

## Experiment 9 (Lab period 10)

### Cell fractionation and isolation of chloroplasts

The green color of the leaves, and sometimes the stems, of plants is due to the presence of the green pigments chlorophyll *a* and chlorophyll *b* in the subcellular organelles called chloroplasts. The remainder of a green cell is typically colorless. The green organelles lie free in the cytoplasm of the cell, unattached to other cellular components such as the wall, the membranes, the nucleus, and the mitochondria. When the cell wall is disrupted, the cell membrane breaks, and the subcellular components are released as separate particles of various sizes and densities.

In this laboratory, the cells of spinach leaves will be disrupted, freeing the untethered organelles, which can then be sorted out from each other by filtration and differential centrifugation as cell fractions. Filtration will remove large debris (e.g., cell walls) and unbroken cells, providing a filtrate that contains organelles (nuclei, chloroplasts, mitochondria, ribosomes), small membrane vesicles, and soluble components; most of these will not be visible in the light microscope. Low-speed centrifugation will sediment remaining large bodies from the filtrate, and moderate-speed centrifugation will sediment chloroplasts, leaving mitochondria and ribosomes and soluble components in the supernatant. (The mitochondrial fraction could be collected by high-speed, and ribosomal and membrane vesicle fractions by ultra-high-speed centrifugation.) Repeated rounds of differential centrifugation can be used to further purify the chloroplasts when highly purified preparations are required for experimentation, but one round of low-then-moderate centrifugation will suffice for the purposes of this exercise.

In this laboratory, you will prepare a crude suspension of chloroplasts and determine several characteristics of the preparation. You will measure the chlorophyll *a* content of the suspension, count the chloroplasts per unit volume of the suspension, and use those data to estimate the quantity of chlorophyll *a* per chloroplast. You will also observe and record the *in vivo* spectrum of the chloroplasts. In the next laboratory, the pigments in the isolated chloroplasts will be fractionated by differential extraction.

## Procedure

**Keep the tissue and all fractions ice cold throughout steps 1 - 6 and 10.**

1. Obtain 8 grams of de-veined leaf tissue rinsed in ice water, blotted and cut into pieces about 1 cm square.
2. Place the leaf pieces in a pre-chilled blender cup containing 40 ml of ice-cold 0.5 M sucrose. Blend for 15 sec. at top speed, pause about 10 sec., then blend again for 10 sec.
3. Remove the ice from the 100-ml beaker, then squeeze the leaf homogenate through four layers of pre-chilled cheesecloth into the cold beaker by twisting the top corners of the cloth around each other.
4. Pour 14 ml of the homogenate into each of two centrifuge tubes and centrifuge at 200g for 5 min.
5. Using a Pasteur pipet, transfer each supernatant (containing the chloroplasts) to a second centrifuge tube and centrifuge at 1000g for 7 min. (Save the pipet.)
6. Using the pipet, discard the supernatant but be careful not to disturb the pellet. Pour 2 ml of phosphate buffer onto the pellet and gently resuspend it by moving it up and down in the pipet. Using a clean Pasteur pipet, add buffer until you have a total volume of 8 ml and mix the diluted suspension using the pipet.

**This is your chloroplast suspension. You should examine it in the microscope.**

Use the suspension for steps 7, 8, and 9, and then prepare the chloroplasts for storage in step 10.

7. Estimation of chlorophyll *a* concentration of the suspension.
  - a. Measure 4.75 ml of 80% acetone into a 13 x 100 mm tube.
  - b. Add 0.25 ml of chloroplast suspension, mix well, and read the Absorbance at 652 nm against 80% acetone as the reference blank.
8. Determination of chloroplast concentration of the suspension.
  - a. Measure 4.75 ml of the phosphate buffer into a 13 x 100 mm tube, and add 0.25 ml of chloroplast suspension, and mix well.
  - b. Prepare the clean, dry hemacytometer with a cover slip in place supported by the frosted-glass shoulders of the chamber.
  - c. Making certain that the chloroplasts are evenly suspended (not settled, not clumped), take up some of the suspension into a clean Pasteur pipet; let part of a droplet from the pipet tip flow under the cover slip of the chamber. When properly delivered, the liquid fills the space between cover slip and etched surface of the chamber and does not overflow into the side troughs beneath the cover slip. (Hint: have a tissue ready, folded to a point; if the chamber fills, but there is still a droplet standing at the side of the cover slip, pull it up gently into the point of the tissue, taking care not to pull any liquid from under the cover slip.) If overflow nevertheless occurs, clean and dry the chamber again and cover slip, wiping finally with alcohol and a Kim-wipe, and begin again with another sample of suspension.

d. Using the high-dry (40x) objective, count the total number of chloroplasts in the large central square of the counting chamber - the square that is bounded by a triple-line border and is itself subdivided into 20 sets of 16 very small squares.

9. *In vivo* absorption spectrum.

Using the dilution of chloroplasts in buffer prepared in step 8, and distilled water as the reference blank, record the absorption spectrum at 10-nm intervals between 400 and 700 nm. When an absorption peak appears, read Absorbance at 5-nm intervals near the peak to determine its location more precisely.

Observe the suspension visually at frequent intervals to be sure that the chloroplasts are not settling. If necessary, mix by inversion against Parafilm in order to keep the chloroplasts as evenly suspended as possible.

10. Storage of the chloroplast suspension. \_

Centrifuge the remainder of the suspension at 1000g for 7 min. Using a Pasteur pipet, aspirate and discard the supernatant. Submit the chloroplast sediment to the instructor for storage (frozen) at -20°C for use next week. Note and record the color of the pellet.

## Calculations

- C1. From step 7 you should have a measure of  $A_{652}$  for your diluted chloroplast suspension. The Molar Extinction Coefficient for chlorophyll a at this wavelength is 34.5 ml/mg so you can calculate the concentration of chlorophyll in this diluted sample. From your knowledge of the dilution you performed in step 7 you can calculate the concentration of chlorophyll a in the original chloroplast suspension. The units will be mg/ml.
- C2. In step 8 above, the volume contained in the chamber over the large square in which you counted chloroplasts was 0.1 ml, or  $10^{-4}$  ml. Therefore, you counted the number of chloroplasts in  $10^{-4}$  ml of the diluted sample. You can now calculate the concentration of chloroplasts/ml of diluted suspension as:
- $$\text{Number of chloroplasts} \times 10^4 \text{ ml} = \text{Number of chloroplasts/ml of diluted suspension}$$
- Using your knowledge of the dilution you performed in step 8 you can calculate the number of chloroplasts per ml in the original, undiluted sample.
- C3. Combining calculations C1 and C2, you can now determine the quantity of chlorophyll a per chloroplast. Dividing the concentration of chlorophyll a (in mg/ml) by the number of chloroplasts per ml will give you the quantity in mg of chlorophyll a per chloroplast.
- C4. The spectrum obtained from step 9 shows the pattern of light absorption by the chloroplast pigments in their native environment, the chloroplast, where they are present in natural proportions to each other and are located in membranes surrounded by an aqueous medium. Plot the spectrum on graph paper, and be prepared to compare this absorption spectrum with the absorption spectra obtained in the next laboratory exercise.