PHOTOSYNTHESIS

Pre-lab Preparatory Information

Know this material prior to attending your lab. You may be quizzed on this material.

Objectives: by the end of the lab, you will be able to...

...determine the role of carotenoid pigments in photosynthesis by:

- determining if they are present
- determining whether they affect the absorption spectrum of a leaf extract
- analyzing the action spectrum of chloroplasts for potential activity by carotenoids within the chloroplasts.

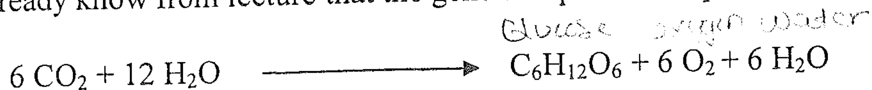
...distinguish an action spectrum from an absorption spectrum.

...explain how DCPIP was used to assay for the rate of the light reaction in photosynthesis.

...explain thin-layer chromatography including the determination of an Rf value.

A. Photosynthesis:

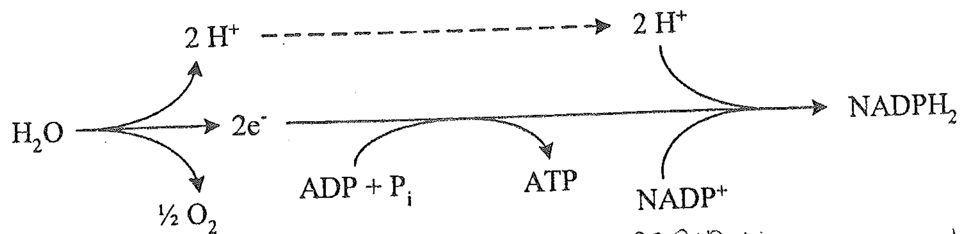
You already know from lecture that the general equation for photosynthesis,



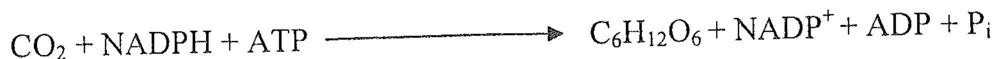
$$6\ CO_2 + 12\ H_2O \longrightarrow C_6H_{12}O_6 + 6\ O_2 + 6\ H_2O$$

actually summarizes two processes that occur in the chloroplast although in different areas:

(1) the <u>LIGHT-DEPENDENT REACTIONS</u>:



(2) the <u>CALVIN CYCLE</u>:



$$CO_2 + NADPH + ATP \longrightarrow C_6H_{12}O_6 + NADP^+ + ADP + P_i$$

(For clarity, the number of molecules has been omitted and NADPH is used rather than NADPH + H^+.)

The RATE of photosynthesis can be measured in several ways. As with respiration, one could determine the rates of gas exchange (CO_2 taken up and O_2 given off). The Calvin Cycle can be followed using radioactive CO_2. In this laboratory you will concentrate on the light reactions, once again using the Spectronic 20 to monitor the rate of color change. Specifically

D. Safety: Potentially hazardous substances, including flammable solvents, are used in this week's activities. Refer to Appendix C for details.

E. Skim the remainder of the laboratory so that you are familiar with the activities.

In-Laboratory Exercises:

I. Sample Preparations

Although the leaf extract samples used in the activities will be prepared for you, you should know something about the processes. For the leaf extract used in Parts II and III, leaves were homogenized in 80% acetone. The nonpolar compounds were then extracted from this homogenate using petroleum ether (a mixture of small hydrocarbons). Some of this sample was highly concentrated for chromatography (Part III).

To isolate chloroplasts for Part IV, the leaves were homogenized gently in ice-cold buffer containing sucrose (to maintain the osmotic balance, recall Lab 1!). The suspension was centrifuged at relatively low speed to sediment the chloroplasts, but not the smaller mitochondria, etc. The pellet was re-suspended in the sucrose solution and kept on ice until ready to use.

Do not get these three samples mixed up! Be sure to use the correct materials.

Part II: uses dilute extract, in **sealed** Spec 20 tubes
Part III: uses concentrated extract, in small vial with capillary tube, located in the hood
Part IV: uses isolated chloroplasts, on ice

II. Absorption Spectrum

Start w/ 400 adjust … then blank

Work in groups of four, making certain that everyone gets to make some of the readings.

DO NOT remove Parafilm from tops of Spec 20 tubes. DO NOT discard samples.

First look at the leaf extract. What color is the sample? What color(s) does it NOT absorb? What color(s) does it absorb? What color(s) does it transmit?

1. Obtain one of the tubes of leaf extract diluted as directed in part I. Since the solvent is clear, use water for a BLANK.

2. Set the spectrophotometer at 400 nm. After you have adjusted the meter reading to zero absorbance with the blank, determine the absorbance of the leaf extract. Record your results in Table 5.1.

3. Advance the setting to 425 nm, re-zero with the blank, and reread the absorbance for the extract. Record the number.

4. Continue this process, advancing the wavelength by 25 nm increments, until you reach 700 nm. [The phototubes in some of the Spec 20s can only read to ~600-650. If you have one of these instruments, get the remaining data from someone else.]

5. Graph your results in Figure 5.1. What colors were absorbed the most? The least? Was this consistent with your predictions before you started?

What color does each wavelength represent? Review your notes from Lab 3 and refer to your textbook to verify these colors.

Now compare your absorption spectrum for the leaf extract to the following absorption spectra for purified pigments. [Absorbance is a ratio (comparing the intensity of light before and after it is transmitted through the sample) and therefore has no units. However, the relative absorbances are the same no matter what the actual numbers.]

a. Does chlorophyll *a* absorb in the red, green, and/or blue region of the spectrum?

b. Does β-carotene (a carotenoid) absorb in the red region of the spectrum? If your absorption spectrum indicated absorption in the red region, was it due to chlorophyll, carotenoids, or both?

c. Is the absorbance in the blue region due to chlorophyll, carotenoids, or both?

d. How does this help you determine whether carotenoids are present in the leaf extract?

Figure 5.1. Relative absorption of chlorophyll a (left) and B-carotene (right).

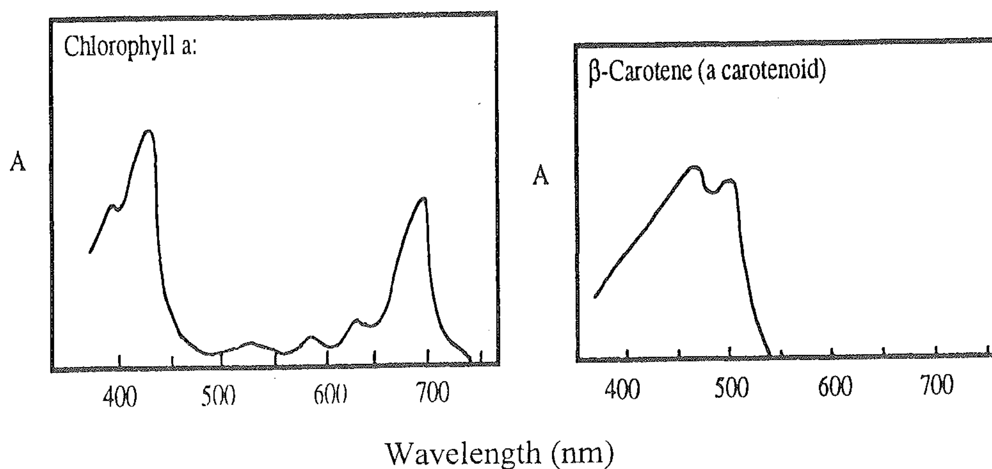


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Table 5.1. Absorption spectrum for pigments extracted from bean leaves.

Wavelength (nm)	Color	Absorbance
400		1.4
425		1.2
450		.9
475		.58
500		.32
525		.2
550		.14
575		.09
600		.12
625		.15
650		.23
675		.48
700		.5

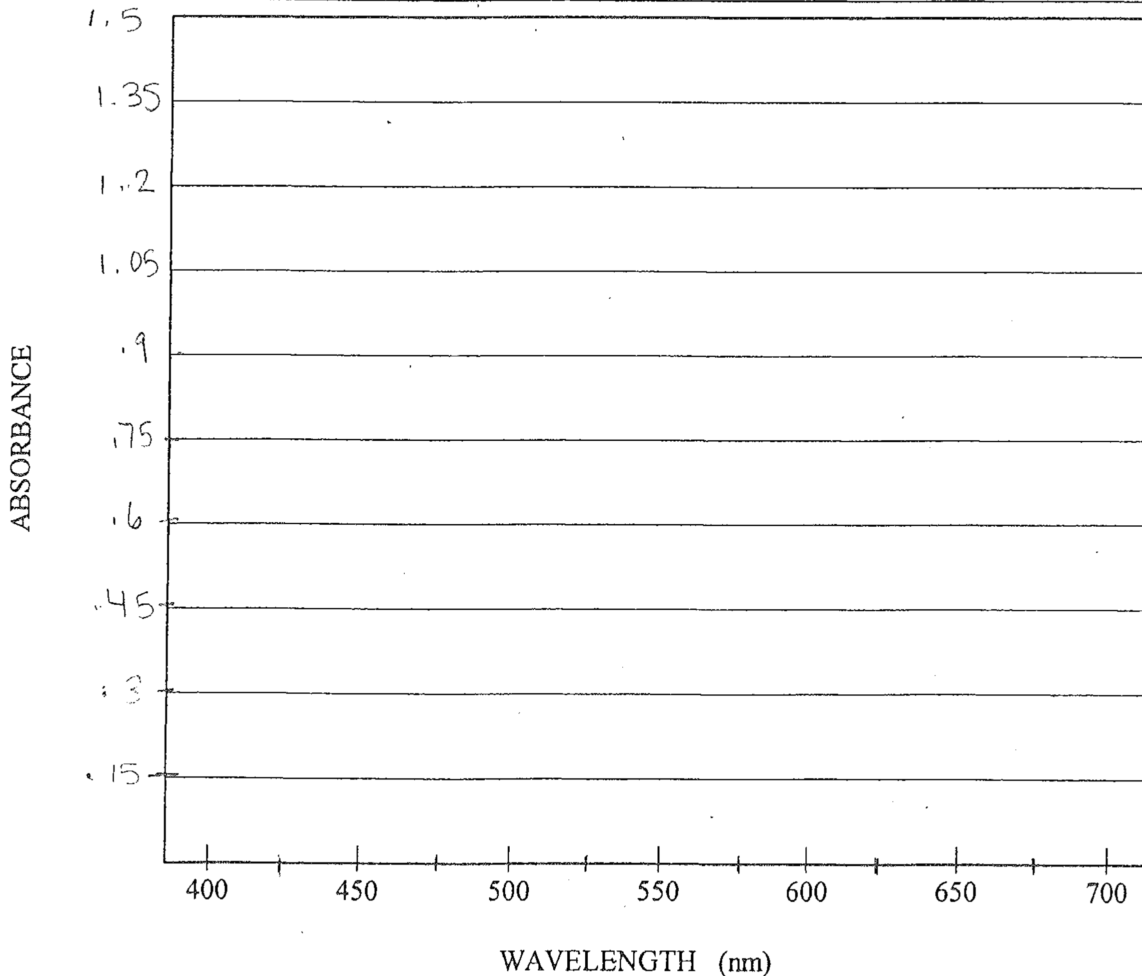


Figure 5.2. Absorption spectrum for leaf extract.

Indicate the color that corresponds to 400, 500, and 600 nm using your Table 1 from Lab 3.

III. Separation of Leaf Pigments: Thin-layer Chromatography

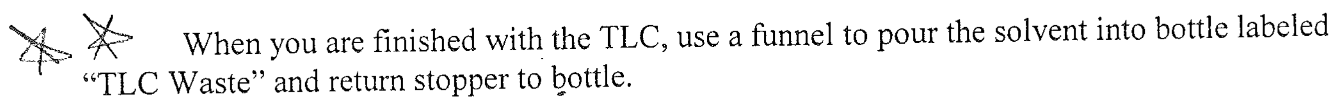
Work in groups of four. In this section you will use thin-layer chromatography (TLC) to separate the hydrophobic compounds extracted from the bean leaves. You will also determine the Rf of each component as a reminder of how many pigments were separated, and as a tentative method of identifying the pigments. (More accurate identification could be achieved if pure pigments were also chromatographed with the extract.)

Before you start, refer to Appendix C to note whether either solvent is potentially dangerous.

acetone:

petroleum ether:

Since the TLC solvent is 4 volumes petroleum ether to 1 volume acetone, what precautions should you take while handling this mixture?



When you are finished with the TLC, use a funnel to pour the solvent into bottle labeled "TLC Waste" and return stopper to bottle.

> SAFETY NOTE: The chromatography must be performed in the hood. Wear your chemical splash-proof safety goggles when adding the solvent, and when adding or removing the TLC strip.

1. Obtain one Eastman silica gel chromatography strip. ONLY handle the strip by the edges; do NOT touch the surface.

2. Place the dull side up on a paper towel.

   Use a capillary pipette to place small spots of colored extract 1 cm from the bottom. This will be the "origin". You will need to repeat this process many times, allowing the solvent to completely evaporate between applications. The sample at the origin MUST be dark green BEFORE you start, or you will not be able to see the separate bands later.

3. Add approximately 2 mL of TLC solvent to the Coplin jar, enough to cover the bottom about 5 mm deep. Put the lid back on the jar and wait a few minutes so the jar's atmosphere becomes saturated with the solvents.

4. Remove the lid and lower the strip into the cylinder. The solvent must NOT cover the sample.

5. Periodically check to see how far the solvent front has moved. When it nearly reached the top of the strip, OR when the two chlorophylls have separated, remove the strip from the jar. QUICKLY draw a pencil line across the strip to show how far the solvent had moved (called the "**solvent front**"). You must do this before the solvent dries!

6. Make a sketch of your results noting the location and color of each band.

7. Make a table to record the color and location of each band. Determine the location of each by calculating the **Rf**, or position relative to the front.

ratio doesn't matter how big — theory in same in any compound even if you use a meth

Rf = **distance between origin and band** / **distance between origin and solvent front**

no units — how far solvent front travelled

For example, if the solvent front was 10 cm above the origin and a yellow band was 7 cm above the origin, the Rf would be 0.7.

This number is used to characterize different compounds when separated by the same procedure (solvents, etc.), and can be used to identify the compound when used with the proper standards.

8. The number of pigments varies, dependent upon the organism, its environment and the extraction. However, you may tentatively identify each band using the following guide:

color	pigment
solvent front	β-carotene (a very hydrophobic carotenoid)
other yellow bands	other carotenoids (may be more than one)
dark green	chlorophyll a
yellow-green	chlorophyll b
yellow	xanthophylls (another type of carotenoid)
origin	

$\frac{5.5}{6}$ = — 6

IV. Action Spectrum

rate of photosyn — looking @ how much light absorbed — which wavel creating most photosyn reactn

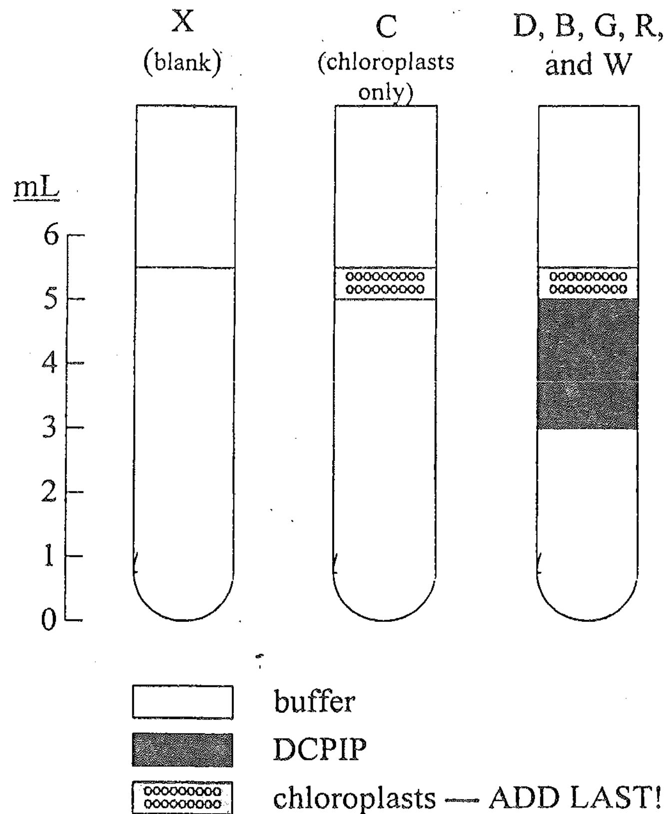
Work in teams of four.

Before you start, predict which tubes should not have a change in absorbance. Why?

$\lambda = 620$ nm

more phosy reac occuring → clearer soln

Which tubes are the controls? Why are these tubes important?



1. Label seven Spec 20 tubes: X, C, D, B, G, R, and W (see Table 5.2). Place 5.5 mL of buffer in tube X (your BLANK). Also place your group number on each tube. (Three tubes will be in each treatment, and you need to retrieve yours.)

2. Add 3 mL of buffer and 2 mL of DCPIP to tubes D, B, G, R, and W. Add 5 mL of buffer to tube C.

3. Under subdued laboratory light, add 0.5 mL of chloroplast suspension to each tube except for the BLANK (tube X). Cover tube with a small piece of Parafilm, and mix by inversion. (zero time = ______o'clock)

   Immediately determine the absorbance (A) at 620 nm for each tube and record your results in Table 5.2.

4. Place tube D in the beaker covered with aluminum foil. Place tubes B, G, and R in beakers covered with blue, green, and red cellophane. Cover the top of each beaker with aluminum foil. Tube W goes into the clear beaker, along with tube C.

5. After 3 to 5 minutes, determine the absorption in tube W (in white light). If the absorbance has decreased more than 0.3 A, then determine the absorbance for all tubes. Enter your results in Table 5.2. If not, return the sample to white light and repeat at 5-minute intervals.

   If necessary, determine the absorbance of all tubes up to 30 minutes. Enter these results in Table 5.2. If there is no change by this time, the chloroplasts are inactive.

   [Considering the fact that you are using organelles isolated from the rest of the cell, it is not surprising that this section of the lab is at times unsuccessful.]

6. Calculate the rate of the reaction by subtracting the initial from the final absorbances and convert to A/15 minutes. (Ignore the sign.) Enter these calculations in Table 5.2.

7. Place your calculated rates on the blackboard. When everyone has completed this table, copy the class results onto table 5.3. Show your calculation for the tube in red light below your data.

8. Graph means for the class data in Figure 5.2.

   [The tube in white light allows you to monitor the activity of the chloroplast preparation. Since you changed the conditions every time you read the absorbance, it is not comparable to samples in red, green, and blue light.]

COMING ATTRACTIONS:

In this lab you determined both the action spectrum of photosynthesis and the absorption spectrum of the leaf extract, as well as separating the pigments in the extract by TLC. This week finishes a three-part series on uses of a spectrophotometer.

Next week you will begin a four-part series on evolution, starting with reproduction of plant-like protists (algae) and fungi. Remember that you can preview and/or review many of the skills in that laboratory by using the VIRTUAL LABORATORY FOR BIOLOGY 101.

Name ______________________; Section _________; Date ______________

Table 5.2. Action spectrum for photosynthesis.

Tube	D	C	B	G	R	W
Color of light shining on sample:	(dark)	(chloroplasts only)	blue	green lower reflect	red	white
Absorbance at 620 nm:						
initial	1.3	.24	1.5	1.5	1.5	1.5
final	1.3	.25	.85	1.3	.45	.27
7 min						
Rate (decreased A per 15 min):	0	-.0214	1.3928	.42857	2.25	2.6357

Sample calculation:

D $\frac{1.3-1.3}{7-0} \times 15$ C $\frac{.25-.24}{7-0}$ = B