Chapter 8

Diversity of Photosynthetic Pigments

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Introduction

Photosynthetic organisms -- plants, algae, and some bacteria -- rely on a variety of pigments to capture light energy from the sun. These colored compounds are particularly diverse in the algae, and their prominence in the biology of algae is indicated by the very names of many algal divisions. Indeed, photosynthetic pigments reveal much about the physiology of these organisms and their likely evolutionary relationships. In this lab exercise students extract photosynthetic pigments from red, brown, and green algae as well as from a cyanobacterium ("blue-green alga") and from an angiosperm (flowering plant). The pigments are then separated by means of thin-layer chromatography to yield patterns that can be interpreted to make ecological and evolutionary inferences, using the information and questions at the end of the exercise as a guide. Extraction of the pigments, running the chromatograms, and discussion of the results all fit comfortably into a two and a half hour lab period. The exercise is well suited to an introductory level biology or plant diversity course. The set up is moderately time-consuming because of the variety of materials that are needed for each lab group.

Materials

- Centrifuge, small clinical (1)
- Flask, side-arm, fitted with long stem funnel and attached to aspirator at sink (1 or 2 per lab room)
- Flask, side-arm, fitted with Buchner funnel and attached to vacuum line (1 or 2 per lab room)
- Jar for chemical waste jar to receive used chromatography samples and extracts (1 per lab room)

Each group of 4-6 students will need the following:
- Graduated cylinders, 5- or 10-mL (2 or 3)
- TLC strips in glass jar or other lidded container (15-20 strips in each of 2 or 3 jars)
- Test tube racks (3)
- Test tubes, 15-mm diameter (5/student)
- Rubber stoppers, # 0 (5/student)

- Forceps (1/student)
- Scissors, fine pointed (1/student)
- Acetone in labeled 125-mL squirt bottle with cap for nozzle (1) except for *Spirulina* group
- TLC solvent in labeled 125-mL squirt bottle with cap for nozzle (1)
- Capillary tubes (1 vial with 8-10 tubes)
- Spinach leaves, fresh (1/student)
- Coins (2 or 3 - optional)

Separate groups of students extract pigments from different algae for use by all groups in the class. Materials specific for each group are listed below.

Cyanobacterium (*Spirulina*) group

- *Spirulina* powder (about 3-4 g) in small bottles or jars (3 bottles)
- Test tubes, 25 mm diameter screw-top (1/student)
- "Scoopulas" (3)
- Stirring rods, glass (1/student)
- Percale cloth, 10 x 10 cm pieces (4)
- Filter paper circles, Whatman #1, 7-cm diameter (1 box)
- Beakers, 50-mL, labeled "*Spirulina*" (2)
- Beakers, 10-mL, labeled "Cyanobacteria" (1 for each group of students in class)
- Vials (2 or 3 dram), screw top, labeled "*Spirulina*" (1 or 2)
- Extraction solvent for non-polar pigments (2:1 methanol and petroleum ether) in labeled 125-mL squirt bottle with cap for nozzle (1)

Red alga group

- *Porphyra* or other red alga, fresh, frozen, or dried; if fresh or frozen, in large (20 cm) bowl labeled with "red alga" and appropriate genus name (1)
- Mortars, small porcelain (70 mm), and pestles (1/student)
- Sand, fine, in small container with plastic teaspoon (1)
- Percale cloth, 10 x 10 cm pieces (2)
- Beaker, 100-mL (1)
- Beaker, 50-L, labeled "red alga" (1)
- Beakers, 10-mL, labeled "red alga" (1 for each group of students in class)
- Vials (2 or 3 dram), screw top, labeled "red alga" (1 or 2)

Brown alga group

- Brown alga (e.g., (*Fucus* or *Ascophyllum*) in large (20 cm) bowl labeled with "brown alga" and appropriate genus name (1)
- Mortars, small porcelain (70 mm), and pestles (1/student)
- Sand, fine, in small container with plastic teaspoon (1)
- Beaker, 50-mL, labeled "brown alga" (1)
- Beakers, 10-mL, labeled "brown alga" (1 for each group of students in class)

Green alga group

- Green alga (e.g., *Ulva* or *Spirogyra*), fresh or frozen, in large (20 cm) bowl labeled with "green alga" and appropriate genus name (1)
- Mortars, small porcelain (70 mm), and pestles (1/student)
- Sand, fine, in small container with plastic teaspoon (1)
- Beaker, 50-mL, labeled "green alga" (1)

- Beakers, 10-mL, labeled "green alga" (1 for each group of students in class)

Student Outline

Pigment Extraction Procedures

Listed below are the procedures for extracting pigments from an assortment of species. Each lab group will prepare extracts from one division of algae (or the cyanobacterium), with each person in the group carrying out one of the described procedures, as directed by your instructor. The non-polar extracts will then be shared with other groups to enable everyone in the lab to analyze a sample from each species.

Extraction from Cyanobacteria

The cyanobacteria contain both water-soluble (polar) and water-insoluble (non-polar) photosynthetic pigments. Both types can readily extracted from *Spirulina*, a marine species. Conveniently for our purposes, it is cultivated in large saltwater ponds, where it is collected and dried for sale in powdered form as a nutrient supplement.

To extract the polar (water-soluble) pigment proceed as follows:

1. Add ~2 g of *Spirulina* powder to a 25 mm diameter screw-top test tube. This much powder will be about 1 cm deep in the tube.
2. Add 10 mL of water and mix it into the powder with a stirring rod. The *Spirulina* will absorb the water and become a paste.
3. Mix in an additional 20 mL of water to form a slurry. Then tighten the screw cap of the tube *firmly* and shake the tube vigorously for 4-5 minutes.
4. Arrange a single layer of wet percale cloth in a filter mounted on a side-arm flask. Pour the contents of the test tube onto the filter and attach the flask to a vacuum line. Stir the slurry around on the percale cloth with the mouth of the test tube to help the water extract go through more easily. The cloth filter will remove the large algal particles and leave a cloudy solution in the flask. Pour it off into a 50-mL beaker.
5. Clarify the cloth-filtered pigment solution by spinning it in a small, clinical centrifuge for ~5 minutes at the highest speed. Decant the supernatant and, if necessary, filter it through a #1 filter paper in a funnel mounted on a flask attached to a vacuum line. Pour the clear solution into a **vial** labeled *Spirulina.*
6. Thoroughly clean the test tube, funnels, beaker, and flasks. Rinse the percale cloth and spread it out to dry.

To extract the non-polar pigments of Spirulina proceed as follows:

1. Add ~1 g of *Spirulina* powder to a 25 mm diameter screw-top test tube. This amount of powder will fill the tube ~0.5 cm deep, to just above the level of the curved bottom.
2. Add 20 mL of the extraction solvent, a 2:1 mixture of methanol and petroleum ether. Tighten the screw cap of the tube *firmly* and shake the tube vigorously for 3-4 minutes. Allow the contents to settle for 1 minute, then shake for an additional 2 minutes.
3. Place a piece of #1 filter paper into a filter mounted on a side-arm flask. Attach the flask to an aspirator at the front sink. *Do not use a vacuum line when filtering volatile organic solvents like*

pet ether or acetone. Moisten the filter paper with extraction solvent, and filter the contents of the test tube. If the filtrate still contains particles from the *Spirulina* powder, filter it again.

4. Pour the filtered methanol/pet ether extract from the flask into a 50-mL beaker. (Everyone in a group extracting the non-polar pigments members should use the same beaker.) Divide the filtered extract equally into the 10-mL beakers labeled "Cyanobacteria."
5. Thoroughly clean the test tube, funnel, and side-arm flask.

Extraction from Rhodophyta
The red algae also contain both water-soluble and water-insoluble photosynthetic pigments. These will be extracted from *Porphyra* ("laver") or *Palmaria* ("dulse"), both edible seaweeds, or other common genus, such as *Chondrus* ("Irish moss").

To extract the <u>*polar*</u> *(water-soluble) pigments from the red algae proceed as follows:*

1. If using rehydrated pieces of dried *Porphyra* or *Palmaria*, measure out ~8-10 cm^3 of the algae into one of the 10-mL beakers. If using fresh or frozen red algae, cut the tissue into small pieces (~1 cm^2) until the mortar is about one third full. Add ~2 cc of sand and 10 mL of water.
2. With the pestle, grind the algae vigorously for 3-4 minutes to thoroughly macerate the tissue.
3. Add 5 mL more water and grind for 1-2 additional minutes, until a strongly pinkish-colored fluid is produced.
4. Scrape and pour the entire mortar contents into a piece of percale cloth draped over a 100-mL beaker. Draw the cloth around the wet tissue into a ball, then squeeze as much liquid as possible through the cloth and into the beaker.
5. Clarify the liquid either by: (a) spinning it a small clinical centrifuge for ~5 minutes at the highest speed, or (b) running it through a #1 filter paper in a Buchner funnel attached to a vacuum line. Collect the clear extract in a vial labeled "red alga."
6. Clean the mortar and pestle, funnel, beaker, and centrifuge tubes. Rinse the percale cloth and spread it out to dry.

To extract the <u>*non-polar*</u> *pigments from the red algae proceed as follows:*

1. If using rehydrated pieces of dried *Porphyra* or *Palmaria*, measure out ~8-10 cm^3 of the algae into one of the 10-mL beakers. Squeeze out any excess water, blot it dry with a paper towel, and place the alga in the mortar. If using fresh or frozen red algae, blot the clumps of tissue dry with paper towels. Cut the tissue into small pieces (~1 cm^2) until the mortar is about one third full. Add ~2 cc of sand and 6 mL of acetone to the mortar.
2. Grind the tissue vigorously for ~4 minutes, *or* as long as necessary for the tissue to be thoroughly macerated and the solvent to become a dark green. Pour off the solvent extract into a 50-mL beaker labeled "red alga." (All group members should pour their extract into the same 50-mL beaker.)
3. Add an additional 5 mL of acetone to the mortar and grind for 3 more minutes, or until the extract is once again dark green. Then carefully pour all of the remaining extract into the labeled 50-mL beaker. You may need to use the bottom of the pestle to help squeeze out as much solvent as possible from the ground tissue.

4. Clarify the extract by centrifuging it for 1-2 minutes at the highest speed to sediment out the particulate matter. Decant the clear extract into the 10-mL beakers labeled “red alga.”
5. Thoroughly wash the mortar, pestle, and centrifuge tubes.

Extraction from Phaeophyta

To extract pigments from a brown alga you will use either *Fucus* or *Ascophyllum*, two intertidal seaweeds from cold waters, or *Sargassum*, a warm-water genus. Proceed as follows:

1. Take a clump of fresh alga and blot it dry with paper towels. If using *Fucus*, look for receptacles, slightly knobby-textured reproductive structures that form at the tips of the blades during the winter months. If these are present, remove them. They contain gelatinous colloids that will gum up your grinding.
2. Cut the alga over a small mortar into pieces ~0.5-1.0 cm long until the mortar is about one third full. (You may find it easier to put an intact clump of algae into the mortar and cut it up in place with the tips of your scissors.)
3. Add ~5 cc of sand to the cut pieces of alga and grind with the pestle for 1-2 minutes to tear the tissue.
4. Now add 8-10 mL of acetone and grind vigorously for ~4 minutes, or as long as necessary for the solvent to become a dark green. Carefully pour the solvent extract into a 50-mL beaker labeled with the name of the genus you used. (All group members should pour their extract into the same beaker.)
5. Add an additional 4 mL of acetone, and grind for 2 more minutes or until the solvent again becomes dark green. Pour the extract into the same 50-mL beaker. Let the contents of the beaker settle.
6. Wash the mortar and pestle thoroughly.
7. Carefully decant into 10-mL beakers labeled “brown alga” the clear acetone extract that forms the upper layer of the fluid in the large beaker. *Do not* pour out the sediment or the cloudy, aqueous solution from the bottom layer.

Extraction from Chlorophyta

As an example of the green algae, use either *Ulva* (sea lettuce) or a freshwater, filamentous species such as *Spirogyra*. Proceed as follows:

1. Blot the sheets or clumps of algae dry with paper towels. For filamentous species put enough dried mass into a small mortar to completely cover the bottom. For *Ulva* cut the sheet into small pieces (~1 cm^2) until the mortar is about one third full.
2. Add ~2 cc of sand and 5 mL of acetone. Grind with the pestle for 3-4 minutes, or for as long as necessary for the solvent to become a dark green. Carefully pour the solvent extract into a 50-mL beaker labeled with the name of the genus you used. (All group members should pour their extract into the same beaker.) If there is too little extract to pour, add 2-3 mL of acetone to the ground tissue.
3. Add an additional 4 mL of acetone and grind for 1 minute, or until the solvent again becomes dark green. Pour the solvent extract into the beaker, then use the bottom of the pestle force out as much extract as possible. Let the contents of the beaker settle.

4. Wash the mortar and pestle thoroughly.
5. Carefully decant into 10-mL beakers labeled “green alga” the clear acetone extract that forms the upper layer of the fluid in the 50-mL beaker. *Do not* pour out the sediment or the cloudy, aqueous solution from the bottom layer.

Chromatography procedures

The organic solvent (acetone or methanol/pet ether) extracts contain a mixture of pigments, which you will separate by thin layer chromatography, or TLC. This process uses a thin plastic plate coated with silica gel. A sample of a mixture of compounds is deposited near the bottom of the plate, and the plate is then placed vertically in a suitable solvent. As the solvent is wicked up, it passes the sample and starts to carry the compounds upward with it. Different compounds dissolved in the solvent adsorb to the silica gel to different degrees; the more polar a molecule, the more strongly it is adsorbed. (Note: *adsorb* means adhere to the surface of another compound without forming a chemical bond.) As a result, some compounds, the relatively more polar ones, remain near the bottom of the plate, while other, less polar ones, are carried by the solvent nearer the top.

Each student will make separate chromatograms from the *organic solvent* (acetone or methanol/pet ether) extracts of the cyanobacteria, red, brown, and green algae, and from leaves of a representative flowering plant, spinach. You will *not* make chromatograms from either of the aqueous extracts.

1. Take five plastic TLC strips, and check to be sure that each one will fit into the stoppered 15-mm diameter test tubes you will use as chromatogram chambers. The top of the strips should not touch the rubber stopper when it is inserted, and the edges of the strips should clear the sides of the test tube by at least 1 mm. If a strip is too wide or too tall, trim it to fit with scissors, or choose another one.
2. Near the top edge of each TLC strip, on the silica gel (rough) side, label the pigment source, e.g., "**C**" for cyanobacteria, "**R**" for red alga, “**B**” for brown alga, “**G**” for green alga, and “**A**” for the angiosperm, spinach. Do this in pencil *very gently* otherwise the silica gel will crack off.
3. Using a capillary tube, apply a row of spots of the appropriate extract along a line 2 cm from the bottom of TLC strip. (See diagram below, Figure 1.) To control the size of the spots, hold your finger firmly over the top of the tube, then gently touch it to the strip so that only a small amount of extract is wicked out. Allow the spots to dry *completely*; this may take 30 seconds to a minute. Then apply another row of spots on top of the first one. The more times you spot the strip the brighter your chromatogram will be, but if you don't allow the spots to dry between repeat applications the bands of separated pigments may be blurred. The diagram on the next page shows the number of applications recommended for each type of sample. Your instructor may modify the recommendations depending on the concentration of your extracts.
4. Use a different, simpler technique to transfer the spinach (angiosperm) pigment to the TLC strip. Place a leaf over bottom end of the strip, with the coated side of the strip *up* and the underside of the leaf *down*. Using the edge of a coin (a nickel or a quarter works well), crush a line of tissue onto the TLC strip two cm from the bottom edge. Roll the coin firmly enough that you apply a thin layer of tissue to the strip but not so hard that you tear through the leaf or break the silica gel coating. Repeat using a fresh surface of leaf until you have made a distinct, uniform band about 2 mm wide. Two or three applications should be sufficient. Try to avoid getting spots from the

bruised leaf tissue on parts of the strip other than the intended band, as they will make the chromatogram blurry.

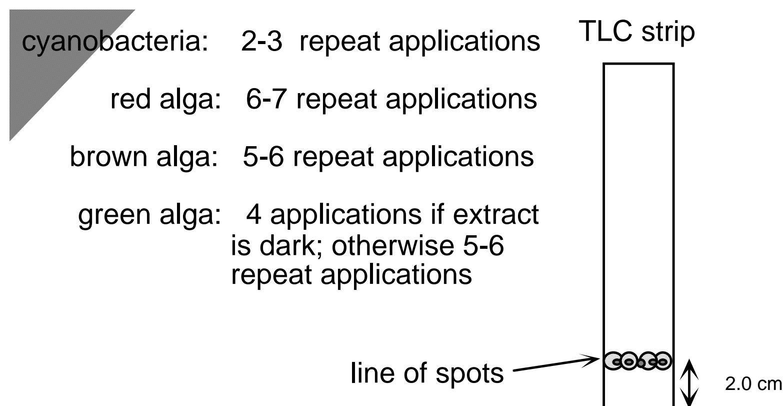

Figure 1. Diagram showing application of acetone extract to thin-layer chromatography (TLC) strips.

5. While you are waiting for the acetone extract spots to dry, set up five 15-mm diameter test tubes in a test tube rack so that they are completely vertical. Add a *small* squirt of the chromatogram solvent to the bottom of each tube. The solvent should just fill the curved bottom of the tube. Avoid letting the solvent run down the sides. If you are not yet ready to insert a TLC strip, stopper the tube.
6. Lower one prepared TLC strip into each tube, being careful not to let it slide down the sides of the tube. You may find this easier to do if you hold the top of the strip with forceps. Leave the tube in the rack while inserting the strip; if you pick up the tube you risk splashing the solvent over the pigment spots thereby ruining the chromatogram. After inserting a strip, stopper the tube *gently*.
7. When all the chromatograms have been started, watch the solvent fronts move and the pigments separate. Remove the strips when the solvent front comes to within 1-2 cm of the top.
8. When all the chromatograms are complete, *pour all of the solvent from the tubes into the designated chemical waste jar*. **Do not** rinse the empty chromatogram tubes with water.

Identifying photosynthetic pigments

There are three broad classes of photosynthetic pigments: chlorophylls, carotenoids, and phycobilins. Each class includes several pigments with similar chemical structures. Different pigments absorb light at different wavelengths, and hence differ in color. (Why?) Because they differ in chemical structure, they also vary in their solubility in water and degree of polarity and adsorbance to silica gel. This information is summarized in Table 1 on the next page.

Sketch the banding patterns you obtained on your chromatograms on the diagram on the next page, Figure 2. You should do this as soon as you remove the strips from the tubes because some of the fainter bands may fade quickly under fluorescent lights. Using the information in Table 1, identify the pigment responsible for each band, and fill in the right-hand column of the table. Use this summary of the distribution of photosynthetic pigments in different groups of organisms to help you answer the questions in the next section.

Table 1. Characteristics of photosynthetic pigments.

Pigment	Color	Water soluble?	Position on TLC strip*	Occurs in:
phycobilins:				
phycoerythrin	red	yes	- *(not applicable)*	
phycocyanin	blue	yes	- *(not applicable)*	
carotenoids:				
carotene	orange	no	very high	
xanthophylls	yellow, orange	no	moderate to low	
chlorophylls:				
chlorophyll *a*	bluish green	no	high	
chlorophyll *b*	yellow green	no	slightly lower than chl *a*	
chlorophyll *c*	light green	no	very low	

* Note: A high position -- nearer the top of the chromatogram -- means the pigment adsorbs weakly to the silica, while a low position -- nearer the origin -- means it adsorbs strongly.

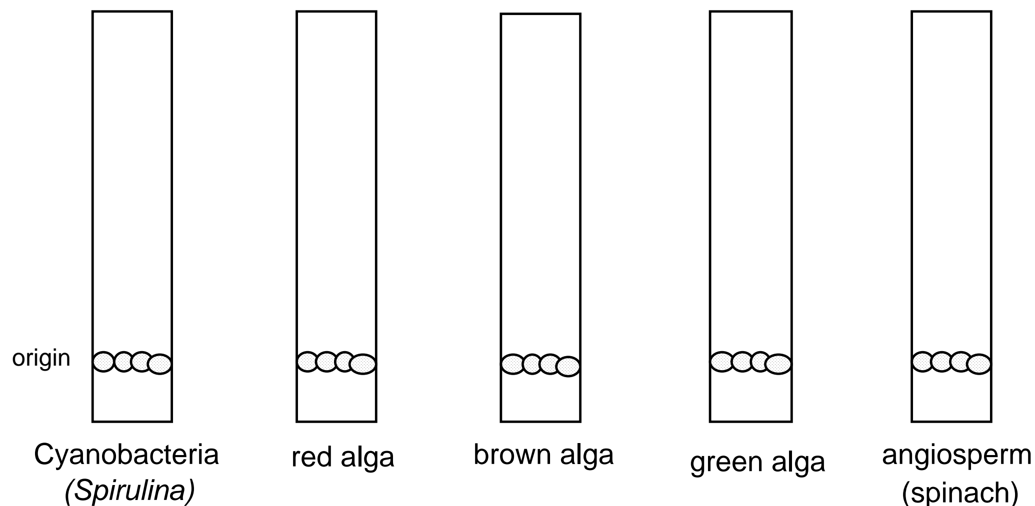

Figure 2. Patterns of photosynthetic pigments on TLC strips for organic solvent extracts from five different sources.

Interpreting patterns in photosynthetic pigments

Isolation of photosynthetic pigments from many species clearly shows that the pigments do not occur in different groups at random. Knowing this, you can use your chromatogram results to (1) infer evolutionary relationships among groups, (2) detect evidence of possible multiple origins of photosynthetic capability in eukaryotes, and (3) evaluate the ways different groups have solved the physiological problem of obtaining light for photosynthesis in different habitats. Refer to your chromatograms and Table 1 to answer the following questions.

1. Notice that all of the groups have chlorophyll *a*. If, as fossil evidence indicates, the Cyanobacteria evolved before the algae or plants, what could you conclude about the evolutionary origin of this pigment in the chloroplasts of eukaryotes?
2. Do any of the chromatograms share the same banding pattern? If so, which ones? What does this suggest about common ancestry of these groups? Why?
3. What evidence from the pigments suggests that the photosynthetic apparatus of red algae evolved independently from that of green or brown algae?
4. The distinctive orange-brown band in your *Fucus* or *Ascophyllum* chromatogram is **fucoxanthin**. This pigment is characteristic of the Phaeophyta. It also occurs in the Chrysophyta (golden brown algae), Bacillariophyta (diatoms), and in some dinoflagellates. So does another characteristic phaeophyte pigment present in the brown algal chromatogram. What is this other pigment?

   The similarity of pigments in these divisions suggests a common origin for their chloroplasts and photosynthetic apparatus, but one of them is probably *not* closely related to the others. Which three are probably closely related, and what evidence are you aware of, besides photosynthetic pigments, that suggests a common ancestry?
5. Chlorophyll *a* is the primary pigment participating in the chemical reactions of photosynthesis. The others are all **accessory pigments**, which capture light at different wavelengths than chlorophyll *a* then transfer the light energy to it. You can get some idea how accessory pigments work from the aqueous solutions of phycobilins.

   Hold the vials in the sunlight or other strong light source. How does their appearance change? What you are seeing is **fluorescence**. The light absorbed by the pigment molecules in solution excites electrons that subsequently fall back to their resting energy level, and in doing so emit photons of light. It is these electrons that in an intact cell transfer light energy to the chlorophyll *a* molecules.
6. The Euglenophyta have the same photosynthetic pigment distribution as the Chlorophyta, but their chloroplasts are enclosed by a *triple* membrane, rather than the usual double membrane found in chloroplasts of green algae. From this information and your observations earlier in the lab, offer a hypothesis that explains how the Euglenophyta may have acquired their photosynthetic apparatus.
7. Knowing that the longer (red) wavelengths of light are absorbed preferentially as light travels through water, what benefit might be conferred by the fucoxanthin in brown algae and phycoerythrin in red algae for species living in deep water?
8. A common alga of fresh, brackish, and marine waters is *Vaucheria*. It is a bright yellow-green color, and belongs to a group called the yellow-green algae. Because of its color and filamentous growth form it might be mistaken for a green alga upon casual examination. However, its chloroplasts lack chlorophyll *b* but have chlorophyll *c*. Based on this evidence to which division of algae do the yellow-green algae actually belong?
9. The obvious color of a seaweed specimen does not always tell you in which division it should be classified. Species of Rhodophyta can sometimes be yellow, brownish, black, or green. For example, along the coast of the southeastern U.S. there are two different color morphs of a common alga called *Gracillaria*; one is red, the other is green. Both are the same species, but

the green morph has a mutation that blocks the production of phycoerythrin. How could you demonstrate from pigment analyses that a green specimen of *Gracillaria* belonged in the division Rhodophyta and not Chlorophyta?

Notes for the Instructor

Introduction

As indicated by the student Introduction and the questions at the end, the exercise as presently written emphasizes algal diversity. The procedures take about two hours to complete and thus can be effectively complemented by providing live and preserved specimens for students to observe. These can include not only the species used in the chromatography (*Spirulina* and *Spirogyra* are both delightful under the microscope) but also additional taxa to illustrate other growth forms, e.g., unicellular green algae, crustose and filamentous red algae, and large-bladed brown algae such as the kelps. Specimens of other prominent algal groups, such as diatoms, dinoflagellates, and *Euglena*, could also be included for even greater variety.

Another way to use the exercise is to adapt it for a photosynthesis lab to emphasize the variety and properties of accessory photosynthetic pigments. For example, the pigment bands on the TLC strips can be readily cut apart and the pigments individually re-solubilized in acetone. Bands from about 15-20 strips placed in about 5 mL of acetone yield a colored solution sufficiently concentrated to measure an absorption spectrum with a spectrophotometer. To save time in the lab, the extracts can be prepared ahead of time for the students and stored in a refrigerator in tightly sealed, dark containers. If time permits using only one group in addition to angiosperms, a brown alga is probably best because of the distinctive chlorophyll *c* and the strikingly orange fucoxanthin band.

Prep Notes

Students should be divided into groups, with each group responsible for one alga. Groups should include enough students that they can supply acetone extract to all of the groups in the lab; for a lab of 24 students four groups of six students works well. The amount of extract one student should produce is somewhat more than a group of 4-6 students would need to spot their TLC strips, but having more than the minimum number of students making an extract provides insurance against spills or weak solutions. If groups sizes are unequal or the total number of students is small, the *Spirulina* and red algal groups will need more help because these groups produce both organic solvent and aqueous extracts. (In the green and brown alga groups all students produce acetone extracts.) Because the finished aqueous extracts are delivered to the instructor for demonstration purposes, no more than two students per *Spirulina* and red alga group need to be involved in their production.

Individual TLC strips are 8-12 mm wide and 83-87 mm long and are cut with scissors from precoated 20 cm x 20 cm sheets. The "Silica Gel 60" plastic-backed TLC plates manufactured by E. Merck work well. (See Appendix A for sources.) To avoid contaminating the sheets, the people cutting the strips should wash their hands first and/or wear thin cotton gloves. Cut TLC strips are stored in double zip-locked bags before lab to reduce hydration of the silica gel. If absorption of atmospheric moisture is likely to be a problem, the strips should probably be kept in a desiccator. Fresh strips are put out as necessary for each day of the lab.

Chromatogram tubes are emptied of solvent (in the designated solvent waste jar) but are *not* washed with water at the end of lab because of the difficulty in drying them completely before the next lab period. Percale cloth filters are rinsed and reused.

The most important points to watch for in this exercise are: (1) grinding the algae sufficiently vigorously to produce strongly-colored extracts, (2) ensuring that all extracts are carefully decanted so that they are not contaminated with water or sediment, and (3) controlling the size of the spots applied to the TLC strips by letting spots dry between applications and by keeping a finger firmly over the end of the capillary tube so that only a small amount of solvent is drawn out onto the strip. It is especially important to make the extracts as darkly colored as possible. Obtaining strongly colored extracts of the red alga requires very vigorous grinding, especially if dried *Porphyra* is used. To a certain extent, dilute extracts can be compensated for by spotting the TLC strips more frequently (the higher applications rate shown on Figure 1 for the red and brown algae reflects the sometimes weaker concentration of these extracts), but that takes more patience and increases the difficulty of controlling the spot sizes.

Other potential student problems to be aware of include strips that are too wide and wedge into the bottom of the tube (the pigments form streaks along the edge of the strip), too much TLC solvent in the test tube or spots too low on the strip (the extract is immersed in the solvent and dissolves off the strip), and using acetone instead of the TLC solvent (the pigments do not separate).

Solvent preparation and safety notes

About 200-250 mL of acetone is needed per lab section (24 students) for extraction of non-polar pigments. About 50-75 mL of the methanol and petroleum ether (2:1) extraction solvent is needed for the *Spirulina* group. This mixture should be made fresh each semester because the pet ether is itself a mixture of solvents with different boiling points and so can change composition over time.

Allow about 0.7 mL of TLC solvent for each chromatogram. With 5 chromatograms per student and 24 students per lab, a total of about 100 mL of solvent is needed for each section. Three TLC solvent combinations can be used; each has advantages and disadvantages as described below. Because they are mixtures of compounds with different volatilities, and the ratio of parts is critical for the chromatography, they should be made up fresh (ideally no more than a few weeks in advance of the lab) and stored in tightly sealed containers.

a) *6 parts toluene : 4 parts acetone* (volume : volume). This mixture gives the fastest run times and the greatest separation between individual pigment bands. The solvent front carries the carotene band as a narrow, but distinctly yellow-orange line to within 2 cm of the top of the TLC strip in 6-11 minutes, although the individual pigments are clearly separated before then. The carotene band is very sharp while the chlorophyll and xanthophyll bands are usually distinct, but sometimes with blurring of the chl *a* and *b* bands on their trailing edges.

    A potential disadvantage of this mixture is its use of the aromatic hydrocarbon toluene. References on the hazards of organic compounds describe somewhat lower permissible exposure concentrations for toluene than for non-aromatic solvents such as cyclohexane, acetone, or pet ether, but indicate that toluene is not nearly as hazardous as the most familiar aromatic solvent, benzene (Sax, 1975). Indeed, much of the toxicity attributed to toluene in industrial settings may instead be due to benzene contamination. It is therefore important that toluene used in the lab be at least reagent grade.

b) *8 parts cyclohexane : 1 part 2-propanol (isopropyl alcohol)*. The run times for this mixture are slower than for toluene/acetone, 15-20 minutes for the solvent front to reach 2 cm from the top.

Although the bands are not as widely separated as with the toluene/acetone mixture, they tend to be quite clear, and the chlorophyll bands are often more distinct. However, compared with the toluene/acetone mixture, the carotene band is more diffuse and appears more yellow, and it runs far ahead of the chlorophyll and xanthophyll bands. Also, the breakdown products of chlorophyll (see Appendix B: Expected Results) seem to be more visible with this mixture, not necessarily an advantage when students are interpreting their results.

c) *5 parts petroleum ether : 1 part acetone*. This mixture requires about 15 minutes for the solvent front to advance within 2 cm of the top. The xanthophyll and chlorophyll bands are less widely separated than in the toluene/acetone mixture and less distinct than in the cyclohexane/propanol mixture, with the chl *b* band sometimes very close to the top xanthophyll band. Also, compared with the other mixtures pet ether/acetone yields a carotene band that is more yellowish and less distinct, and for the brown algae, a chl *c* band that remains even closer to the origin. Due to the greater volatility of this mixture, the rubber stoppers often pop out of the test tubes before the chromatograms are finished. In addition, because the pet ether is a mixture of compounds with different boiling points its composition is likely to change if not used promptly or kept stored in tightly sealed containers.

All of the TLC mixtures, and the acetone, present a flammability hazard and are potentially harmful if breathed in high concentration or left in prolonged contact with skin. They should thus not be used in the presence of open flames, and the lab rooms and prep spaces should be well ventilated. The procedures in the exercise are designed to minimize the total amount of solvents used, but any liquid that is left in the chromatogram tubes or in beakers of extract should be poured into a chemical waste jar. If a hood is available, the chemical waste should be collected there and the chromatogram tubes placed inside the hood to dry after they are emptied. (Solid waste, e.g., TLC strips, filter papers, and ground algal tissue from mortars, can be disposed of in the trash.)

Answers to Discussion Questions

Brief answers to the questions are given below. For an informative but not overly technical discussion of algae, consult Raven *et al*. (1999). A more thorough treatment of algal photosynthetic pigments is given in Rowan, 1984. For general information on the characteristics of photosynthetic pigments and on photosynthesis consult Salisbury and Ross (1985), a standard plant physiology text.

1. Eukaryotes most likely obtained Chl *a* from a prokaryote, probably some kind of cyanobacterium or very similar organism. The presence of Chl *a* in cyanobacteria and in the chloroplasts of all eukaryotic organisms supports the theory that chloroplasts arose by endosymbiosis. (Note: The original chloroplast symbionts were not *Spirulina* itself, but rather some species that lived billions of years ago.)

2. Green algae and plants (e.g., spinach) both have the same pattern: carotene, Chl *a* & Chl *b*, and similar xanthophylls. This supports the theory that the ancestor of plants was a green alga (e.g., something like a modern charophyte such as *Nitella*) rather than a brown alga, which lacks Chl *b* and has Chl *c*.

3. The pattern of photosynthetic pigments in red algae corresponds closely to that of cyanobacteria, with the presence of phycobilins and the absence of Chl *b*. This suggests that red algae may have captured their own cyanobacteria symbiont independently of brown or green algae, both of which lack phycobilins and have an alternate form of chlorophyll. (Note: Biochemical evidence

suggests that the green algae and brown algae may also have acquired chloroplasts independently of each other.)

4. The other distinctive pigment is Chl *c*. Although diatoms and dinoflagellates are both unicellular and have armored cell walls, the dinoflagellates are probably *not* very closely related to either chrysophytes, diatoms, or brown algae. Dinoflagellates store food as starch (unlike diatoms and brown algae), have unusual chromosomes and a distinctive form of mitosis, and are sometimes heterotrophic. Diatoms, chrysophytes, and brown algae, however, probably do share the same common ancestor as reflected in their carbohydrate storage compounds, their shared distinctive types of flagella, and very similar photosynthetic pigment distribution.

5. Note: This can done as a demonstration by the instructor or by students themselves in groups. When viewed by reflected light or by transmitted light that is not too strong, the aqueous *Spirulina* extract is a translucent blue due to the dominance of the phycocyanin, and the red algal extract is a translucent pink due to the dominance of phycoerythrin. When the extracts are placed in bright light, such as that of a high-intensity reading lamp, and viewed from the side so that the light is not shining in the eyes, the extracts appear to change color and are not longer translucent. This is a result of fluorescence. The light absorbed by the pigment molecules excites the electrons used in photosynthesis, which in the absence of an intact chloroplast, fall back to their resting level and emit photons of light at a characteristic wavelength. The light-stimulated *Spirulina* extract thus turns red (or a glowing light blue with long-wave UV light) and the red algal extract turns orange. The fluorescence is evidence that phycocyanin and phycoerythrin are used in photosynthesis. These pigments can be contrasted with another common aqueous pigment found in plants, anthocyanin. This pigment occurs in vacuoles, where it gives red onions their color, and does not fluoresce. (Note: Fluorescence of the chlorophyll molecules on the TLC strips can be observed in a darkened room by illuminating the strips with a long-wave UV lamp.)

6. One hypothesis is that an ancestral euglenoid acquired a unicellular green alga as a symbiont, and it in turn provided chloroplasts that allowed the euglenoid to become phototrophic. Thus the chloroplasts of a *Euglena* not only have the characteristic double membrane of chloroplasts and mitochondria but also a third membrane derived from the cell membrane of green algal symbiont.

7. Fucoxanthin and phycoerythrin, because they appear orange and red (i.e., reflect colors at the longer wavelength end of the visible light spectrum), absorb most strongly at the shorter wavelengths, green and blue. These wavelengths pass most readily through water and are the ones most available in deep water. Algae in such habitats that have accessory pigments that can absorb shorter wavelengths might thus be more efficient at photosynthesis. It turns out, however, that other physiological responses, including the total amount of all photosynthetic pigments, are at least as important in determining algal distribution with depth. For an engaging review of the significance of accessory pigments in seaweed ecology and the distribution of red algae, see Saffo, 1987.

8. Yellow-green algae such as *Vaucheria* are probably most closely related to brown algae, the other group of multicellular algae that possess chlorophyll *c*. (These algae also contain a xanthophyll related to the fucoxanthin found in brown algae – vaucherixanthin – that contributes to their color.)
9. Green algae have Chl *b* but red algae do not.

Acknowledgments

The procedures using *Spirulina* are adapted from an exercise developed by Kathleen Nolan of St. Francis College, Brooklyn, NY. Her original work is gratefully acknowledged.

Literature Cited

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Appendix A: Sources of Materials

The "Silica Gel 60" plastic-backed TLC plates manufactured by E. Merck work well. On source is their subsidiary, EMD Chemicals <http://www.emdchemicals.com/analytics/EMD_Analytics.asp>. A box of 25 sheets (20 cm x 20 cm) lists for about $154.00 US and should yield over 1,000 strips.

Spirulina powder is readily available from health food stores and is used just as is. A convenient source of red algae is dried "laver" (*Porphyra umbicalis*) or “dulse” (*Palmaria*) from Maine Coast Sea Vegetables (Franklin, Maine 04634; *<http://www.seaveg.com/whleaf.html>* or phone 207-565-2907). The most economical package is one pound for about $15.00 US, an amount sufficient for many hundreds of students. (Note: The thin sheets of dried "nori" that are sold in the Oriental food section of groceries are not recommended as the pigments have been too degraded during processing.) To save time grinding dried *Porphyra* or *Palmaria* in the mortars, the dried blades are first chopped briefly in a blender into fragments several millimeters across. These are then rehydrated at the start of the lab by adding just enough water for

the dried pieces to absorb without becoming dripping wet. It is much more difficult to grind the dried material without first rehydrating it. In principle, any fresh, non-filmentous red seaweed should also work. Fresh *Porphyra* and the morphologically similar *Palmaria* both yield strong extracts. We have had some success with *Chondrus crispa* (“Irish moss”), a common intertidal species in the North Atlantic, although sometimes the acetone extract is a bit weak. *Porphyra, Palmaria,* and *Chondrus* can be ordered from the Marine Biological Laboratory's Department of Marine Resources (see below).

Ascophyllum or *Fucus* can sometimes be obtained free from local seafood markets, because these seaweeds have traditionally been used as a packing material for lobsters, clams, and mussels shipped from New England. It is best to check availability with several merchants well ahead of the lab period. Both species can be stored for several weeks in a refrigerator in buckets of seawater, including "Instant Ocean" mix, with no ill effects. An alternative source for *Fucus* (and possibly *Ascophyllum*) is the Marine Biological Laboratory's Department of Marine Resources (Woods Hole, Massachusetts 02543; *<http://www.mbl.edu/marine_org/marine_supply.html>,* or phone 508-548-3705 ext. 375). An approximately 3-liter quantity of either genus cost about $30.00 US in 2003 (but with a minimum order charge of $75.00 US) and is available year round. For pigment extraction purposes this volume should be enough for 3-4 sections of 24 students; the amount needed for one section is roughly one large handful. Fresh *Sargassum* can be substituted for *Fucus* or *Ascophyllum* -- it is also a member of the Fucales and produces a strong fucoxanthin pigment band -- but it seems to be more susceptible to contamination by epiphytes in other algal divisions. Dried kelp, *Laminaria*, does not work well.

Spirogyra is common in farm ponds, although it is a good idea to check on potential sources in advance as the abundance in a given location may shift unexpectedly. It should be collected fresh for the lab, especially in warmer months, cleaned of mud or any dead plant debris, and stored in a refrigerator for at most a few days to a week. Although other filamentous green algae should also work, *Spirogyra* is easy to recognize and collect because of its bright green color and slippery texture. (It is also aesthetically pleasing for students to observe under the microscope.) Fresh *Ulva* (sea lettuce) can be substituted for a freshwater, filamentous form, although care must be taken to avoid including epiphytic algae from other divisions. It does not store in a refrigerator as well as the brown algae -- a week to 10 days is about the maximum -- and is sometimes tougher to grind than filamentous forms. *Ulva* is also available from the Marine Biological Laboratory in two forms, as food (for sea urchins) and as student observation material. The latter is more expensive ($30.00 US a bucket) but is sorted to reduce contamination with other species. For both the *Spirogyra* and *Ulva* the amount needed for a section of 24 students is a large handful, or about the quantity that could be wadded into a 250-mL beaker.

Recently, Carolina Biological Supply Company (2700 York Road, Burlington, North Carolina, 27215-3398 or PO Box 187 Gladstone, Oregon 97027-0187; phone (800) 334-5551) has included living, collected seaweed in their catalog, including *Ascophyllum*, *Fucus*, *Ulva*, and *Porphyra*. They offer a "generous portion, freshly collected and shipped from Maine" for about $26.00 US.

Note: Excess quantities of all of the cold water seaweed species can be frozen while wet with saltwater, then thawed for use at a later time without at all adversely affecting their photosynthetic pigments.

Appendix B: Expected Results

Three types of pigments are extracted in this exercise: phycobilins, carotenoids, and chlorophylls. The phycobilins occur only in the cyanobacteria and the red algae. In contrast to the carotenoids and chlorophylls, the phycobilins are polar and water-soluble. When they are extracted they remain associated with the proteins that help determine their absorbance characteristics and hence their color. The two most abundant phycobilins are phycocyanin (blue) and phycoerythrin (red). They occur together in the same organism, although as the names of the groups imply, phycocyanin predominates in cyanobacteria and phycoerythrin predominates in red algae. This is evident in the aqueous extracts from *Spirulina* and *Porphyra*. No attempt is made in the exercise to separate different kinds of phycobilins in the aqueous extracts.

The carotenoids are long-chain hydrocarbons and are of two types: the carotenes and xanthophylls. The only carotene of importance in photosynthesis is the widely occurring beta-carotene, the familiar source of color in carrots. Xanthophylls are much more variable than carotenes, and all are distinguished from carotenes by having at least one oxygen attached to them. A particularly common xanthophyll (prominent in plants and green algae) is lutein. Like many xanthophylls it is yellow. A distinctive, more orange-colored xanthophyll is fucoxanthin, the pigment that gives the brown algae and related groups (such as the diatoms) their characteristic color.

The chlorophylls are the dominant non-polar photosynthetic pigments. (Notice that all of the non-polar extracts, including those from cyanobacteria and red alga, are distinctly shades of green.) Chlorophylls consist of a magnesium-containing porphyrin group typically attached to a hydrocarbon chain, the phytol tail (see Salisbury and Ross, 1985, for illustration). Chlorophyll *a* is the form that participates most directly in the light reactions of photosynthesis and is found in all of the groups. Chlorophyll *b* is found in plants and green algae, and is very similar to Chlorophyll *a,* differing only in the substitution of an aldehyde for a single methyl group on the porphyrin ring. This small change is sufficient to alter the absorption properties of the molecule and makes Chlorophyll *b* appear yellow-green while chlorophyll *a* looks blue-green. Chlorophyll *c*, which occurs in the brown algae and related groups, is identical to chlorophyll *a* except that it lacks the long phytol tail.

Figures 3 and 4 below show the expected banding patterns for TLC strips developed in the toluene/acetone and cyclohexane/propanol mixtures, respectively. Note that the order in which the pigments separate is the same for both solvent mixtures. At the top is beta-carotene, which is the most non-polar and fastest moving pigment. In the toluene/acetone mixture, beta-carotene forms a distinct thin, orange line that travels with the solvent front. In the cyclohexane/propanol it forms a more diffuse yellow band slightly behind the front. It may be very faint or not visible in the red alga strip unless the spotting solution is very concentrated. Chlorophyll *b* comes out just below Chlorophyll *a*, a consequence of the slightly greater polarity conferred by the substitution of the aldehyde for the methyl group. The various xanthophylls, because the oxygens attached to the ends of their chains make them more polar than the beta carotene, sort out below the chlorophylls. Toward the bottom, nearest the origin, is chlorophyll *c*, which because it lacks the long hydrocarbon tail of the other chlorophylls is much more polar and thus strongly adsorbed to the silica gel.

A major difference between the two solvent systems is that the chlorophylls and xanthophylls do not migrate as fast relative to the beta carotene in cyclohexane/propanol as they do in toluene/acetone and as a result the bands in cyclohexane/propanol come out closer together. Nevertheless, the separation between chlorophyll *a* and chlorophyll *b* in this system is usually quite sharp, with less of a blurring below the trailing edge that sometimes occurs with toluene/acetone. Other differences in results between the two solvent systems are that the xanthophyll bands in cyclohexane/propanol are sometimes a paler yellow and harder to distinguish while the bands of the breakdown products of chlorophyll are more obvious. (See comments below.)

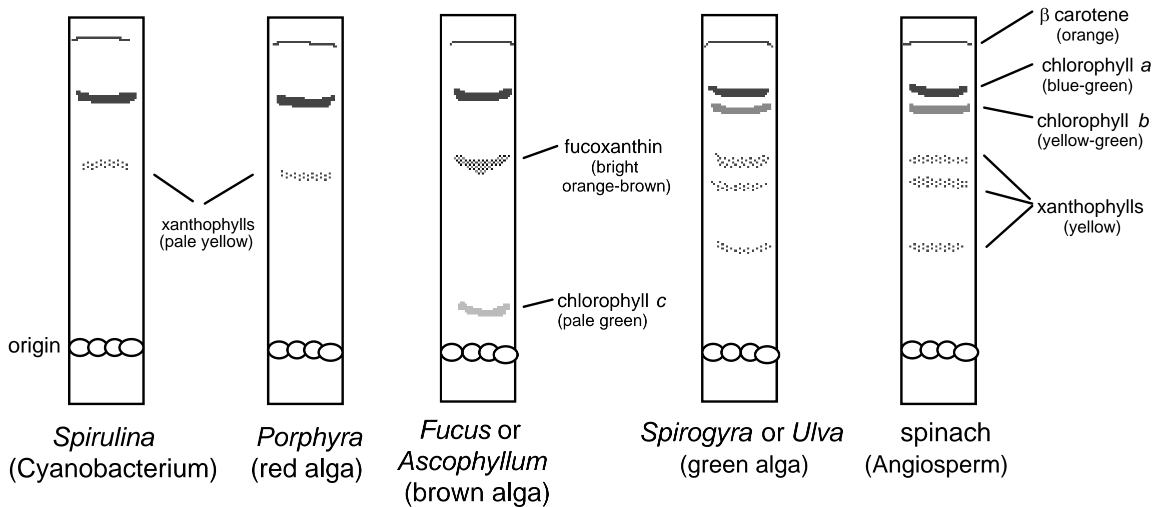

Figure 3. Expected separation of non-polar photosynthetic pigments on silica-gel thin layer chromatography strips using toluene/acetone as the developing solvent.

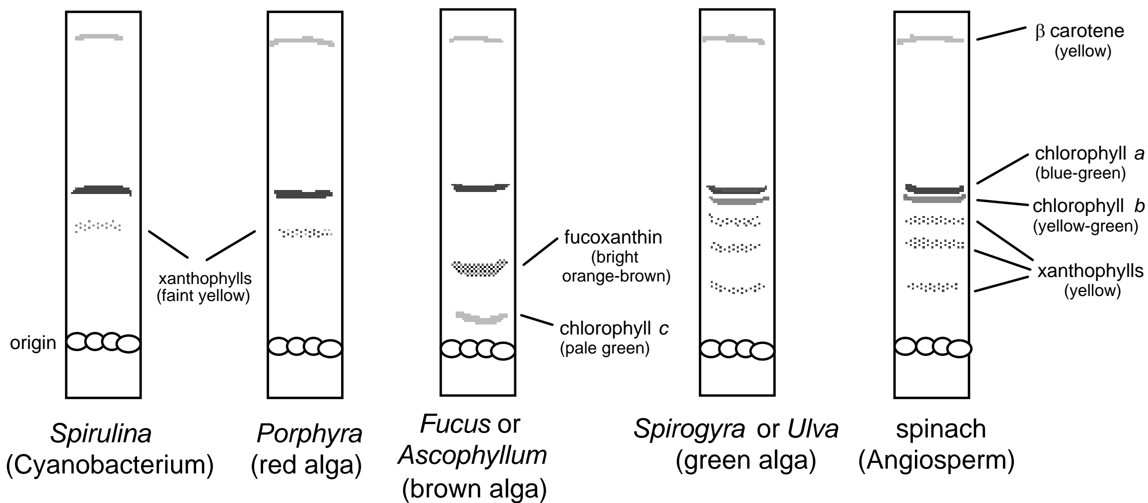

Figure 4. Expected separation of non-polar photosynthetic pigments on silica-gel thin layer chromatography strips using cyclohexane/isopropanol as the developing solvent.

The patterns illustrated above assume no contamination of the pigment sources by algae in other divisions. However, TLC is a very sensitive technique, and faint but noticeable fucoxanthin and chlorophyll c bands can be produced in extracts of fresh *Ulva*, *Porphyra,* or *Palmaria* by diatoms that are too few in number to form a film visible to the naked eye. They can, however, be detected under the compound microscope by making a wet mount of a small piece of the green or red algal thallus. Although the diatoms slightly complicate interpretation of the expected TLC patterns, the presence of these “bonus” organisms provides an opportunity to point out their phylogenetic connection to the macroscopic brown algae, and their direct observation with the microscope provides a way to test the hypothesis that they are indeed the cause of faint, unexpected bands on the red or green algal strips.

In addition to the colored photosynthetic pigments diagrammed on the figures, some TLC strips may also contain bands formed by the breakdown products of chlorophyll. When a chlorophyll molecule loses the magnesium from its porphyrin ring it becomes *pheophytin*, which on a chromatogram appears as a gray band just above the chlorophyll *a* band. If the chlorophyll loses both the magnesium and the non-polar phytol tail it becomes *pheophorbide*, which appears as a gray band at about the same height on the TLC strip as chlorophyll *c*. The gray pheophytin and pheophorbide bands are most likely to occur in the extracts of *Spirulina* and dried *Porphyra* because both of these pigment sources have been subjected to the conditions likely to degrade the chlorophyll (including heating and drying) than the fresh materials. (For more information about chlorophyll degradation products see Rowan, 1984).

Lastly, the chromatograms from *Spirulina* run with toluene/acetone sometimes have what appears to be a double blue-green band in the chlorophyll *a* position when the methanol:pet ether extraction solvent is used. The reason for this is unknown. It does not occur when acetone is used as the extraction solvent, although sometimes a very faint, light green band occurs in the chlorophyll *b* position, which could be caused by a small amount of green algae harvested with the *Spirulina*. Acetone is much less effective at extracting pigments from the dried *Spirulina,* so if this solvent is used instead of methanol:pet ether, add 2-3 g of powder (instead of 1 g) to the screw-top test tube, and spot the TLC strip 6-7 times.