305. CRASSULACEAN ACID METABOLISM

(Leave the Windows Open at Night, it's Cooler)

Photosynthesis consists of two processes. In the first process, light energy is trapped to form the energy- and reducing power-carrying intermediates ATP and NADPH. In the second process, ATP and NADPH are used to power CO_2 fixation, turning CO_2 from the atmosphere into carbohydrate. The most widely distributed pathway for CO_2 fixation is the Calvin-Benson cycle, or C_3 path (POH p. 172-179).

The two parts of photosynthesis have different requirements. Light drives the production of ATP and NADPH, so this process works better in bright light. CO_2 fixation does not require light, but occurs faster and more efficiently the higher the CO_2 concentration. However, in nature, very bright light may be accompanied by high temperature and low water availability. If plants close their stomata to reduce transpirational water loss, they also reduce diffusion of CO_2 into the leaves. If they open their stomata to speed the diffusion of CO_2, they lose more water through transpiration.

Temporary storage of CO_2 as a carboxylic acid can provide a solution to this dilemma. A three-carbon carboxylic acid, phosphoenolpyruvate, accepts CO_2, forming the four-carbon (C_4) dicarboxylic acid, oxaloacetic acid. (The oxaloacetic acid may be modified slightly to form other dicarboxylic acids.) The enzyme that adds CO_2 to phosphoenolpyruvate works well even at very low CO_2 concentrations.

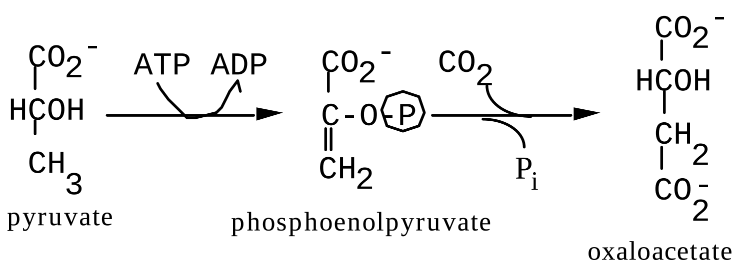


Figure 1. The formation of oxaloacetate by carboxylation of phosphenolpyruvate.

Two groups of plants using C_4 compounds as CO_2 carriers are the C_4 plants and the CAM plants. In both C_4 and CAM plants, the Calvin-Benson cycle is still used to produce carbohydrate.

In C_4 plants, the initial capture of CO_2 and its final assimilation are separated in space. Examples of C_4 plants include most "large" grasses (corn, sorghum, giant cane) and most members of the family Euphorbiaceae (including *Euphorbia*, or spurge). In C_4 plants, the initial capture of CO_2 occurs by the formation of oxaloacetic acid in mesophyll tissue accessible to the stomata. Since the C_4 enzyme works well at low CO_2 concentrations, CO_2 can be assimilated *even when the stomata are closed to conserve water*. The C_4 compounds move to special mesophyll packed around the vascular bundles (bundle sheath mesophyll). In the bundle sheath mesophyll, the C_4 compounds give up the CO_2, which is then used to produce carbohydrate in the Calvin-Benson cycle. It is its ability to conserve water through the C_4 path that makes corn such an important agricultural crop in arid regions.

In CAM plants, the initial capture of CO_2 and its final assimilation via the Calvin-Benson cycle are separated in time. Unlike most plants, CAM plants *open their stomata at the night and close them during the day*. At night, when the stomata are open, CO_2 is assimilated to form oxalacetate. During the day, the stomata can close to conserve water. The C_4 acids are decarboxylated, providing CO_2 which is then assimilated using the Calvin-Benson cycle. The CAM pathway is named after the plant family Crassulaceae. This family includes many succulent species able to grow in deserts or along sea shores where water availability is low. Examples include *Crassula* (jade plants and many other succulents), *Sedum* (stonecrops) and *Dudleya* (hens and chickens). "CAM" stands for "crassulacean acid metabolism". CAM plants are the primary subject of this experiment.

Text References

POH 172-181, The Calvin-Benson Cycle (especially Figure 8.27); 652-654, Leaf Anatomy; 664, Cross-Section of a Dicot Leaf; 665-666, Crassulacean Acid Metabolism

Study Questions

Describe the functions in the cell of ATP, NADPH, chlorophyll *a*, accessory pigments, ribulose bisphosphate, rubisco. Explain how the processes of trapping light energy into chemical energy and of using this energy to fix CO_2 are connected.

Describe the functions of the leaf structures including the cuticle, upper epidermis, palisade mesophyll, spongy mesophyll,vascular bundles, xylem, phloem, and guard cells. Compare the leaf structures of C_3 and C_4 plants.

Compare the C_3, C_4 and CAM photosynthetic mechanisms. Compare the ecology and behavior of the three types of plants.

Purpose

To demonstrate the effect of the light cycle on organic acids in CAM plants and to compare transpiration and leaf structure in CAM, C_4 and C_3 plants.

<u>Effect of light cycle on organic acids in CAM plants.</u>

From the Text References and the introduction to this experiment, predict on the worksheet how the acid level and pH of CAM plant leaf tissue will vary over a day/night cycle.

Materials: CAM plant; small plastic bags; mortar and pestle; pH meter; 10 ml beaker; phenolphthalein (0.05% in 50% ethanol); 10 ml 0.01 M NaOH (**Caution; caustic**); 10 ml burette; ring stand and burette clamp

Determine how many leaves of the available CAM plant are necessary to provide 1 ml of liquid when crushed. (For donkey-tail sedum, seven leaves are needed.) Take home a plant and place it near a window where sunlight is plentiful. Leave it there for one day to acclimate. Early in the morning of the second day, remove enough leaves to provide 1 ml of liquid. Put the leaves into a small plastic bag labeled with the time. Store the leaves in a freezer until they are to be analyzed in the laboratory. Repeat this process at regular intervals, storing the leaves from the different sampling times in separate labeled bags. Continue sampling through the night, again labeling the bags with the time. *Record the times of sunset and sunrise.* On the day of your laboratory, pack the bags of leaves in ice and store them in the laboratory freezer until class time.

Thaw the leaf samples and grind each sample separately with a clean, nonporous mortar and pestle. Tilt the mortar so the sap forms a pool separate from the tissue. (If you have difficulty separating the liquid from the solid material, you may briefly centrifuge the samples.) *Use a small piece of pH paper to determine the pH values of the samples.*

Pipette 1 ml of the sap into a very clean 10 ml beaker and add three drops of phenolpthalein indicator. Titrate the sap with NaOH, one drop at a time, swirling the beaker gently (see Appendix). Stop the titration when a stable red-brown color is reached. Be aware that other compounds present in the sap may cause sudden color changes as the titration end point is approached.

Calculate the concentration of dicarboxylic acids (DCA) in the sap. Two moles of base (NaOH) are required to neutralize one mole of dicarboxylic acid:

$$\frac{\text{mmoles DCA}}{\text{liter}} = \frac{\text{ml NaOH used}}{\text{ml sample used}} \text{ X } \frac{\text{1000 mmoles}}{\text{mole}} \text{ X } \frac{\text{0.1 moles NaOH}}{\text{liter NaOH}} \text{ X } \frac{\text{1 mole DCA}}{\text{2 mole NaOH}}$$

Transpiration by CAM and other plants

Terrestrial plants lose water through transpiration. However, the special adaptations of C_4 and CAM plants alter their patterns of transpiration. To detect transpiration from individual leaves, we will measure the weight of water lost through the leaf surfaces.

Materials: CAM plant; C_3 plant; C_4 plant; small vials; vegetable oil; balance; light; microscope

Obtain a leaf from each type of plant, selecting leaves of similar size if possible. Carefully remove the leaves from the plants underwater so as not to break the water column. Position each leaf with its petiole in a vial of water (Figure 2). Pour a small amount of vegetable oil on the water to reduce evaporation from the water surface. *Weigh the vials with the leaves.* Place the vials in the light for several hours, and then *reweigh*.

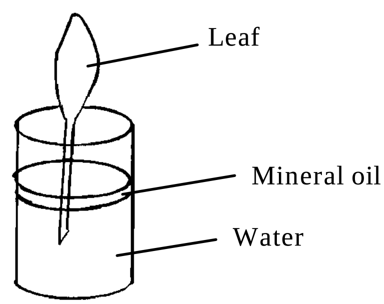


Figure 2. Using a single leaf to measure transpiration.

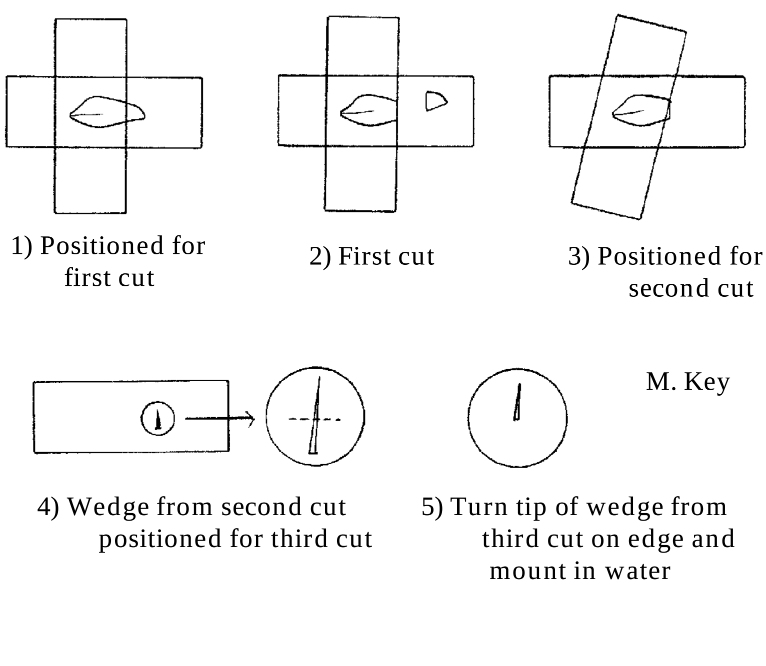
During the incubation period, peel a small piece of the lower epidermis from a fresh leaf of each type. (Use new leaves, not the ones you are incubating.) Make a wet mount of the epidermis and *count the number of stomata in three distinct randomly selected fields of view of your microscope. From the area of the field of view of your microscope, calculate the number of*

stomata per mm^2. The field of view of your microscope is 15.9 mm^2 at 40X magnification, 2.41 mm^2 at 100X magnification and 0.15 mm^2 at 400X magnification.
Now place the vials in the dark for about sixteen hours. At the end of the dark incubation, *reweigh the vials*.
Remove the leaves and wipe them dry. *Calculate the area of each of the leaves.* You can trace the leaves on graph paper and count the number of squares they cover. However, you may find it quicker to photocopy the leaves, and cut out and weigh their images. To convert from paper weight to area, weigh an 8.5" by 11" (or 21.6 cm by 27.9 cm) sheet of paper. Divide the weight of the sheet of paper by its area to obtain g/cm^2 of the paper. Divide this conversion factor into the weights of your leaf images to obtain their areas. *Express the transpiration rates of the leaves during the light and dark periods in grams of water per cm^2 per hour.*

Leaf structure of CAM and other plants

Materials: prepared cross-section of *Ligustrum* leaf; fresh *Ligustrum* leaves; prepared cross-section of C_4 plant leaf; fresh leaves of C_4 plant species; fresh leaves of CAM plant; razor blade (student supplied); Petri dish; slides and coverslips; toluidine blue dye

Observe prepared cross-sections of *Ligustrum* and C_4 plant leaves at 400X magnification under the microscope. Sketch and label diagrams of your observations, locating the cuticle, upper epidermis, palisade mesophyll, spongy mesophyll, vascular bundle, bundle sheath cells, xylem, phloem, lower epidermis and guard cells. Compare the two types of leaves.
Make your own wet mounts of *Ligustrum*, C4 and CAM plant leaf cross sections as follows. Sandwich a leaf between two slides, pulling the upper slide back to expose part of the leaf (Figure 1). Cut the leaf using the upper slide as a straight-edge. Now pull the upper slide back very slightly at an angle exposing a narrow triangle of leaf. Shave off the triangle, holding the razor blade as vertically as possible. Curl the triangle on its cut edge on another slide. (If you make several sections, keep them wet until you are ready to continue with them.) Slice away and discard the wider end of the triangle. The tip of the triangle should narrow down to a thin point just a few cells thick. Make a wet mount of the thin point (cut edge down), adding a drop of water and gently topping with a coverslip.



1) Positioned for first cut
2) First cut
3) Positioned for second cut
4) Wedge from second cut positioned for third cut
5) Turn tip of wedge from third cut on edge and mount in water

M. Key

Figure 1. Preparing a leaf cross section.

Observe the specimens at 100X and then at 400X magnification. *Sketch and label diagrams of your observations. Compare the three types of leaves.*

Cleanup

Discard used plant material. Wash all glassware. Return the ring stand and clamp to their proper locations. Discard coverslips in the glass discard and dull razor blades in the "Sharps" container.

Additional References

Salisbury, F. and C. Ross. 1992. Plant Physiology. Wadsworth Publishing Co., Belmont, CA

Walker, J.R.L. and J.A. McWha. 1976. A Simple Demonstration of Carbon Dioxide Fixation and Acid Production in CAM Plants. J. Biological Education 10:169-172.

305. CRASSULACEAN ACID METABOLISM (25 PTS)

Name   Lab day and time ____________________________

PRELAB PREPARATION:

1. Procedural outline:

2. Will your sap sample be the *titrand* or the *titrant* in this experiment? (See "Titration" in Appendix A.)

3. Predict how the acid level and pH of CAM plant leaf tissue will vary over a day / night cycle.

4. Which type of plant (CAM, C_3 or C_4) will have the highest transpiration rate in the light? In the dark? Defend your predictions.

5. Which type of plant will have the most stomata per cm^2? Defend your prediction.

RESULTS:

6. Time of sunrise:__________   Time of sunset:__________

7. pH of CAM plant over time

Time	pH	ml of NaOH used	concentration of DCA

8. On the same graph, plot the pH of CAM tissue fluid and the leaf acid content against time of day. Indicate on your graph the times of sunset and sunrise.

9. Are your results consistent with your predictions? Explain any discrepancies.

10. Comparison of transpiration rates of CAM, C4 and C3 plants.

Plant type	original weight (mg)	weight after light (mg)	weight after dark (mg)	leaf area (cm^2)	transpiration rate in light (g/cm^2)	transpiration rate in dark (g/cm^2)

11. Comparison of stomatal density of CAM, C4 and C3 plants.

Magnification:_________ Area of microscope field:________

Plant type	stomata per microscope field			stomata per mm^2
	Field 1	Field 2	Field 3	

12. Compare light and dark transpirations rates and the stomatal densities for the three types of plants. Do the results follow your predictions? Explain any discrepancies.

13. Which type of plant appears to be most efficient at minimizing water loss? Defend your answer.

14. Sketch and label diagrams of the prepared slides.

Ligustrum C4 plant

15. Sketch and label diagrams of the leaf cross-sections you prepared.

16. In which plant tissues did you observe chloroplasts?

17. From your observations and readings, give the function of each of the leaf structures you observed.

18. In a table, summarize the major physiological and structural differences between C_3, C_4, and CAM plants.