The Hill Reaction in Chloroplasts

Photosynthesis is a cellular process that occurs within the chloroplasts of plants, some algae, and some bacteria. Photosynthesis consists of a series of chemical reactions in which the electrons from water and the energy from sunlight are used to convert CO_2 into organic molecules, such as glucose. In the *light reactions*, excited electrons (from the photosynthetic pigment chlorophyll) are moved among a series of electron carriers that are embedded in the *thylakoid membrane* of the chloroplasts. The energy obtained by this electron transport drives the synthesis of ATP in the *stroma*, and the final step in the light reactions is formation of NADPH. In the *dark reactions*, the ATP and NADPH are used to convert CO_2 to carbohydrate. The dark reactions begin in the stroma but continue in the cytosol.

The *Hill reaction* is the portion of the light reactions in which electrons from water are transferred to an electron acceptor, reducing the acceptor. This reaction was first observed by Robert Hill in 1937 and it was he who demonstrated that isolated chloroplasts can produce O_2 in the absence of CO_2. This established that the source of the electrons used in the light reactions was indeed water. In chloroplasts, the final electron acceptor is $NADP^+$, which is reduced to form NADPH, but the Hill reaction can be studied in the laboratory by using an artificial electron acceptor. The dye 2,6-dichlorophenolindophenol (DCIP) is a useful acceptor because it changes color as it reduced, from blue (oxidized form) to colorless (reduced form). This permits the progress of the Hill reaction to be monitored in isolated chloroplasts.

The Hill reaction can be inhibited by various chemicals that interfere at different steps of the processes of electron transport and phosphorylation. The two inhibitors to be used here are ammonia and DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, a herbicide.

There are three objectives for this laboratory. (1) To isolate active chloroplasts from spinach leaves. (2) To measure the rate of the Hill reaction. (3) To examine the effects of two inhibitors on the rate of the reaction.

Procedures

A. Isolation of Chloroplasts

Note: *It is important to keep all solutions and labware cold during the extraction.*

1. Weigh out 4 g of fresh, deveined spinach leaves. (About 5 good-sized leaves).

2 Cut the leaves into small pieces with scissors and place in a *chilled* mortar. Add 15 ml ice-
cold Tris-NaCl solution along with a pinch of sand. Grind the tissue for approximately two minutes.

3. Pour the homogenate through a few layers of cheesecloth into a chilled 15-ml centrifuge tube; squeeze the cloth to get as much of the extract as possible. After balancing, centrifuge the tubes at 200*g* for 1 minute.

4. Decant the supernatant into a clean, chilled centrifuge tube and spin at 1300 *g* for 5 minutes.

5. Remove and discard the supernatant and add 10 ml ice-cold Tris-NaCl to the pellet. Resuspend the pellet *thoroughly* with a Pasteur pipet.

6. Transfer 4 ml of the chloroplast suspension to a clean , chilled test tube and add 6 ml ice-cold Tris-NaCl. Cover the tube with Parafilm and invert to mix. This is the chloroplast suspension that will be used for the following experiments. *Keep the chloroplast suspension on ice at all times.*

G. Measuring the Rate of the Hill Reaction

The experiments will be conducted in a semi-darkened room so that ambient light will not influence the reactions taking place in the test tubes. The rate of the Hill reaction will be measured with a spectrophotometer. Make sure the instrument is allowed to warm up for at least five minutes. Your instructor will show you how to use the spectrophotometer.

DCIP will revert to its oxidized state (blue) as soon as the chloroplast suspension is removed from the light path. It is important to take all absorbance readings as quickly as possible. Make sure you have a good understanding of what you are doing before you begin the experiments. It will be necessary to coordinate your activities with the rest of your group and with other groups in the class.

Since the rate of the Hill reaction is affected by temperature, a "heat-sink" will be placed between the light source and the reaction tubes. This should keep the temperature fairly constant over the course of the measurements.

1. Place a test tube rack 25 cm from the light source (with the heat-sink in between). Keep the light off until the start of each measurement.

2. Label five cuvettes as shown below; prepare a data sheet with spaces for recording the absorbance of each tube at 1-minute intervals, from 0 to 10 minutes.

Tube	Tris-NaCl	DCIP	Ammonia	DCMU	Water	Chloroplast Suspension*
Blank	3.5 ml	—	—	—	1.0 ml	0.5 ml
1	3.5 ml	0.5 ml	—	—	0.5 ml	0.5 ml
2	3.5 ml	0.5 ml	—	—	0.5 ml	0.5 ml

3	3.5 ml	0.5 ml	0.5 ml	—	—	0.5 ml
4	3.5 ml	0.5 ml	—	0.5 ml	—	0.5 ml

* Be sure to keep the chloroplast suspension on ice; mix the suspension before dispensing by covering the tube with Parafilm and inverting.

Prepare the tubes by adding the solutions in the order given. After all solutions have been added, cover each tube with Parafilm and invert to mix. *Do not prepare a tube until you are ready to conduct your readings on that tube.* Remember to adjust the spectrophotometer with the blank before taking the absorbance readings.

3. Wrap tube 1, the non-illuminated control, in two layers of foil and cover with a foil top. Set this tube aside. You will take a reading on this tube only after 10 minutes.

4. Prepare tube 2 (make sure the contents are mixed) and *immediately* take an absorbance reading at 600 nm (do not expose the tube to light for this 0 minute reading). Record the absorbance on your data sheet.

5. Place tube 2 in the test tube rack and turn on the light. After 1 minute, wipe the outside of the tube with a KimWipe and take a second reading on tube 2; record the absorbance on the data sheet.

6. Return tube 2 to the rack and continue taking readings at one-minute intervals for a total of ten minutes. *Make all readings as quickly as possible, as DCIP will revert to its oxidized state as soon as the sample is removed from the light.*

(Remember to take a reading on tube 1 after 10 minutes have elapsed).

7. When finished with tube 2, prepare tube 3 by mixing the solutions as described in the table above.

8. Take an initial (0-minute) reading before exposing tube 3 to light, and continue taking readings at one-minute intervals for ten minutes. Record the readings in the data sheet and work as quickly as is practical. Remember to adjust the spectrophotometer for the blank before taking the absorbance readings.

9. When finished with tube 3, prepare tube 4 and proceed with absorbance readings as done on tubes 2 and 3 above. Record the readings on the data sheet.

Data presetation

For tubes 1 - 4, calculate a *delta A* value for each time interval. *delta A* is calculated for each time point by subtracting the absorbance reading at a specific time for that tube from the initial absorbance reading for that tube (at 0 minutes). Present the *delta A* values for each tube in the form of a table.

Plot the cummulative change in absorbance *delta A*, ordinate) versus time (minutes, abscissa) for tubes 2 - 4. For each tube, assume the 0-minute value for *delta A* to be 0 and include that point on your graph.

Points to ponder (as you write your lab report)

Explain the centrifugation steps in part A. (Remember that the goal is to isolate chloroplasts).

In which tube (1 - 4) does the reaction proceed most rapidly? Explain your answer.

How do the shapes of the curves for the inhibitors (tubes 2 and 3) compare to the shape of the curve for tube 1? Does this tell you anything about the mechanisms of inhibition?

Adapted from: Bregman, A. *Laboratory Investigations in Cell and Molecular Biology*, third edition. J. Wiley & Sons, New York.