Chapter 5

Measurement of Photosynthetic Activity in Plant Cell Fractions

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Contents

Introduction

Photosynthetic electron transport in chloroplasts involves the transfer of electrons from water to $NADP^+$ (Allen and Holmes, 1986; Amesz, 1987; Govindjee and Coleman, 1990; Greenberg, 1991; Hoober, 1984). This process is carried out by two photosystems, photosystem II (PSII), and photosystem I (PSI). PSII transfers electrons from water to plastoquinone, and PSI transfers electrons from plastocyanin to $NADP^+$. The activity of PSII is relatively easy to measure *in vitro* if an artificial electron acceptor such as dichlorophenol indophenol (DCPIP) is used (Allen and Holmes, 1986; Kyle et al. 1984; Peterson and Arntzen, 1982). An assay that measures the change in absorbance of a reaction mixture containing DCPIP can demonstrate a number of biological and biochemical principles in the undergraduate laboratory. These include success of a sub-cellular fractionation (as described here), aspects of photosynthesis, the phenomenon of electron transport, bioenergetics, and redox chemistry.

This technique is taken from a comprehensive series of exercises in which, over the course of four 3-hour laboratory periods, students fractionate the cells of pea plants and then test the fractions and a sample of the homogenized tissue from which they were made for protein and chlorophyll content, and for the activities of photosynthesis and the mitochondrial electron transport chain (Arnon, 1949; Rocha and Ting, 1970). The results of these assays are analyzed and values found for the distribution of enzymatic activities among the fractions. Further calculations yield the specific activities of these enzyme systems (units of activity per unit of protein), percent recovery of the protein, chlorophyll and enzymes originally present in the homogenized plant material, and the distribution of these values in the fractions. From this last set of figures, it is possible to derive a measure of how successfully the organelles were isolated into separate fractions. For example, comparison of the photosynthetic activity in the chloroplastic fraction with that in the cytosolic fraction reveals whether the final centrifugation step may have been too long. This type of comparison is especially illuminating for students who may have made assumptions about the contents of the fractions based on the names they are given. Should the fraction labeled **NUCLEI & DEBRIS** have photosynthetic activity? And if it does, why? (Because it contains unbroken cells.)

This laboratory exercise allows instructors either to use it as described, or to adapt the assay for other systems in which a reliable measure of the activity of photosystem II is required. If you use the protocol as it is given here, the plant material will first have to be fractionated (a 3-hour procedure). On the other hand, if you want to apply this assay to your own samples, some modification will be involved, mostly to find the concentrations of plant material and reagents that will give the best results. We have found this to be a robust assay, giving good values as long as the ratio between the concentration of chloroplasts and the concentration of DCPIP is within a reasonable range. (For background information on cell fractionation, see Edelman et al., 1987; Rocha and Ting, 1970; on photosynthesis, see Allen and Holmes, 1986; Amesz, 1987; Hoober, 1984; and Salisbury and Ross, 1991.)

The assay for photosynthetic activity as given in the Student Outline usually takes 2–2.5 hours. Our experience with this laboratory exercise shows that it is suitable for undergraduate biology or

biochemistry majors in the last or second-last year of their programs (i.e., juniors and seniors). An introductory course in cell or general biology, and courses in organic chemistry and biochemistry are considered essential for understanding the basics of the laboratory exercise.

Materials

Preparation of Fractions

Pea plants for each 5–6 pairs of experimenters (1 flat)
Shears or scissors for cropping the pea plants (1 pair)
Top-loading balance and double-pan balance (1 of each)
Waring blender (1)
Distilled or deionized water (5 liters)
Crushed ice and ice buckets
Test tubes, 15-ml (16 mm × 125 mm Baxter Dispo round-bottomed culture tubes, or equivalent) (the total number will depend on how this preparation is conducted).
Cheesecloth (1 large roll)
Wide-mouth funnel, large (1)
Ehrlenmeyer flask, 100 ml (1)
Graduated cylinders, 100 ml (2)
Graduated cylinder, 10 ml (1)
Screw-top vials, 10 ml to 20 ml, for storage of the fractions, with masking tape and elastic bands (4)
Benchtop clinical centrifuge (optional, it may be more efficient to do all of the centrifuging in a preparative centrifuge) (1)
Sorvall refrigerated preparative centrifuge with an SS34 rotor (we use model RC5C) (1)
Sorvall centrifuge tubes, 50 ml (2)
Automatic pipettor (to measure 1 ml) (1)
Pasteur pipets and bulbs
Paper towels and kimwipes
All-purpose paintbrush, small (3 mm) (1)
Grinding buffer: 500 mM sucrose, 10 mM $MgCl_2$, 1 mM EDTA. Dissolved in 50 mM Tris buffer, with pH adjusted to 7.5.

Assay of Photosynthetic Activity (by 12 two-person experimental teams)

Test tubes, 20 ml (252)
Test tube racks (24)
Automatic pipettors (with replacement tips) to measure 25-µl volumes (12)
Benchtop lamps (one 40W or 60W bulb each) (12)
Ice buckets containing crushed ice (12)
Spectrophotometers (e.g., Spec 20s) (12)
Benchtop clinical centrifuges (12)
Parafilm and scissors
Distilled water (1 liter)
DCMU solution (10 mM dissolved in 100% methanol) (10 ml) (This substance degrades slowly in solution; make it up fresh every 5 days.)
DCPIP solution (5 mM dissolved in 95% ethanol) (10 ml) (This substance degrades slowly in solution; make it up fresh every 5 days.)
Grinding buffer (500 ml)

Notes for the Instructor

Preparation of Cell Fractions from Pea Plants (*Pisum sativum*)

1. The pea plants are grown for 2–3 weeks and kept in the dark for 24 hours before use. (The 24-hour dark period depletes the starch in the chloroplasts.)

(a) Cut the plants just above the lowest true leaves (about 3 cm above soil level) and chop them into segments about 1 cm long. Wash the pieces twice in distilled or deionized water, and blot dry with paper towels.

(b) Weigh the chopped pea plants and divide them into batches of about 30 g each. Put each batch in turn into a chilled Waring blender with grinding buffer (about 3 ml per g of tissue), and grind the material for 20 seconds (i.e., start the 20-second count once the entire mass of plant material and buffer is in motion).

Note: The instructions from step 2 to step 5 are those used in our teaching laboratory, and describe the centrifugation of 75-ml aliquots of the homogenized plant material in benchtop clinical centrifuges. Following this plan will yield enough material for four groups; you may find it more efficient to convert this part of the procedure for use in a larger-capacity centrifuge.

2. Put 12 empty 15-ml test tubes and the six metal "buckets" from your benchtop centrifuge into an ice bucket filled about halfway with ice, and leave them to cool. Carry out all of the following steps at 4°C.

3. Filter 75 ml of the homogenate from step 1b through eight layers of cheesecloth in a wide-mouthed funnel into a 100-ml ehrlenmeyer flask set in ice. When the liquid has stopped running, remove the cheesecloth and squeeze it gently to press out the remaining liquid. Measure the volume of the filtered homogenate in a graduated cylinder. Measure out and save about 1 ml of this into each of four vials labeled **HOMOGENATE** and put them on ice. Label these and all other sample vials with a lead pencil on masking tape.

4. Divide the rest of the homogenate evenly among six of the chilled 15-ml test tubes for centrifugation. Be sure to balance the tubes in pairs (i.e., each tube is matched with another that has the same level of liquid in it). Insert the tubes into the iced centrifuge buckets, and put the buckets into the centrifuge with each tube 180° apart from its paired mate. Run the centrifuge at 300 × g for 60 seconds.

5. Pour the supernatant into the remaining six chilled 15-ml tubes (again balancing in pairs), and centrifuge this suspension at 1500 × g for 10 minutes. Invert the original six tubes containing the pellets from the 300 × g centrifugation (nuclei and debris fraction) on paper towelling to drain. These pellets are small and inconspicuous; please take care not to discard them accidentally.

6. Resuspend the nuclear and debris fraction in grinding buffer.

Resuspension has two objectives: removal of the pelleted material from the centrifuge tube, and its dispersion into a uniform suspension. Because it is advantageous to have the pellet suspended in a small volume, a single 1-ml aliquot of buffer will be put into the first tube, and then passed successively to the second tube, the third, etc., suspending as much of the pellet as possible at each transfer. The sequence is repeated with a second 1-ml aliquot.

(a) Put 1.0 ml of grinding buffer into the first of the six tubes from step 5 containing pellets of nuclei and cellular debris, and stir up the pellet with a small paint brush.

(b) Take up all of the liquid into a pasteur pipet and place its tip against the bottom of the tube. Slowly but firmly expel the liquid from the pipet a few times to break up any small clumps of pellet.

(c) Pour the liquid into the next tube, and use the brush and pipet to mobilize and suspend its contents in the same way. Continue through the series of tubes until all the pellets have been suspended.

(d) Pour the suspension into a 10-ml graduated cylinder kept on ice.

(e) Pipet a second 1.0 ml aliquot of buffer into the first tube, and use a pasteur pipet to rinse all remaining particles of pellet from the sides of the tube.

(f) Transfer the liquid to the next tube and repeat the process. Continue until all the tubes have been rinsed.

(g) Pour the liquid into the graduated cylinder with the first suspension (step 6d), mix well, and record the final volume.

(h) Distribute the suspension into a four vials labeled **NUCLEI AND DEBRIS**, and place them on ice.

7. Pour the supernatant from the 1500 × g centrifugation (started in step 5) into two 50-ml centrifuge tubes. Using a twin-beam balance, distribute the liquid so that the two tubes are of equal weight, and then centrifuge them for 20 minutes at 21,000 × g (15,000 rpm in the Sorvall centrifuge with a SS-34 fixed-angle rotor).

8. Resuspend the six pellets from the 1500 × g centrifugation (**INTACT CHLOROPLASTS**) in grinding buffer as in step 6. When all the pellets have been suspended, add 8.0 ml of grinding buffer to the suspension, mix well, and measure its total volume. Divide it evenly among four labeled vials and put them on ice.

9. Pour the supernatant (**CYTOSOL**) from the 21,000 × g centrifugation (step 7) into a 100-ml graduated cylinder, save about 3 ml of it in each of four labeled vials on ice, and discard the remainder.

10. Resuspend the two pellets from the 21,000 × g centrifugation (a mixture of **BROKEN CHLOROPLASTS, MITOCHONDRIA AND OTHER ORGANELLES**) in 8 ml of grinding buffer, and distribute it among four labeled vials on ice.

11. Freeze all of the labeled vials.

The Assay

DCPIP is a redox indicator; each molecule is reduced by accepting two electrons and two hydrogen ions, changing structure as indicated in Figure 5.1. In either form, DCPIP is somewhat hydrophobic and can insert into biological membranes. Within the thylakoid membranes of chloroplasts it accepts electrons and hydrogen ions from plastoquinone, one of the components of the chloroplastic electron transfer chain (Figure 5.2; Anderson and Andersson, 1988; Fujii et al., 1990). DCMU can stop the transfer of electrons to plastoquinone by attaching to its binding site on the PSII complex (Inoue et al., 1986; Izawa, 1980).

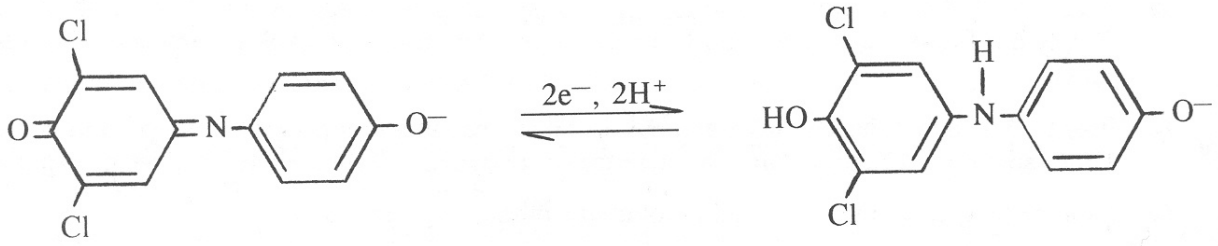

Figure 5.1. Structures of the reduced and oxidized forms of DCPIP.

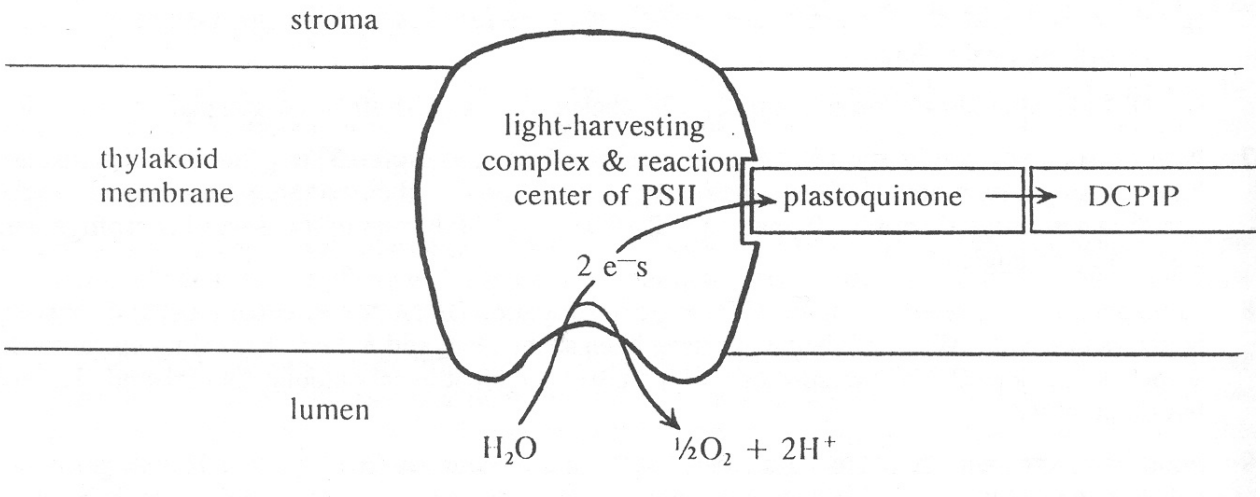

Figure 5.2. Transfer of electrons in the thylakoid membrane of chloroplasts from PSII to DCPIP.

Since DCPIP can also be reduced by high-energy electrons from other sources, it is necessary for the calculations that give a measure of photosynthetic activity to be based on the *changes* in absorbance between the illuminated samples and those kept in the dark. The intention is to exclude any decrease in absorbance caused by the reduction of DCPIP by non-photosynthetic electron donors. (This is the answer to question 1 in the Student Outline.)

The reduced form of DCPIP is at a higher energy level than its oxidized form, and spontaneous reoxidation occurs easily. The degree of this reoxidation is diminished by degassing the buffer included in the reaction mixtures, by keeping them from contact with atmospheric oxygen with the use of parafilm seals, and by limiting the agitation of the samples while mixing as much as possible. These measures minimize the level of dissolved oxygen. Even with these safeguards, the colorless solutions containing the illuminated samples of chloroplasts will begin to turn blue over a period of several minutes following the addition of DCMU, especially once their parafilm covers are removed. (The answer to question 2.) This spontaneous reoxidation can be used as an opportunity to illustrate two facts: first, a change in oxidation state is often accompanied by a change in energy state; and secondly, it is important for experimenters to be familiar with the nature of the reactants in their assay. Since the change in the absorbance of the redox indicator is temporary, it is necessary to measure it as quickly as possible.

Safety

Both DCPIP and DCMU can be toxic and tumorigenic and should be handled with the usual precautions: avoidance of skin contact with the solids and solutions, and inhalation of their dust. Please see the Material Safety Data Sheets of these two compounds for more details.

Student Outline

Introduction

Light drives electron transport through the two photosystems in chloroplast thylakoid membrane (Amesz, 1987; Govindjee and Coleman, 1990; Greenberg, 1991). The process can be studied *in vitro* if an electron acceptor is provided (Allen and Holmes, 1986; Kyle et al., 1984; Peterson and Arntzen, 1982). In this exercise, the oxidized form of DCPIP (2,6-dichlorophenol indophenol) accepts two electrons per molecule from photosystem II, as represented by the following reaction:

$$H_20 + DCPIP_{ox} \xrightarrow[PSII]{LIGHT} \tfrac{1}{2}\, O_2 + 2H^+ + DCPIP_{red}$$

The oxidized form of DCPIP is dark blue and the reduced form is colourless. In this assay, the degree of reduction of DCPIP is determined by measuring the change in absorbance of light at 590 nm with a spectrophotometer.

Procedure

The samples assayed in this procedure are four cellular fractions and a cell homogenate prepared by the following technique: 2- to 3- week-old pea plants were homogenized in a Waring blender; the resulting mixture was strained, centrifuged three times, and the pellets resuspended according to the flow chart in Figure 5.3.

1. Put five test tubes into a test tube rack, and label them as follows: **HOM**, **N/D**, **InCP**, **BrCP**, and **CYTO**. Dispense 3 ml of degassed grinding buffer into each test tube, avoiding turbulence as much as possible, and add 25 µl of DCPIP solution (5 mM in 95% ethanol) to each.
2. Put 25 µl of homogenate into the test tube labeled **HOM**. With a fresh tip each time, transfer 25 µl of each of the other fractions into the appropriately labeled test tubes. Seal the top of each tube with parafilm. Mix the contents of each test tube gently but thoroughly. *Immediately* place the rack of test tubes on your benchtop under illumination by a pair of lamps. Note the time so that the reactions occurring in the tubes can be stopped after 20 minutes.
3. Repeat steps 1 and 2 with a second rack of test tubes, but instead of putting them under illumination, put the rack into your bench drawer or cabinet to keep it in the dark (dark controls). It is convenient if the dark incubation begins about 5 minutes after the light-incubation. Note the time.
4. Remove the set of buckets from your centrifuge and place them on ice.

5. After 20 minutes incubation of the set of test tubes from step 2 (i.e., the light-incubated samples), peel back the layer of parafilm of each tube and add 25 µl of DCMU to stop the reactions. Re-seal the tubes after the DCMU has been added and gently swirl the contents to ensure that they are well mixed. Press any loose ends of parafilm against the sides of the tube so that the seals are not dislodged while they are being centrifuged. Place the tubes into the chilled buckets and centrifuge them for 5 minutes at 1000 × g in a benchtop centrifuge to remove suspended particulate material. While the centrifuge is running, make up a zeroing blank of 3 ml grinding buffer, 25 µl of DCMU, and 50 µl of distilled water. When the centrifuge has stopped, carefully decant the supernatant from each tube into a labeled test tube. Reseal these tubes with parafilm.
6. Repeat step 5 for the dark-incubated tubes. Zero the spectrophotometer at 590 nm with the zeroing blank, and using a single spectrophotometric cuvet, read the absorbances of both sets of tubes.

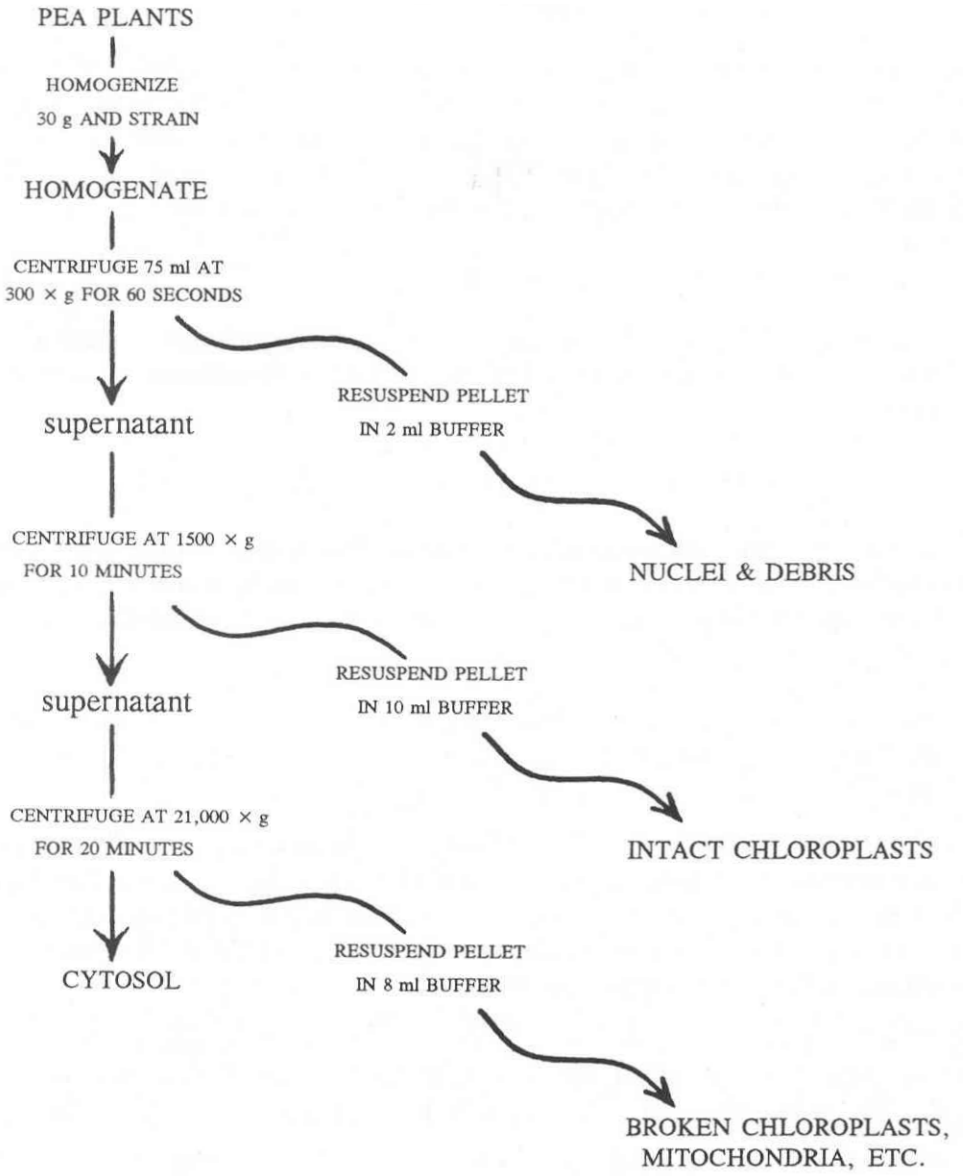

Figure 5.3. Flow chart of the fractionation procedure for pea plants.

Calculations

Subtract the absorbance of the illuminated sample from that of the corresponding dark-incubated sample to give the difference in absorbance (ΔA) for the homogenate and for each fraction. The molar extinction coefficient for this assay is 16×10^3; that is, a change in the concentration of the oxidized form of DCPIP of 1 mole per liter changes the absorbance of the solution by 16×10^3. As an example, let us use a value for ΔA of 0.50:

ΔA	INVERSE OF MOLAR EXT. COEFF.	VOLUME IN SPEC. TUBE				
	$\frac{\text{moles}}{\text{liter}}$	$\frac{\text{ml}}{\text{ml/liter}}$		$\frac{\mu\text{moles}}{\text{mole}}$	$\frac{\text{minutes/hour}}{\text{minutes}}$	ml^{-1}
$0.50 \times$	$\frac{1}{16 \times 10^3}$	$\times \frac{3.075}{10^3}$	$\times$	10^6	$\times \frac{60}{20}$	$\times \frac{1}{0.025}$
	gives conc. of DCPIP in spec. tube in moles/liter per 20 min	converts to actual no. of moles of DCPIP in tube		converts moles to μmoles	converts to activity per hour	adjustment for size of aliquot used

Equals 11.53 micromoles of DCPIP reduced per ml of sample per hour. As the reaction indicates, for every 2 moles of DCPIP that are reduced, 1 mole of O_2 is generated.

The total activity (amount of oxygen produced) in each sample can now be derived by multiplying the activity per ml by the *total volume* of the fraction. The sum of the activities in all the fractions except the homogenate can be compared to the homogenate's activity to find the *percent recovery*. Determine the percent recovery of activity for the fractionation and the *distribution* of recovered activity in each fraction.

Percent recovery of activity for the fractionation = (Total amount of activity in all fractions) ÷ (Amount of activity in the homogenate) × 100%

Distribution of recovered activity found in each fraction = (Amount of activity in that fraction) ÷ (Total amount of activity in all fractions) × 100%

Questions

1. The purpose of the dark controls is to verify that the reaction under study is light-dependant. Why is it important to do this?
2. You may have seen some of the illuminated reaction tubes that had become colourless reverting to blue sometime after you added DCMU. What reaction was taking place? Why?

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