BISC 367   Plant Physiology Laboratory   Simon Fraser University

Chloroplast photosynthesis

Effects of varying light intensities and wavelengths on the activity of the oxygen evolving complex

Photosynthesis can be divided into two major pathways, the "light reactions and the carbon reduction reactions". The light reactions consisting of the light regulated splitting of water and subsequent transport of electrons takes place in the grana of the chloroplast. The rate of this reaction can be monitored by several methods. For example, the rate of NADPH production (the end product of the electron transport chain) can be monitored with a dye such as Neotetrasolium chloride. Another frequently used method to detect chloroplast activity is to monitor the rate of oxygen evolution. Although the final electron acceptor *in vivo* is NADP, in isolated chloroplasts, the activity of this compound is quite low. Therefore artificial electron acceptors such as potassium ferricyanide or methyl violagen are used to sustain the transport of electrons, received from the splitting of water at photosystem II, across the electron transport chain.

In this exercise we will be using isolated spinach chloroplasts to study the effects of quantity (different light intensities) and quality (different wave lengths) of light on the rate of oxygen evolution. Also, the effectiveness of two photosystem inhibitors on O_2 production will be compared. The mode of action of these inhibitors is different. DCMU (3-3,4 dichlorophenyl-1.1 dimethyl urea) is an herbicide that accepts electrons from one of the intermediates in the electron transport chain (ETC). FMN (flavin mononucleotide) on the other hand inhibits oxygen production by accepting electrons and transferring them to O_2 (Mehler reaction). Figure 1 shows the arrangement of electron carriers in the ETC.
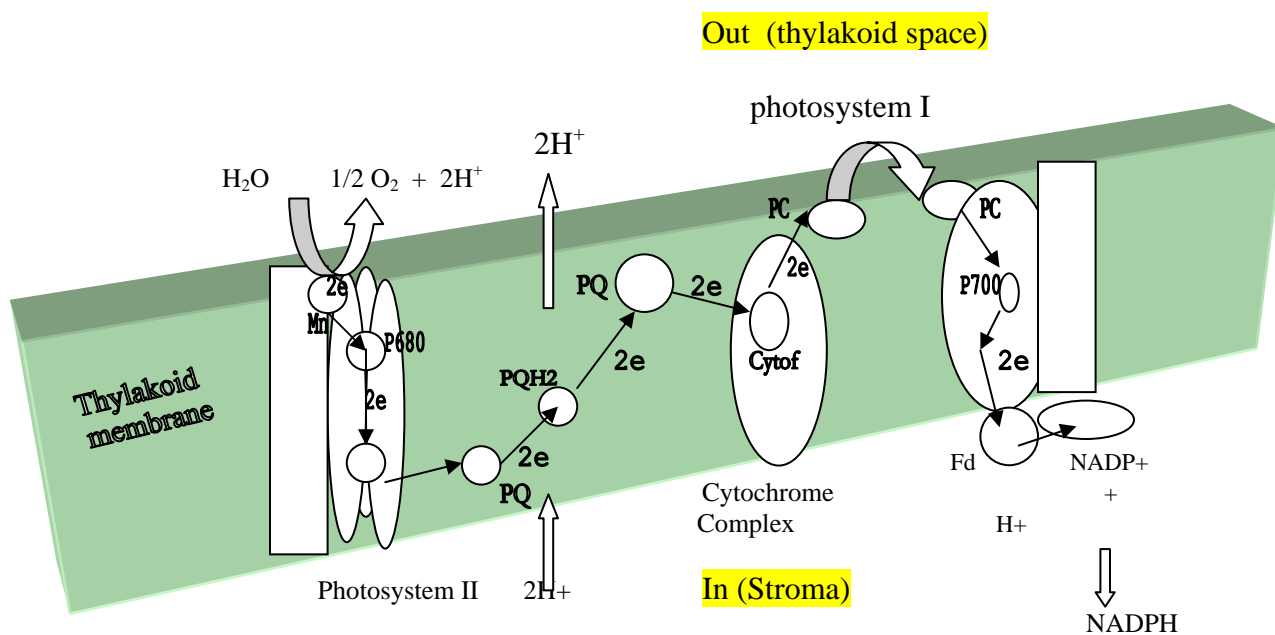

Figure 1. Arrangement of electron carriers in the thylakoid membrane

Oxygen electrode reaction:

Dissolved O_2 can be measured electrochemically with a Clark O_2 sensor. In the Clark O_2 sensor, the cathode or negative electrode, is made of Gold (Au) or Platinum (Pt) while the anode or the positive electrode is silver (Au). At the cathode, molecular O_2 is consumed along with the electrons. The electrodes are immersed in saturated KCl as the electrolyte.

Cathode reaction: $O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$

Anode reaction: $4Ag + 4Cl^- \rightarrow 4AgCl + 4e^-$

The movement of electrons from the anode to the cathode creates a current which can be measured with a sensitive ammeter. For each molecule of oxygen that comes into contact with the cathode, a proportional current travels through the circuit. Constant stirring is essential since oxygen is constantly being consumed at the cathode. With constant stirring, a change in current indicates a change in O_2 partial pressure in the solution.

OBJECTIVES:

At the end of this exercise you should;
a. Know how to use the blender (medium shearing force) to homogenize spinach leaves and how to use the high-speed centrifuge to isolate chloroplasts.
b. Know the importance of each component in the buffer used for isolating chloroplasts.
c. Understand the theoretical aspects of the Clark O_2 electrode, how to calibrate and use it.
d. Know how to use the Li-cor Inc. photon meter and the units of photosynthetic photon flux (PPF)
e. Know how to extract chlorophyll and determine chlorophyll concentration spectrophotometrically.
f. Know how to analyse your data and express the rate of oxygen evolution in mg or µg $l^{-1} mg^{-1}$ chlorophyll min^{-1}
g. Be able to define and calculate the quantum yield of oxygen evolution.
h. Know the role of DCMU.
i. Know the role of FMN and pseudocyclic electron transport (Mehler reaction).
j. Know how to use the phase contrast microscope to determine the intactness of chloroplasts.

MATERIALS:

a. Fresh spinach leaves, balance, cheese cloth and beakers in ice in a dark ice bucket.
b. Waring blender, rubber policemen and graduated cylinders.
c. Ice cold 0.4 M sucrose in 0.05M Tris buffer at pH 7.8 with 0.01M KCl.
d. Ice cold 0.1M potassium ferricyanide (K_4FeCN_6 – artificial electron acceptor).
e. Ice cold 0.01M riboflavin phosphate (FMN).

f. Ice-cold 1 mM DCMU
g. Cold centrifuge tubes and graduated cylinders.
h. Set up of O_2 electrode, reaction chamber and chart recorder.
i. Licor Light meter and Plexiglas filters.
j. Acetone, test tubes and rack, glass funnel and glass wool for extraction of chlorophyll.
k. Spectrophotometer with glass cuvette for chlorophyll determination.
l. Phase contrast microscope, slides and coverslips

PROCEDURES:

WEEK 1

A. Preparation of chloroplasts:

IT IS ESSENTIAL THAT THE CHLOROPLASTS BE KEPT COLD DURING THIS ENTIRE PROCEDURE

1. Blend 80g of spinach leaves (the leaves have been prepared for you with the midrib and stalks removed) in 160 mL of ice-cold sucrose-buffer for 15-20 sec. at top speed in the blender.

2. Strain the mixture through 8 layers of cheese-cloth into a chilled beaker, pour the extract into 4 chilled centrifuge tubes labeled 1-4. Balance in pairs.

3. Centrifuge at 2000 x g (~ 4000 rpm in the Sorvall SS 34 rotor) for 1-2 min. (refer to the table with the centrifuge to obtain the correct rpm values for a given rotor). You will need the sediment or the pellet.

4. Decant the supernatant of tubes 1 to 3 and add 26 mL sucrose-buffer. Resuspend your pellets gently by stirring with a glass rod covered with a rubber policemen. These tubes are now ready for use and MUST be kept in the dark.

5. To the pellet in tube # 4, add 20 mL of $1/10^{th}$ dilution of the sucrose buffer in water and stir. After 20 min on ice, centrifuge as above and resuspend in 26 mL of $1/4^{th}$ dilution of sucrose-buffer. This procedure produces broken chloroplasts (how?). Keep all the preps in a covered ice bucket to prevent damage from light.

B. Calibration of Oxygen meter:

1. Transfer 25 mL of cold aerated buffer into the reaction chamber. Add a stir bar and stir well. Make sure cold water is running through the outer jacket of the chamber.

2. Select the zero position using the select knob of the oxygen meter. Turn the O_2 zero knob to read 0.00.

3. Turn the select knob to the temperature position and record the temperature. Then turn the same knob to % position and set to 100% (using the O_2 calibration knob). This represents **100%** air saturation.

C. MEASUREMENT OF OXYGEN PRODUCTION:

1. Make sure to reserve 1 mL of each sample for determination of chlorophyll concentration. Put this into a labeled glass tube.

2. Fill the reaction flask for the oxygen electrode with 25 mL of your chloroplast preparation from tube # 1. Remember to insert a magnetic stir bar and make sure stirring is continuous. Be sure water is flowing through the outer cooling chamber. Stop the stirring so that you can place the lid on the oxygen electrode. Make sure that you eliminate air bubbles through the groove in the side of the lid. Resume stirring.

3. Measure the O_2 concentration in the dark for 1 min and then in the light for 1 min.

4. Using a 1 mL syringe, add 0.1 mL of Ferricyanide in the dark and repeat step 3. BE VERY CAREFUL FERRICYANIDE IS VERY TOXIC.

5. Add another 0.1 mL of Ferricyanide solution and measure the change of oxygen concentration for several minutes in the light. Measure the light intensity (PPF) using a quantum meter. We will insert 2 pieces of plexiglass between the light source and the quantum meter to approximate the light level inside the oxygen electrode.

6. Add 0.1 mL of FMN and continue to measure O_2 concentration for several minutes. FMN accepts electrons from PSI and passes them to O_2 to produce H_2O_2. This means that O_2 is being removed from the preparation at the same time as it is being evolved.

7. By gradually adding more FMN determine approximately how much FMN must be added to a chloroplast preparation to completely inhibit net oxygen production. Record how much has been added when O_2 production is finally blocked. For this value to be meaningful it must include a measurement of chlorophyll content. Refer to section on chlorophyll determination.

8. Repeat steps 2 to 6 using DCMU as the inhibitor and a **fresh sample** of chloroplast (from tube # 2).

9. Compare the effectiveness of FMN and DCMU (note the concentrations used).

D. MEASUREMENTS WITH BROKEN CHLOROPLASTS

1. Measure the O_2 production as in the previous section (C) using the broken chloroplasts. Are whole chloroplasts necessary for O_2 production?

2. Observe a sample of your unbroken and broken chloroplasts by placing a drop of each on a slide and place a cover slip on the drop. Remove excess solution using a Kleenex. Observe under 40x magnification using PH2 position of the phase selector. An intact chloroplast has both membranes intact and contain all stromal contents etc. and appears yellowish and bright structureless with a black border. The broken chloroplasts appear dull green and flat.

E. CHLOROPHYLL DETERMINATION

1. Mix 0.5 mL of your reserved chloroplast suspension with 9.5 mL of H_2O / acetone (20 mL of acetone + 4.5 mL H_2O) in a glass tube.

2. Filter through glass wool into a beaker.

3. Transfer to a 3 mL glass cuvette (Why not use a disposable plastic cuvette?) and read absorbance at 652 nm. Remember to zero the spectrophotometer first with the acetone / H_2O mix.

4. Determine the chlorophyll content (µg/mL) for the dilute sample using,

$$\text{Concentration (C)} = \frac{A_{652}}{0.0345} \quad \text{(this is the extinction coefficient for chlorophyll at this wave length)}$$

5. Calculate the concentration of chlorophyll in the reaction flask for each experiment. Be sure to correct for the dilution factor that you made in step 1.

6. Calculate the rate of oxygen evolution (Photosynthesis) as mg or mmol/mg Chl. min. (refer to the sample calculation given below)

WEEK 2

1. Blend **40** g of spinach leaf blades (as for week one) in **100** mL of sucrose-buffer for 15-20 sec at top speed in the blender (you may have to repeat blending another two times).

2   Strain the mixture through 8 layers of cheese-cloth into a chilled beaker, pour the extract into 3 chilled centrifuge tubes labeled 1 to 3. Balance tubes 1 and 2 and have

another tube with water to balance against tube 3.

3   Centrifuge at 2000 x g (~ 4000 rpm in the Sorvall SS 34 rotor) for 1-2 min. (refer to the table with the centrifuge to obtain the correct rpm values for a given rotor). You will need the sediment or the pellet.

4   Decant the supernatant of tubes # 1 and 2 and add 26 mL sucrose-buffer. Stir with a glass rod covered with a rubber policemen. These tubes are now ready for use and MUST be kept in the dark.

5   To tube # 3, add 20 mL of $1/10^{th}$ dilution of the sucrose buffer in water and stir. After 20 min on ice, centrifuge as above and resuspend in 26 mL of $1/4^{th}$ dilution of sucrose-buffer. Keep all the preps in a covered ice bucket to prevent damage from light.

6   **Make sure to reserve 1 mL of each sample for determination of chlorophyll concentration**

7   Using a fresh chloroplast prep (tube # 1) repeat steps 2-5 (**in section C page 4**) using green, blue and red filters (in that order, increase the light intensity when the filters are in place). Also, measure oxygen evolution in the light but ensure that the light level is not set to the maximum. Measure the light intensity (PPF) for each filter and for the white light using the quantum meter.

8   Using another fresh prep (tube # 2), measure the effects of low to high light intensities (use cheese cloth frames A-D as filters) on the rate of oxygen evolution. Start from the lowest light intensity (with all the filters together) and end with no filter.

9   Calculate and compare the **quantum yields** (rate of oxygen evolution per quanta of light) at different wavelengths and at different light intensities of white light.

10 Determine the chlorophyll concentration in each sample according to section E of week 1.

Calculations

You should now convert the % saturation value to partial pressure (kPa) using the given barometric pressure.

Example:
>   If the barometric pressure = 100.5 kPa and
>   if you get the water vapor pressure (wvp) @ $5^{o}C$ from the tables
>   provided,

partial pressure of oxygen $p(O_2)$ in the air equilibrated system is,

$p(O_2)$ = 0.209(100.5 - wvp @ 5°C).

This value represents 100 %.

Any other % values can then be converted to partial pressures using this value.

The rate of oxygen evolution can be obtained as a % change in a given time interval (slope of the line) and converted to partial pressure.

To convert the partial pressure to concentration ppm or mg/l) you have to use Henry's law.

ppm = k_H . $p(O_2)$

Since Henry's constant k_H depends on the temperature and salinity of the medium first you have to calculate k_H using the solubility of oxygen at that particular temperature and salinity. This value is obtained from the tables provided.

$$\frac{\textbf{ppm}}{\textbf{p}(O_2)} = k_H .$$

Now that you know the k_H and the pO_2, you can calculate the change in oxygen concentration (mg/l) for a given time period.