, you can use hot isopropanol to speed the extraction. Plug the mouths of the 15 mL centrifuge tubes loosely with cotton balls and place the tubes in the 250 mL beaker full of tap water heated in the microwave to about 80 °C (**Caution! HOT!**). Much higher than 80 °C will cause the alcohol to boil too vigorously; much lower will slow extraction. If the alcohol boils too vigorously, just add some cold tap water to cool the water bath. If the water cools too much, add a small amount of boiling water to the bath.

Allow the extraction to continue at or near the boiling point of the alcohol until most of the color is extracted from the leaves, which may require several minutes or more, depending on the type of leaf and the size of the pieces. When extraction is complete, use the test tube clamp to transfer the centrifuge tubes to the test tube rack and allow them to cool.

5. Use the forceps to remove one piece of leaf from the first centrifuge tube and transfer it to a microscope slide. Center the specimen on the stage and observe it with low magnification. Depending on the thickness and level of decolorization of the leaf, you may or may not be able to see significant detail using transmitted light. If not, illuminate the specimen from above with a high-intensity desk lamp or similar external illuminator. Use medium and/or high magnification to reveal additional details. Note your observations in your lab book, and shoot an image if you are equipped to do so.

6. Transfer one drop of Gram's iodine stain to the specimen, allow it to work for 30 seconds or so, and then draw off any excess stain using the corner of a paper towel. Repeat your observations and note any differences. If starch is present in the leaf, it will be visible as intensely blue-black clumps. Once again, record your observations in your lab notebook and shoot an image if possible.

7. Repeat the preceding two steps for each of your other leaf specimens.

The next step is to use paper chromatography to separate the leaf pigments. Depending on the type of leaf, time of year, and other factors, your developed chromatograms may show that anything from two or three to half a dozen or more different pigments are present in an extract.

Some or all of the following pigments may be visible in your chromatograms after you develop them:

- Anthocyanins (red and violet pigments)
- Carotenes (yellow to yellow-orange carotenoid pigments)
- Chlorophyll A (bluish-green pigment)
- Chlorophyll B (yellow to yellowish-green pigment)
- Pheophytin A (bluish-gray pigment)
- Pheophytin B (bluish-gray pigment; often visible only under UV light)
- Xanthophylls (yellowish carotenoid pigments)

1. Prepare as many chromatography vessels as you have extract solutions by filling 50 mL centrifuge tubes to about the 5 mL line with 70% isopropanol and capping them. (If you have more extracts than tubes, don't worry; you can simply develop the chromatograms in two passes.)

Handle the chromatography paper with gloves. Skin oils can interfere with the chromatography process.

2. Cut one chromatography strip about 2.5 x 9 cm for each of your extract solutions. About 1 cm from one end of each strip, draw a light pencil line across the width of the strip. At the far end of the strip, label it, e.g., "extract A."

3. Spot each strip with the corresponding extract. To do so, place the strip on a clean flat surface. Dip the tip of a clean plastic toothpick into the extract solution and transfer the tiny drop on the tip of the toothpick to the center of the pencil line, ideally with the pencil line bisecting the spot. The goal is to make the smallest possible spot on the paper. Allow the spot to dry.

4. Repeat the preceding step several times, transferring more of the extract to the same spot on the paper. The goal is to reinforce that spot, making a small spot with a high concentration of the pigments present in the extract.

5. Repeat the spotting procedure for each of your other extract solutions. Allow all of the spotted paper strips to dry completely.

6. Uncap all of your chromatography vessels and quickly transfer each spotted paper strip to one of the vessels. Immediately recap the vessels.

7. Keep an eye on the development progress, which may take 15 minutes or more. As development progresses, the isopropanol is drawn up the paper strip by capillary action and the pigments begin to separate. When the solvent front reaches nearly the top of the strips, use forceps to remove the strips from the chromatography vessels. Place the strips on a paper towel and immediately make a pencil mark to indicate the furthest extent of the solvent front (which will be invisible after it dries).

8. For each strip, measure the distance from the index line to the solvent front line and record it in your lab notebook. (These values should be nearly identical for all strips, since they were placed into and removed from the chromatography vessels nearly simultaneously.)

9. Measure the distance from the index line to the center of each pigment spot on each chromatogram and record those values in your lab notebook.

If you have a UV (black light) source available, use it to view the chromatograms in a darkened room. Some pigment spots that are invisible under white light may be quite prominent under UV light. (UV LED flashlights are readily available for $10 or less, and are useful not just for biology but for chemistry and forensic science studies.)

As long as you're at it, use the UV lamp to illuminate one of the tubes of pigment extract. Note your observations in your lab notebook.

10. Calculate the R_f value for each pigment spot by dividing the distance from the index line to the pigment spot by the distance from the index line to the solvent front line. For example, if a pigment spot migrated 2.5 cm from the index line and the solvent front line is 7.5 cm from the index line, you'd calculate the R_f value for that pigment as (2.5 / 7.5) = 0.33. Record these values in your lab notebook.

R_f value is a dimensionless number that can be compared to R_f values for other chromatograms only if the combination of substrate (such as this particular chromatography paper) and solvent is identical. For example, if you've run several chromatograms in this procedure, the R_f value for the intense blue-green spot of chlorophyll A should be identical (or nearly so) for each of the extracts. If we ran chromatograms using a different solvent (or a different substrate), the R_f value for chlorophyll A might be very different.

REVIEW QUESTIONS

Q1: In procedure IV-1-1, why did we use a combination of tubes with and without carbon dioxide, Elodea sprigs, and exposure to sunlight?

Q2: In procedure IV-1-1, why did we add a small amount of hydrochloric acid to tubes B and E?

Q3: In procedure IV-1-1, based on your observations of the six tubes, what do you conclude?

Q4: In procedure IV-1-1, propose an explanation for the changes you observed in one or more of the tubes. Why did these changes not occur in all tubes?

Q5: In procedure IV-1-1, what would you expect to happen in tube A if you continued to observe the tube over a period of days, weeks, or months? Why? Would you expect a different result if you also introduced a snail into that tube?

Q6: In procedure IV-1-2, why did we use two tubes at 0.25 meters from the light source, one with and one without a sprig of Elodea?

Q7: In procedure IV-1-2, what did your observed data indicate about the effect of light intensity on photosynthesis rate?

Q8: In procedure IV-1-2, would you expect the (approximate) linearity in photosynthesis rates to remain true at light intensities much, much higher or lower than those you tested?

Q9: The light source we used also produces considerable heat, as do most intense light sources. It is possible that the tubes nearer the light source were warmed by the light source, and that that higher temperature increased the rate of photosynthesis. Design an experiment to determine what effect, if any, different temperatures have on photosynthesis rate.

Q10: In procedure IV-1-3, we implied that the extracts obtained in cold isopropanol and hot isopropanol were for practical purposes identical. What assumption did we make and how might you test that assumption? If that assumption is false, what evidence would you expect to see?

Q11: If you used the UV light in procedure IV-1-3, what results did you observe when you viewed the extract solution? Using Internet resources, research this phenomenon and propose an explanation.

Q12: Using the materials and resources from this group of procedures, design an experiment to determine if photosynthesis requires only chlorophyll, carbon dioxide, and light, or if it can occur only within a plant.