Chapter 3

Extraction of Chloroplasts from Plant Tissue and Their Use in Demonstrating the Hill Reaction

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Introduction

The purpose of this laboratory experiment is multi-fold: (1) to consider photosynthesis as a physiological system, (2) to consider the role of light in cellular functions, (3) to become familiar with some of the basic methods used in the disruption and fractionation of cells, and (4) to become familiar with the concept of synthetic electron acceptors/donors and their use in physiological studies. I have used this laboratory in a junior-senior undergraduate level general physiology course at Virginia Commonwealth University and in a senior undergraduate-basic graduate level cell biology course at the University of North Carolina at Chapel Hill. The students perform all of the procedures on their own, either individually or in groups of two to four people.

In the differential centrifugation procedure the following points are emphasized: (1) the principle of differential centrifugation and its use in a purification procedure, (2) the significance of expressing centrifugation data as the relative centrifugal force (RCF) rather than as revolutions per minute, (3) the different types of centrifuges and rotors which are used and why they are used, and (4) the proper and safe use of the centrifuge.

I also use the study to illustrate the significance of buffers, osmotic pressure, and ions in physiological experiments. The concepts of buffers and how they function is reviewed in lecture before the laboratory. The concepts of osmotic pressure and solute reflection coefficients, which are also covered in lecture before the laboratory, are illustrated by the presence of sucrose in the buffer. The significance of ions and ion balance is also discussed and is shown by the use of KCl in the buffer system. In most instances the instructor prepares the buffer for class use 24 to 48 hours before it is needed. I, however, do require that the students calculate and describe the procedures used in the preparation of the buffer; this assignment is submitted to the instructor for review. This provides the student with some ideas concerning the methods and procedures used in the preparation of buffers. During the laboratory (usually during one of the waiting periods in the centrifugation procedure) I review the principles and use of the pH meter with the students. In those cases where the class size is small (ten people or less) and the laboratory meets two to four times per week it is possible for the students to prepare the buffer themselves. Each student again calculates and describes the procedures for preparing the buffer, and then proceeds to make up 500 ml of the buffer for use in the study. This allows the student to have first-hand experience in the preparation of buffers and the use of the pH meter.

This study assumes that the student is familiar with the theory and use of the spectrophotometer. To assure this I have several laboratory experiments preceding this one which utilize the spectrophotometer extensively.

The entire experiment can be completed within a three-hour laboratory period. The time required to prepare the materials for the experiment if the instructor prepares the buffer is two to four hours. If the students prepare the buffer the preparation time of the instructor is reduced to one hour, although some time is required to assist students while they are preparing the buffer. For lower level classes or other instances where equipment, time, and/or laboratory space are limited, the instructor can use this experiment as a demonstration. The chloroplasts can be prepared several hours beforehand and will retain activity if kept in the dark at 4°C.

The following "Introduction" section of the laboratory handout, which is given to the students, reviews/reinforces concepts and principles of photosynthesis previously covered in lecture.

Student Materials

Photosynthesis is the mechanism by which light energy is converted into chemical energy, and can be summarized by the following overall equations:

(1) $nH_2O + nCO_2 \longrightarrow (CH_2O)_n + nO_2 + H_2O$

for those organisms which use water as an electron donor and produce molecular oxygen (includes the higher plants and algae).

(2) $nH_2S + nCO_2 \longrightarrow (CH_2O)_n + nS + H_2O$

for those organisms which use H_2S as the electron donor and do not produce molecular oxygen. Other electron donors include H_2, lactate, and thiosulfate. This group includes the photosynthetic bacteria.

Photosynthesis can thus be generalized in the following equation:

(3) $nH_2D + nCO_2 \longrightarrow (CH_2O)_n + nD + H_2O$

where H_2D is the electron donor and CO_2 is the final electron acceptor. Other electron acceptors are also used, e.g., NO_3^- and N_2 in the nitrogen-fixing bacteria.

Although it was postulated many years ago that there were two processes involved in photosynthesis—(1) a reaction which is dependent on light and (2) a reaction which can occur in the absence of light—this was not conclusively shown until Robert Hill demonstrated light-dependent oxygen evolution in cellular preparations from photosynthetic organisms. Hill's preparations contained the cellular organelles known as chloroplasts (Hill 1937). Chloroplast preparations in the presence of light and an artificial electron acceptor (e.g., ferricyanide or other reducible dyes) demonstrated the evolution of oxygen and the simultaneous reduction of the electron acceptor according to the following equation:

(4) $H_2O + A_{ox} \longrightarrow AH_{2_{red}} + O_2$

where H_2O is the electron donor and A is the electron acceptor; water is the only electron donor required. It was further shown that CO_2 was not required for this reaction nor was it accumulated in a stable form. Equation 4 is known as the *Hill Reaction,* and the acceptor A is a *Hill Reagent.* Subsequent work showed that NADP was a Hill Reagent, and since NADP was already recognized as an electron donor in cellular biochemical reactions this result supports the idea that a reducing agent is one of the products of the light-dependent reaction. The role of NADP as a Hill reagent is summarized in the following equation:

$$(5)\quad H_2O + NADP \longrightarrow NADPH + H^+ + O_2$$

It was also found that isolated chloroplasts could form ATP from ADP and P_i (inorganic phosphate) in the presence of light, a process known as *photosynthetic phosphorylation* or *photo-phosphorylation.* Based on this and subsequent work it can be concluded that the light reactions of photosynthesis constitute a photoinduced electron transport system which results in the production of NADPH and ATP; the NADPH and ATP provide the energy for the "dark reactions" in which CO_2 and H_2O are converted to carbohydrates.

The light reaction in those cells which evolve oxygen apparently consists of two photosystems which together constitute a coupled electron transport system. The electron flow in this system is both cyclic and non-cyclic—the non-cyclic flow results in the photoreduction of NADP and the photophosphorylation of ADP to produce NADPH and ATP, and the cyclic electron flow results in the photophosphorylation of ADP only. The concept of the two light reactions and the cyclic/non-cyclic electron flow is summarized in Figure 3.1.

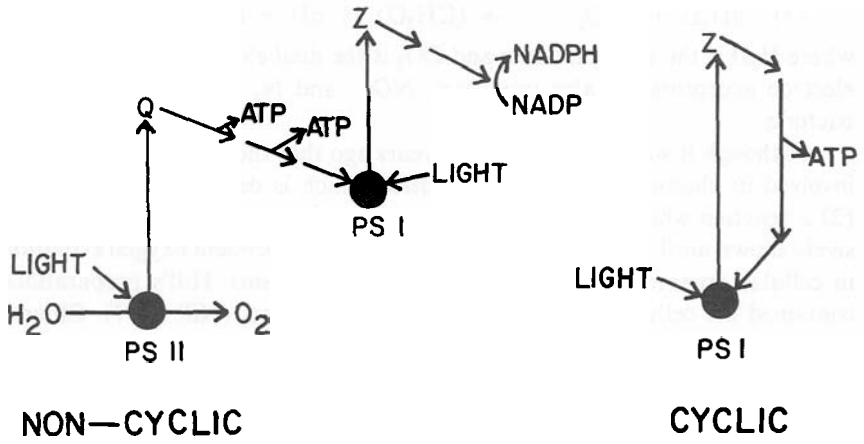

Figure 3.1. Non-cyclic and cyclic electron flow in photosynthesis (see text for additional information).

In this experiment the coupled electron transport system of photosynthesis will be studied using the Hill Reaction and a Hill reagent. The Hill reagent which will be used is 2, 6-dichlorophenolindophenol (DPIP). The acceptor has a blue color in the oxidized state; when it is reduced the acceptor becomes colorless. The equation of the reaction is summarized below:

(6) $DPIP_{ox} + H_2O \longrightarrow DPIP \cdot H_{2_{red}} + O_2$

The reaction can be quantitatively measured via determination of the rate of disappearance of $DPIP_{ox}$ by measuring the absorbance at 620 nm. $DPIP_{ox}$ absorbs strongly at 620 nm whereas $DPIP \cdot H_{2_{red}}$ does not. Although it is possible to measure the change in NADP directly by determining the rate of NADPH production (NADPH absorbs strongly at 340 nm whereas NADP does not), this method is subject to interference from proteins and oxidation of the NADPH. It is thus considerably easier to use artificial electron acceptors—the use of artificial electron acceptors can be easily extended to other systems where oxidation/reduction is involved, e.g., coupled enzyme reactions, mitochondrial electron transport.

In this experiment the effect of an agent which uncouples Photosystem I and Photosystem II is also studied. The uncoupler used is 3-(3, 4-dichlorophenyl)-1, 1-dimethyl urea (DCMU, a pre-emergent herbicide). The use of reagents which uncouple linked systems has been very useful in studying the mechanisms by which coupled reactions and reaction systems are linked. The effect of heating on the electron transport system will also be used to examine the chemical/structural aspects of the photoinduced electron transport system. The reactions of photosynthesis and DPIP and the role of DCMU is summarized in Figure 3.2.

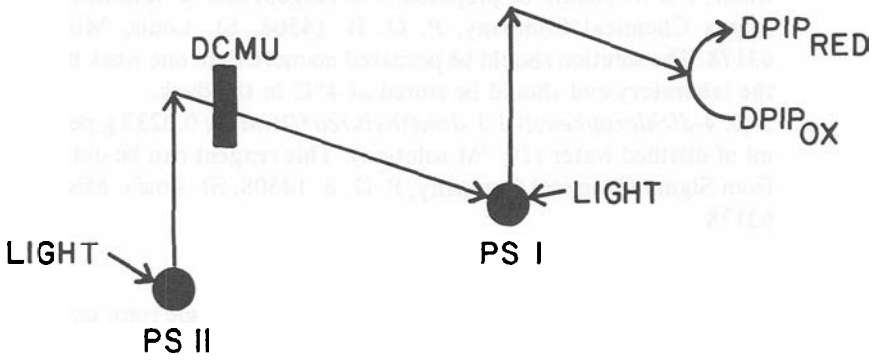

Figure 3.2. Reduction of DPIP and effect of DCMU on photosynthesis (see text for additional information).

Instructor's Materials

The quantities of material given are for twenty students working independently. The amounts can be adjusted accordingly when student groups are used.

1. PLANT TISSUE: Fresh spinach provides the best source of chloroplasts for the experiment. Approximately 400 g of spinach are required; frozen spinach cannot be used. If fresh spinach cannot be obtained, fresh collard greens can be substituted although the photosynthetic activity of the chloroplasts will not be as high. In an emergency almost any plant tissue that does not have thick cell walls or other factors which interfere with disruption can be used. I have successfully used red maple (*Acer rubrum*), pigweed (*Chenopodium album*), may-apple (*Podophyllum peltatum*), Virginia creeper (*Parthenocissus quinquefolia*), and wild ginger (*Hexastylis virginica*). These species are readily collected throughout the southeastern United States during the spring time, but the photosynthetic activities of the chloroplasts may not be as high as those obtained with spinach. Again, 400 g of material are adequate.

2. CHEMICALS:

A. BUFFER: 0.05M sodium-potassium phosphate, pH 7.3, made to 0.4M sucrose and 0.01M KCl (for preparation see Appendix I). Refrigerate at 4°C; 2 liters are required. The buffer should be prepared no more than 48 hours before the laboratory to minimize the chances of bacterial and fungal contamination.

B. 2, 6-dichlorophenolindophenol (DPIP): 8 mg per 100 ml of distilled water; 150 ml should be prepared. The reagent can be obtained from Sigma Chemical Company, P. O. B. 14508, St. Louis, Missouri 63178. The solution should be prepared no more than one week before the laboratory and should be stored at 4°C in the dark.

C. 3-(3, 4-dichlorophenyl)-1,1-dimethylurea (DCMU): 0.0233 g per 100 ml of distilled water (10^{-3}M solution). This reagent can be obtained from Sigma Chemical Company, P. O. B. 14508, St. Louis, Missouri 63178.

3. SUPPLIES AND EQUIPMENT

A. Centrifuge: A refrigerated centrifuge with a fixed angle rotor capable of holding eight to twelve 50-ml centrifuge tubes is required. Centrifuge rotors which meet this requirement include the Beckman JA-20, Dupont/Sorvall SS-34, International 816, 823a, and 870, and Lourdes 9RA. One centrifuge is required.

B. *Centrifuge tubes:* 50-ml plastic or glass tubes with round bottoms and no caps are needed. Tubes which meet this requirement include the Nalgene 3110 and 3117. The Nalgene 3117 is a polycarbonate tube that is transparent and is easier for the students to work with since they can readily see the pellet. A minimum of twenty centrifuge tubes are required. These tubes can be obtained from most scientific supply houses including Fisher Scientific Supply, Arthur H. Thomas and Scientific Products.

C. *Mortars and Pestles:* Five to ten glass mortars and pestles are required as a minimum. Glass is preferred to porcelain since it is more easily cleaned. These can be obtained from most scientific supply houses including Fisher Scientific Supply, Scientific Products, and Arthur H. Thomas.

D. *Waring Blender Units:* Waring blender units can be used in place of mortars and pestles in disrupting the cells. The commercial glass jars supplied with the power bases or an Eberbach 360-ml stainless steel container can be used. Two to three blender units are required as a minimum. The blender units can be obtained at the local store; the Eberbach container can be obtained from most scientific supply houses including Fisher Scientific Supply, Scientific Products, and Arthur H. Thomas.

E. *Plastic Funnels:* Ten to twenty plastic funnels with a 10-cm diameter (minimum) are needed. These funnels can be obtained from local stores or any scientific supply house.

F. *Cheesecloth*

G. *pH Meter:* A Corning Model 5 or equivalent pH meter with a plastic-sheathed combination electrode is required. One to three pH meters will be adequate. I have found the plastic-sheathed electrode to be quite durable and not easily broken.

H. *Light Source:* Any incandescent light source which can produce a light beam with an intensity of 1000 ft. cdls. at 20 cm is adequate. Light sources which meet this requirement include slide projectors, some high-intensity lamps, and some microscope lamps (Fisher 11–986, from Fisher Scientific Supply).

I. *Pipets:* Glass or plastic 1-ml, 2-ml, 5-ml, and 10-ml pipets are needed. Safety pipet fillers are recommended so that mouth-pipetting is not required.

J. *Test Tubes:* One hundred glass test tubes, 15 × 125mm or equivalent are required. These can be obtained from any scientific supply house.

K. *Spectrophotometer, Visible Range:* Four to five spectrophotometers and 40 curvettes are required. Spectrophotometers which can be used include the Bausch and Lomb Spectronic 20, Spectronic 70, and Spectronic 88, Coleman Model II Junior, Coleman Model 44, Turner Model 330, and Turner Model 350. The spectrophotometers and cuvettes can be obtained from most scientific supply houses including Fisher Scientific Supply, Arthur H. Thomas and Scientific Products.

L. *Teflon-Glass Tissue Grinders:* Two to five tissue grinders, 30-ml to 50-ml capacity, are required. These are used to resuspend the pellets during the differential centrifugation procedure. The author considers the use of a teflon pestle to be safer than an all glass system. The use of the tissue grinder is optional and can be replaced by using a stirring rod and vortex mixer. The stirring rod-vortex procedure will not resuspend the pellet as uniformly as the homogenizer, however. The tissue grinders and vortex mixers can be obtained from most scientific supply houses including Fisher Scientific Supply, Scientific Products and Arthur H. Thomas.

4. PREPARATION OF ISOLATED CHLOROPLASTS

NOTE: All operations should be carried out at 4°C; all solutions and containers should be pre-chilled. Do not expose the chloroplast suspension to bright light.

Grind 10 to 15 g of fresh spinach leaves (with midribs and petioles removed) by either of the two following methods:

(1) In an ice-cold mortar and pestle with 30 ml of 0.05M phosphate buffer pH 7.3 (made to 0.4M sucrose and 0.01M KCl).

(2) In a Waring Blender with 30 ml of 0.05M phosphate buffer pH 7.3 (made to 0.4M sucrose and 0.01M KCl). Grind at high speed for 1 minute.

Filter the homogenate through several layers of cheesecloth. If the blender method was used to disrupt the leaf cells, it may be necessary to further homogenize the preparation using a glass teflon homogenizer; this can be determined by examining the preparation microscopically for the degree of cellular disruption. After grinding in a teflon-glass homogenizer, again filter the preparation through cheesecloth.

Centrifuge the filtrate at 1500 × g for ten minutes. Discard the supernatant and resuspend the pellet in 35 ml of the sucrose-phosphate buffer using a glass-teflon homogenizer. Centrifuge the suspension at 1500 × g for ten minutes. Discard the supernatant and resuspend the chloroplast pellet in 15 ml of the sucrose-phosphate buffer. This is the stock chloroplast solution; keep the suspension at 4°C in subdued light.

5. PREPARATION OF ASSAYS

Using the procedure for tube #2 below adjust the chloroplast concentration from the stock suspension with the sucrose phosphate buffer to have the reaction proceed to completion in about 5 minutes. Use this diluted chloroplast suspension for all of the assay tubes.

Prepare 5 tubes according to the following protocol. PREPARE AND KEEP ALL TUBES IN SUBDUED LIGHT.

1. 3.0 ml sucrose-phosphate buffer
   2.0 ml diluted chloroplast supsension
   1.0 ml distilled water
   This tube will serve as a dye control to determine when the reaction has gone to completion. Use this tube to set the spectrophotometer to 0.0 absorbance (100% transmittance).
2. 3.0 ml sucrose-phosphate buffer
   2.0 ml diluted chloroplast suspension
3. 3.0 ml sucrose-phosphate buffer
   2.0 ml BOILED diluted chloroplast suspension
   The boiled chloroplast suspension is prepared by boiling the 2-ml aliquot in a boiling water bath for 5 minutes, cooling it to room temperature, and then adding the sucrose-phosphate buffer.
4. 2.8 ml sucrose-phosphate buffer
   2.0 ml diluted chloroplast suspension
   0.2 ml 10^{-3}M DCMU
5. **3.0 ml sucrose-phosphate buffer**
   2.0 ml diluted choloroplast suspension

When all of the tubes have been prepared add 1.0 ml of DPIP to tubes 2, 3, 4, and 5. Keep all of the tubes in the dark until you are ready to assay them for activity.

6. ASSAY PROCEDURE

The assay procedure is performed on each tube individually; do not run the assays simultaneously. Set the spectrophotometer wavelength selector to 620 nm.

(1) Transfer the contents of the tube #1 to a cuvette and set the spectrophotometer to 0.0 absorbance (100% transmittance) using this tube. Transfer the contents of tube #5 to a cuvette and measure its absorbance at 620 nm; record that value. Then wrap it in several layers of aluminum foil and keep it in the dark until the end of the experiment.

(2) Using tube #1 again set the spectrophotometer to 0.0 absorbance (100% transmittance). Transfer the contents of tube #2 to a cuvette and measure the absorbance of it at 620 nm and record this value; this is the absorbance at '0' time. Then take the cuvette out of the spectrophotometer and expose it to light from one of the light sources at a distance of 15 to 20 cm for 30 seconds. Remove the cuvette from the light at the end of the 30 second period, measure the absorbance of the suspension and record that value. Remove the tube from the spectrophotometer and expose it again to light for 30 seconds (60 seconds total exposure) at the same distance as before; again measure the absorbance at the end of this second 30-second exposure period. Repeat this procedure of 30 second exposures and absorbance measurements until the reaction has gone to completion. The reaction can be considered to have gone to completion when the absorbance in the experimental tube is the same as the absorbance in the control tube (tube #1) or when there is no change in the absorbance over a 2-minute period. (The absorbance may actually begin to increase slightly after it has leveled off and may not reach the exact absorbance of tube #1; when this occurs the reaction can be considered to have gone to completion).

Repeat the procedure with tubes #3 and #4. After the experiments with these tubes are completed take tube #5 out of the dark, unwrap it, and measure its absorbance. Repeat the procedure with tube #5 that was carried out for tubes #2, #3 and #4 to confirm that the reduction of DPIP is in fact light-dependent.

Using a light meter measure the intensity of the light at the point where the experimental tubes were exposed.

7. CALCULATIONS AND OTHER IDEAS FOR CONSIDERATION

For each experimental condition (tubes #2, #3, #4, and #5) prepare a graph of the results plotting the time on the x-axis and the absorbance on the y-axis. From this graph determine the rate of photosynthetic activity for each of the conditions (the rate is expressed as the change in absorbance per minute). I also require that the students utilize the linear regression program available in the University computer or in the departmental statistical calculators to prepare the graphs and determine if the observed correlations are statistically significant. This serves to introduce the students to the use of statistics in the presentation and analysis of biological data.

I have also found the following questions useful for initiating discussion of important ideas. What is the effect of boiling the chloroplast suspension on photosynthetic activity and why? What is the effect of uncoupling Photosystem I from Photosystem II on the reaction and why? How can this be used to develop chemical herbicides? How can this be used to study the relative reaction rates of each photosystem independently? Can oxygen production be used to measure photosynthetic activity? Would oxygen be produced if the

chloroplast suspension were boiled? Would oxygen be produced in the presence of chemical uncouplers such as DCMU? A white light source was used in this experiment; would you expect to find photosynthetic activity at all wavelengths? Why or why not?

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APPENDIX I
Preparation of the Buffer

Preparation of 0.05M sodium-potassium phosphate buffer 7.3 (made to 0.4M sucrose and 0.01M KCl).

Quantities given are for the preparation of 1 liter of buffer.

3.97 g Na_2HPO_4
2.99 g KH_2PO_4
136.9 g sucrose
0.745 g KCl

Dissolve the Na_2HPO_4 in approximately 700 ml of distilled water. When this is completely dissolved, add the KH_2PO_4 and dissolve it. When that salt is completely dissolved add the sucrose and dissolve it. When the sucrose is completely dissolved add the KCL and dissolve it. Add distilled water to give a final volume of approximately 900 to 950 ml. Adjust the pH of the solution with 1N KOH or HCl to a pH of 7.3. When the pH is properly adjusted, bring the final volume of the solution to 1 liter with distilled water. Store the buffer at 4°C.

APPENDIX II
Suggested Studies with the Hill Reaction Procedure

1. Photosynthetic adaptation in sun and shade plants

Many higher plants show a distinct adaptation to their light regime. Species which occupy high-light environments are generally capable of higher photosynthetic rates at light saturation and are termed sun species. Those species which grow normally in shady habitats and which typically do not show high rates of photosynthesis at increased irradiance levels are termed shade species. The high rates of photosynthesis observed in the sun species in high light regimes are due to an adaptation of the photosynthetic apparatus to increase the amount of photochemical energy produced. This results in an increase in the concentrations of some of the photosynthetic molecules and a requirement for more energy to support the system. In a high-light regime the sun species is capable of producing higher net amounts of photochemical energy than the shade species. Thus, the sun species have a competitive advantage in a sunny environment. While the energy needed to maintain the photosynthetic machinery of a sun species can be readily acquired when the light levels are high, its production may be limited by light in shady habitats. The shade species, which exhibit a lower maintainance energy requirement, would have competitive advantage over the sun species in a shady environment. The differences in photosynthetic rates and the adaptation of sun plants to high light regimes and shade plants to low light regimes via differences in the photosynthetic rates can be easily illustrated using the Hill Reaction. The procedures that are described below measure the coupled activity of Photosystem I and Photosystem II, and represent a portion of a Master of Science degree thesis entitled "The Role of Photosynthetic Electron Transport in the Successional Relationships of Several Species Native to Virginia" submitted by Catherine J. Corson to Virginia Commonwealth University.

Materials

(1) PLANT SPECIES: The five plant species used in the study were *Podophyllum peltatum, Parthenocissus quinquefolia, Hexastylis virginica, Chenopodium album* and *Phytolacca americana. C. album* and *P. americana* are sun plants that were collected from full-sun habitats. *P. peltatum, P. quinquefolia* and *H. virginica* are shade area plants and were collected from shaded habitats. Instructors can use any herbaceous species, but the sun plants should be collected from a full-sun habitat and shade plants from a shade habitat. The species noted above are readily collected in the southeastern United States during the spring time. The field-collected plant material should be placed on ice and kept chilled until ready for assay in the laboratory.

(2) MATERIALS FOR THE HILL REACTION: All of the materials described previously.

(3) CHLOROPHYLL CONCENTRATION DETERMINATION:

80% (v/v) acetone
Spectrophotometer, visible range

Procedures

(1) PREPARATION OF TISSUES FOR THE HILL REACTION: Use the procedures described previously to partially purify the chloroplasts. The final stock chloroplast suspension should contain 5 ml of buffer per gram of fresh weight of leaf material. For the Hill Reaction assay with the shade species the solution was diluted with buffer to contain approximately 0.165 mg chlorophyll/ml. For the sun species the activity was so high that it was necessary to dilute the stock solution with buffer to contain approximately 0.0413 mg chlorophyll/ml. If different plant species are used the chloroplast suspension should be diluted so that the reaction will go to completion in approximately 4 to 5 minutes.

(2) DETERMINATION OF CHLOROPHYLL CONCENTRATION: The chlorophyll concentration of the chloroplast suspension is determined via the method of Arnon (1949). 0.5 ml of the chloroplast suspension is mixed with 10 ml of 80% (v/v) acetone. The absorbance of the solution is measured at 645 nm and 663 nm; 80% acetone is used as the blank. The chlorophyll concentration is then determined from the following equation:

$$\frac{\text{mg total chlorophyll}}{\text{g tissue}} = [20.2(Abs_{645}) + 8.02(Abs_{663})] \times \frac{V}{1000 \times W}$$

where Abs_{645} = absorbance of solution at 645 nm
Abs_{663} = absorbance of solution at 663 nm
V = final volume of 80% acetone-chlorophyll extract
W = fresh weight in grams of the tissue extracted

(3) DETERMINATION OF ACTIVITY OF CHLOROPLASTS:

Blank: 1.0 ml diluted chloroplast suspension
1.5 ml sucrose-phosphate buffer
0.5 ml distilled water

This tube will serve as the dye control to determine when the reaction has gone to completion. Use this suspension to set the spectrophotometer to 0.0 Absorbance (100% Transmittance) at 620 nm.

Experimental: 1.0 ml diluted chloroplast suspension
1.5 ml sucrose-phosphate buffer

Set the spectrophotometer to 0.0 Absorbance (100% Transmittance) with the blank. At zero time add 0.5 ml of DPIP to the experimental tube and measure its absorbance at 620 nm. Take the cuvette out of the spectrophotometer and expose the suspension to light from one of the light sources at a measured distance for 30 seconds. At the end of the 30-second time period remove the tube from the light, measure its absorbance at 620 nm and record that value. Remove the cuvette from the spectrophotometer and expose it again to light at the same distance (same light intensity) for 30 seconds (60-second total exposure); again measure the absorbance of the suspension at the end of this second 30-second exposure period. Repeat this procedure of 30-second exposures and absorbance measurements until the reaction has gone to completion. Measure the intensity of the light at the point where the tube was located using a light meter.

Repeat the above procedure at 5 to 8 different light intensities (distances from the light source). The highest light intensity should approximate the light intensity measured in the full-sun environment or, if this is not possible, the highest light intensity available with the light source that is being used. Repeat the entire experiment with several different plant species from sun and shade habitats.

(4) DPIP STANDARD CURVE: The DPIP solution used in the assays has a concentration of 0.28mM. From this solution a dilution series is prepared in the concentration range from 0mM to 0.28mM (dilute with the sucrose-phosphate buffer) and the absorbance of each concentration at 620 nm is measured. A graph is prepared which plots the concentration of $DPIP_{ox}$ (mM) (x-axis) versus the absorbance at 620 nm (y-axis) using a linear regression program. This standard curve will give the concentration of $DPIP_{ox}$ in the suspension.

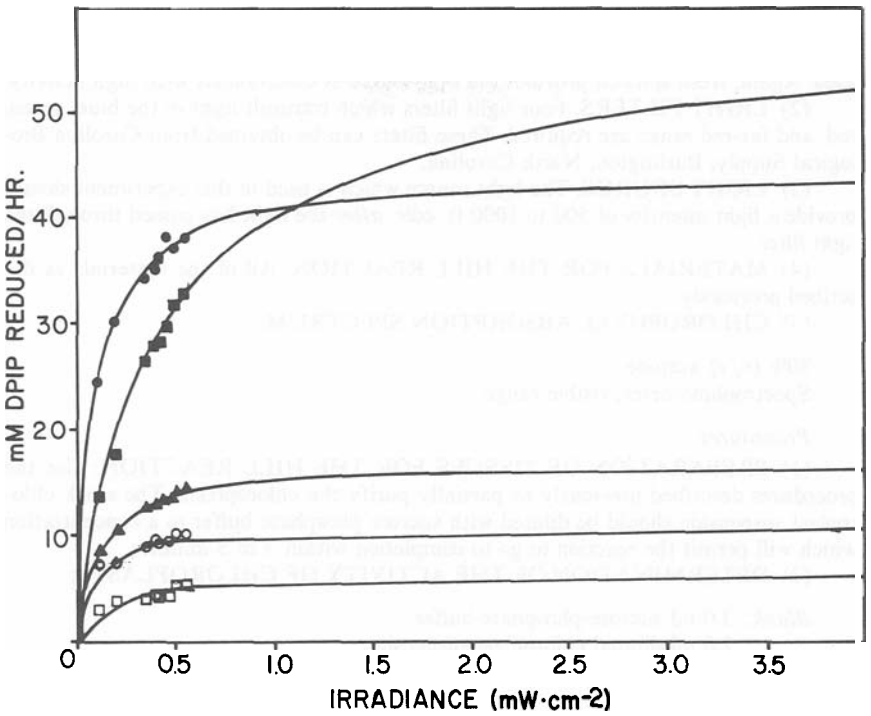
Calculations

(1) DETERMINATION OF THE RATE OF DPIP REDUCTION: For each time interval (with each species and each light intensity) calculate the $DPIP_{ox}$ present in the suspension. Subtract this value from the $DPIP_{ox}$ concentration at time '0'. This will give you the concentration of $DPIP_{red}$ for that time. Prepare a graph of time (in minutes) (x-axis) versus mM $DPIP_{red}$ (y-axis) using a linear regression program. From the graph determine the rate of DPIP reduction per minute; convert this to mM DPIP reduced per hour.

Calculate the mg total chlorophyll present for each species. Express the rate of DPIP reduction for each species at each light intensity as mM DPIP reduced/hour/mg chlorophyll.

(2) PHOTOSYNTHETIC RATE AS A FUNCTION OF LIGHT INTENSITY: Prepare a graph of the light intensity (x-axis) versus the rate of DPIP reduction/hour/mg chlorophyll (y-axis). The data for all of the species tested should be plotted on the same graph. The data should demonstrate a series of rectangular hyperbolas with the sun plants showing higher photosynthetic rates than the shade plants at high light intensities. The data can be analyzed statistically using the method of Bliss and James (1966).

Figure 3.3 illustrates the results obtained with 5 plant species by Corson (1979): *Chenopodium album* (sun species), *Phytolacca americana* (sun species), *Podophyllum peltatum* (shade species), *Parthenocissus quinquefolia* (shade species), and *Hexastylis virginica* (shade species). The results demonstrate that the two sun species exhibited the highest rates of DPIP reduction at high light intensities. *P. peltatum* and *P. quinquefolia* demonstrated intermediate activity, but still saturated at lower light intensities than the two sun species. *H. virginica,* which is an extreme shade species, showed the least activity of all of the species tested.



● = *Chenopodium album*
■ = *Phytolacca americana*
▲ = *Podophyllum peltatum*
○ = *Parthenocissus quinquefolia*
□ = *Hexastylis virginica*

Figure 3.3. DPIP reduction rates per milligram chlorophyll per hour. Irradiance readings given in milliwatts per square centimeter. (From: Corson, C. J. 1979. The Role of Photosynthetic Electron Transport in the Successional Relationships of Several Species Native to Virginia. M. S. Thesis, Department of Biology, Virginia Commonwealth University, Richmond, Virginia.)

2. Action Spectrum of Photosynthesis

The action spectrum of photosynthesis can be easily demonstrated using the Hill reaction procedure, and this reaction will also demonstrate one of the fundamental laws of photochemistry, the Grotthus-Draper Law. The Grotthus-Draper Law basically states that only light which is absorbed will have a photochemical effect; light which is reflected or transmitted is of no consequence. In this experiment the photosynthetic rate is measured in 4 different light wavelength zones: blue, green, red, and far-red. The measured rates of photosynthesis are then correlated with the absorption spectrum of a chlorophyll extract prepared from the plant tissue used in the experiment. The results will demonstrate that significant photosynthesis will occur only at those wavelengths corresponding to the absorption maxima for the tissue extract, or, that only light which is absorbed will have a photochemical effect.

Materials

(1) PLANT SPECIES: Any of the plant species mentioned previously can be used. Again, fresh spinach provides the best source of chloroplasts with high activity.

(2) LIGHT FILTERS: Four light filters which transmit light in the blue, green, red, and far-red range are required. These filters can be obtained from Carolina Biological Supply, Burlington, North Carolina.

(3) LIGHT SOURCE: The light source which is used in this experiment should provide a light intensity of 500 to 1000 ft. cdls. *after* the light has passed through the light filter.

(4) MATERIALS FOR THE HILL REACTION: All of the materials as described previously.

(5) CHLOROPHYLL ABSORPTION SPECTRUM:

80% (v/v) acetone
Spectrophotometer, visible range

Procedures

(1) PREPARATION OF TISSUES FOR THE HILL REACTION: Use the procedures described previously to partially purify the chloroplasts. The stock chloroplast suspension should be diluted with sucrose phosphate buffer to a concentration which will permit the reaction to go to completion within 3 to 5 minutes.

(2) DETERMINATION OF THE ACTIVITY OF CHLOROPLASTS:

Blank: 3.0 ml sucrose-phosphate buffer
2.0 ml diluted chloroplast suspension
1.0 ml distilled water

This tube will serve as the dye control to determine when the reaction has gone to completion. Use this suspension to set the spectrophotometer to 0.0 Absorbance (100% Transmittance) at 620 nm.

Experimental: 3.0 ml sucrose-phosphate buffer
2.0 ml diluted chloroplast suspension

Using the blank set the spectrophotometer to 0.0 Absorbance (100% Transmittance) at 620 nm. At zero time add 1.0 ml of DPIP to the experimental tube and measure its absorbance at 620 nm. Expose the suspension to white light from the light source at an intensity of 500–1000 ft. cdls. for 30 seconds. At the end of this 30-second exposure period remove the tube from the light and measure its absorbance at 620 nm. Remove the tube from the spectrophotometer and again expose it to light at the same intensity (same distance) for 30 seconds (60-second total exposure). At the end of this 30-second exposure period remove the tube from the light source and measure its absorbance at 620 nm. Repeat this procedure of 30-second exposures and absorbance measurements until the reaction has gone to completion.

Repeat the above procedure again for each light wavelength zone (blue, green, red, and far-red). Be sure to adjust the light source so that the light intensity is the same in all phases of the experiment.

(3) DETERMINATION OF THE ABSORPTION SPECTRUM OF CHLOROPHYLL: 0.5 ml of the chloroplast suspension is mixed with 10 ml of 80% (v/v) acetone. The absorbance of the solution is measured at 5 or 10 nm intervals from 325 nm through 750 nm; 80% acetone is used as the blank to zero the spectrophotometer at each wavelength.

Data Analysis

(1) PHOTOSYNTHETIC RATE: For each light wavelength zone and for white light prepare a graph of time (minutes) (x-axis) versus absorbance at 620 nm (y-axis) as described previously. Calculate the rate of photosynthesis for each condition expressed as change in absorbance per minute.

(2) ABSORPTION SPECTRUM: Prepare a graph of wavelength (x-axis) versus absorbance (y-axis)

(3) PHOTOSYNTHETIC RATE (ACTION SPECTRUM) VERSUS ABSORPTION SPECTRUM: Prepare a graph with the following axes: x-axis = wavelength in nm, left y-axis = absorbance of chlorophyll extract, right y-axis = rate of photosynthesis.

(a) Graph the absorption spectrum using the x-axis (wavelength) and the left y-axis (absorbance)

(b) Graph the action spectrum using the x-axis (wavelength) and the right y-axis (rate of photosynthesis). For the light filters obtained from Carolina Biological Supply (Burlington, North Carolina) the average wavelengths which correspond to each filter are: blue = 450 nm, green = 545 nm, red = 650 nm, and far-red = 750 nm. Use these wavelength values for the corresponding photosynthetic rate for each light filter.

Compare the action and absorption spectra. What conclusions can you obtain from this data? Add the individual photosynthetic rates obtained for each light wavelength zone together. Does the total equal the rate measured with white light? Why or why not?