LABORATORY 1

The Hill Reaction

OBJECTIVE

- To determine the photosynthetic activity of chloroplasts using measurement of the Hill reaction.

BACKGROUND

The photosynthetic action of chloroplasts involves an electron transfer chain. Sunlight energy is transferred by chlorophyll to activated electrons that are used to generate ATP from a proton gradient. To quantify the functioning of chloroplasts, the amount of electron transfer occurring can be measured colorimetrically. The activity of the Hill reaction, or electron transfer, in chloroplasts is commonly measured using the chemical DCPIP (2,6-dichlorophenolindophenol.) DCPIP is an electron acceptor that converts from the oxidized to the reduced state with a corresponding decrease in absorbance at 600 nm (color changes from blue to clear). It typically accepts electrons transferred from plastoquinone in the electron chain (see figure below). Any pair of high-energy electrons can cause this transition, however. Since we want to measure photosynthetic activity, it is important to determine only light-induced reduction of DCPIP. Think about how the reported value can be limited to light-induced reduction by chloroplast activity. Identify the control and experimental tubes to clarify what information is being collected in the data from each tube.

Conditions present during the measurement of DCPIP reduction are also important to obtain useful data. Control of temperature, oxygen concentration, and light exposure are crucial to proper measurements. Reduced room lighting and controlled exposure of chloroplasts to light during all phases of work, as well as ice baths, are used. Parafilm is sealed on the tubes, and bubbles are minimized in samples, to reduce reoxidation of DCPIP by oxygen.

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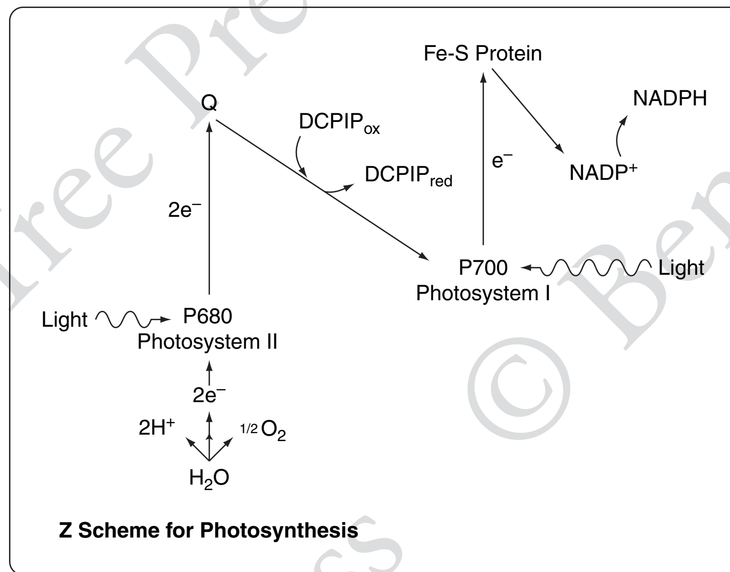

Z Scheme DCPIP

MATERIALS

- Chloroplast suspension
- Heat-killed (inactivated) chloroplast suspension
- Tris buffer (0.1 M Tris, 0.03 M NaCl, pH 6.5)
- 4×10^{-4} M Dichlorophenolindophenol (DCPIP)
- High intensity lamps (60-100 watt)
- Spectrophotometer and cuvettes
- Pipettes and micropipettes
- Beakers for ice baths
- Parafilm

PROCEDURE

1. Prepare isolated chloroplasts as detailed in the chloroplast isolation lab. Dilute the chloroplasts in suspension buffer to yield 0.01 mg/ml chlorophyll, as calculated previously from chlorophyll quantification. *Why is a specific concentration of chlorophyll important?*

2. Take 1.5 ml of diluted chloroplasts and inactivate them by incubation in a boiling water bath for 5 minutes, then cool on ice briefly. *What is the predicted effect of boiling?*

3. Prepare four test tubes as described below, adding all but the 0.5 ml ice-cold chloroplasts (active or heated). *Why does the volume of water vary between tubes?*

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Tube	1	2	3	4
Tris Buffer	1.5 ml	1.5 ml	1.5 ml	1.5 ml
Water	1.0 ml	0.5 ml	0.5 ml	0.5 ml
DCPIP	None	0.5 ml	0.5 ml	0.5 ml
Active chloroplasts	None	None	0.5 ml	0.5 ml
Heated chloroplasts	0.5 ml	0.5 ml	None	None

4. Turn on the spectrophotometer and adjust the wavelength to 600 nm.

5. Be sure chloroplasts are well mixed before pipetting into each tube. Add 0.5 ml heated chloroplasts to tube 1, mix well, and use this tube to blank the spectrophotometer at an absorbance of 0. (Tubes can be mixed by gentle swirling or covering with Parafilm and inverting.)

6. Add 0.5 ml of heated chloroplasts to tube #2, mix, and immediately measure and record the absorbance at 600 nm. Be sure to record the time for each reading.

7. Add 0.5 ml of active chloroplasts to tube #3. Mix and immediately measure the absorbance of the tube.

8. Add 0.5 ml of active chloroplasts to tube #4. Mix and immediately measure the absorbance of the tube. Wrap tube #4 immediately in two layers of aluminum foil to block out light. This tube serves as a "dark" control and the absorbance of this solution will be read again only at the end of the experiment. It is important that no light reach the chloroplasts inside, but the tube is otherwise treated the same as the others. *What information are you collecting from tube #4 that helps with your analysis?*

9. Place tubes #1, #2 and #3 in a beaker of water with ice to maintain constant temperature, and illuminate with a strong light source. Also keep #4 at the same temperature without light exposure.

10. Measure the absorbance of each tube after 5 minutes, 10 minutes, 15 minutes, and finally after 20 minutes, always blanking with tube #1 and mixing tube prior to reading. Be sure to wipe the tubes/cuvettes dry, and clean before inserting them into the spectrophotometer. *Why do this?* Record your results. Time intervals may be shortened to obtain more data points, if desired (e.g., 2- or 3-minute intervals).

11. Create a graph of data for dye reduction versus time.

12. Calculate the total dye reduced (concentration) at your time intervals using the following equation:

$$Cr = \frac{Co(Ao-Ac)}{Ao}$$

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where:
Cr is the concentration of dye reduced in moles/liter
Co is the original dye concentration in moles/liter
Ao is the absorbance of the mixture at the beginning
Ac is the absorbance of the mixture at the end of the time period

How do you adjust your data to remove reduction that is not light dependent?

11. Plot on graph paper the dye reduced over time for tubes 2 and 3. What effect did boiling the chloroplasts have on dye reduction? What information is this tube providing? Was there any DCPIP reduction in tube 4?

12. Discuss what dye reduction means as a measure of chloroplast function.

QUESTIONS TO CONSIDER

1. What conclusions can you make about your chloroplast suspension from your experimental data?

2. What factors might affect the efficiency of chloroplast electron transfer?

3. What would be expected to happen if all the chloroplasts were broken?

4. Would mitochondrial contamination be a concern? Why or why not?

5. What does it mean to uncouple electron transport?

6. What would you predict a higher pH solution would do to the electron transport? What about a lower pH? Why?

Adapted from W. H. Heidecamp, *Cell Biology Laboratory Manual,* chapter 8, Gustavus Adolphus College (homepages.gac.edu/~cellab).

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