Chapter 11

Effects of Irradiance on Photosynthetic CO_2 Uptake and Chlorophyll Fluorescence

Stephen Hunt

Department of Biology
Queen's University
Kingston, Ontario
Canada K7L 3N6
613-533-6384
hunt@biology.queensu.ca

Stephen Hunt is an Adjunct Professor at the Biology Department of Queen's University, where he teaches introductory and advanced courses in plant science. He received a bachelor's degree in Botany from Liverpool University in the UK (1978), and then worked at the John Innes Research Institute in Norwich, UK, where he studied the photosynthetic physiology of C3-C4 intermediate species. He received a Ph.D. from the University of East Anglia, UK in 1986. His current research investigates the physiology of N_2 Fixation, and this has led to the design of several patented instruments for measuring physiological processes in plants. In 1995 he established Qubit Systems Inc., a company based at Queen's University that develops integrated laboratory packages for teaching undergraduate biology.

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Introduction

This laboratory exercise investigates the relationship between light energy incident on a leaf and the rate of photosynthesis measured by CO_2 uptake with an infrared CO_2 analyzer. At the same time, measurement of chlorophyll fluorescence shows students how light energy is transduced during photosynthetic reactions. The exercise is suitable for students taking an advanced course in Plant Physiology. However, the generation of a light response curve for photosynthesis using just the CO_2 analyzer, and its subsequent analysis, is suitable for students taking introductory courses in plant, or general, biology.

Once the user becomes familiar with the equipment, it requires less than 10 minutes to set up, and about the same time to dismantle. The investigation described in this chapter requires about two hours to perform. Data analysis and data plotting in a spreadsheet program such as Excel or Lotus 123 would require another hour. The equipment and software used in this investigation can be used to study many other aspects of photosynthetic, photorespiratory, and respiratory physiology in plants. At Queen's University, students taking the Plant Physiology course in their third or fourth year of a Biology degree program do this exercise, and three other exercises using different equipment to investigate other physiological processes. They then undertake a four-week independent lab study to investigate further whatever physiological process in the course has most interested them. After the initial four labs most students are fully competent in the use of both equipment and software and can proceed with their independent study with minimal supervision from lab instructors.

All the equipment used in this laboratory is manufactured by Qubit Systems, Inc. of Kingston, Ontario, Canada (Phone 1-888-262-2219 from N. America). This company was formed at Queen's University to provide equipment to biology teaching laboratories at a price in keeping with stringent teaching budgets.

Materials

All of the following equipment is supplied as a "laboratory package" by Qubit Systems Inc. Catalog No. COFL1, price $3,805 US.

A higher plant
An infrared CO_2 analyzer
A laboratory stand to which other components are attached.
A light fitted with a 50W halogen actinic light for activating photosynthesis
A light control box with a potentiometer for varying actinic light, a button for producing a saturating pulse of light, and an output for monitoring the amount of light incident on the leaf
A bracket holding an LED light source (for exciting chlorophyll fluorescence) and a fluorescence detector
A chlorophyll fluorometer that monitors chlorophyll fluorescence
A flow-through leaf chamber
A gas pump
A flow meter with needle valve
A nylon-polyethylene gasbag filled with laboratory air
A soda-lime column to scrub CO_2 from the air
A magnesium perchlorate column for drying air
A Universal Lab Interface (analog to digital converter)
A 9 VDC, 1A power supply for the universal lab interface.
A cable to connect the universal lab interface to a computer (Macintosh or PC)

Notes for the Instructor

- It takes 10 – 15 minutes for an experienced instructor to set up and calibrate all the equipment used in this laboratory. Students unfamiliar with the equipment would require 30 – 45 minutes for the same task. The length of the lab, and of the lab protocol, may be reduced considerably by setting up the equipment in advance.
- The Logger Pro Software used in the lab is available for Macintosh or PC computers. The protocol presented in this manuscript describes use of Macintosh software. Instructions differ very little when using PC software.
- The protocol describes the calculation of absolute rates of photosynthesis in units of µmoles $CO_2/m^2/s$. It is not necessary to calculate these absolute rates to generate a light response curve from which a light compensation point and light saturation point can be measured. Relative photosynthetic rates may be used by plotting the difference between the reference CO_2 value and the CO_2 value recorded at each irradiance (i.e. plot irradiance vs δCO_2).

Student Outline

In this experiment you will demonstrate that light is required for photosynthesis, and that the rate of photosynthesis increases with light intensity until a light saturation point is reached. At that point, photosynthetic rate is limited either by the ability of the leaf to transduce the light energy it absorbs to chemical energy, or by the supply of some other factor required for photosynthesis.

Although light may seem to be an abundant source of “free” energy in the environment, there are many ecosystems in which the supply of light is the major factor limiting plant growth. In forests, and in densely planted crops, only the upper leaves are exposed to high light levels, and lower leaves, together with those plants that inhabit the understorey, intercept only the stray light (sunflecks) that pierces the leaf canopy.

Changes in light level on a global basis may have been responsible for one of the major catastrophic events in the earth’s history. It is thought that the impact of an enormous meteorite created an increase in atmospheric particulates that reduced penetration of solar radiation to a level that could not support high rates of photosynthesis. As a result, plant growth was inhibited, and herbivorous dinosaurs starved, causing starvation of their carnivorous predators. It has been suggested that should mankind be foolish enough to make use of nuclear weapons, this could result in an effect similar to that which extinguished the dinosaurs, causing the onset of a "nuclear winter" which would devastate crop production in regions far removed from those directly devastated by warheads.

Meanwhile, degeneration of the ozone layer by atmospheric pollutants is allowing a greater flux of solar radiation to penetrate the atmosphere, with adverse affects both on plants and animals. A study of photosynthesis and irradiation, therefore, has great social, political, economic, and historical relevance, as well as allowing insight into a fundamental process in plant physiology.

Infrared CO_2 Analysis

In this experiment you will measure photosynthetic rate by monitoring the uptake of CO_2 by a leaf enclosed in a leaf chamber illuminated by a halogen light source. The output of this light source will be varied using a potentiometer, and the effect of this variation on photosynthesis will be observed.

CO_2 uptake will be measured using an infrared gas analyzer (IRGA). This instrument contains a tube through which gas is pumped. An infrared light source is located at one end of the tube, and an infrared detector is located at the other end. Carbon dioxide in the gas stream absorbs infrared light so the greater the amount of CO_2 present, the lower the signal from the infrared detector. The detector may be calibrated by passing CO_2-free gas through the instrument to obtain a zero reading, and then passing gas of a known CO_2 concentration through the instrument. The gain control of the IRGA is then adjusted until the value on the digital display corresponds with the CO_2 concentration in the gas stream. Calibration is conducted while monitoring data using Logger Pro data acquisition software, so that the values on the digital display are recorded in Logger Pro.

Chlorophyll Fluorescence and Photosynthetic Activity

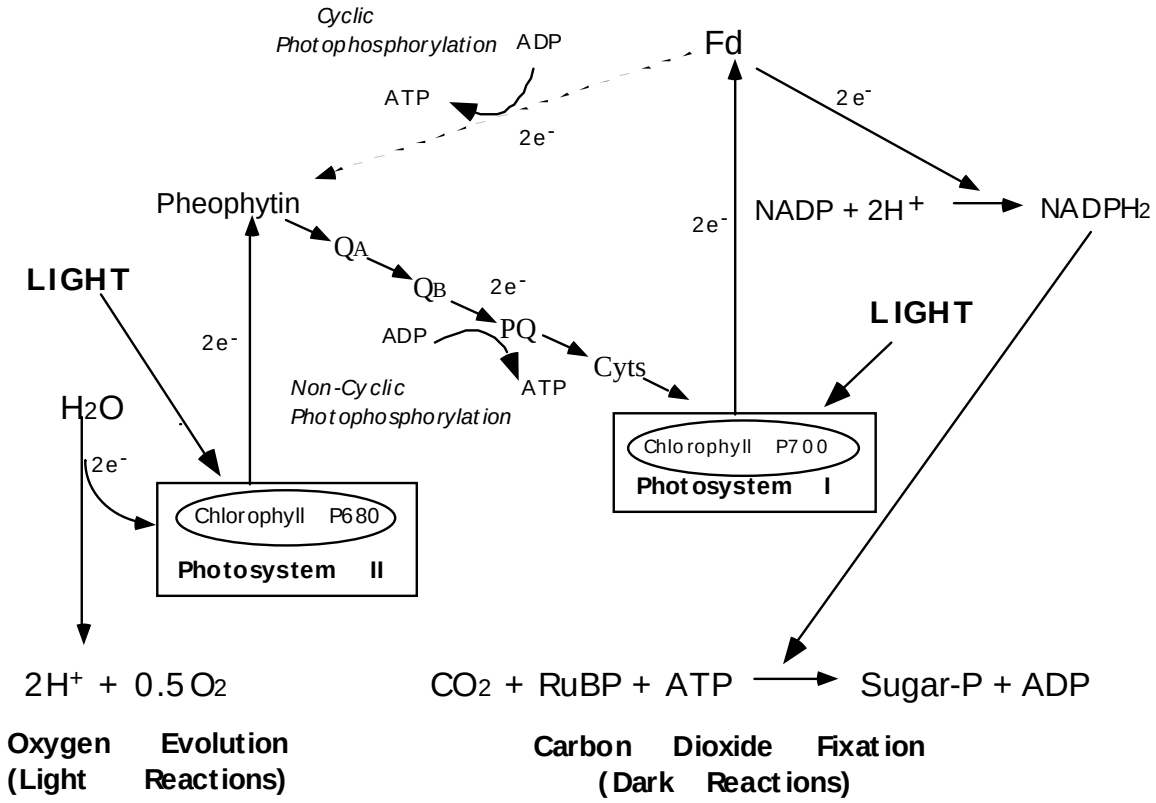
While monitoring CO_2 uptake by the leaf you will also be measuring the efficiency with which the light impinging on the leaf is being used for photosynthetic processes. This is done by measuring energy lost from the leaf in the form of fluorescence.

Photosynthesis involves the conversion of light energy into chemical energy mediated by light sensitive chlorophyll molecules in the leaf. Light-harvesting complexes involving numerous chlorophyll molecules intercept radiant energy and transfer this to a reaction center in Photosystem II (PSII). At the heart of the PSII reaction center a special chlorophyll molecule called P680 becomes excited and an electron from P680 is raised to a higher energy level. Under ideal conditions for photosynthetic activity this electron reduces an acceptor molecule called pheophytin (Fig.1).and is then transferred through a series of electron carriers including Q_A, Q_B, Plastoquinone

(PQ), and cytochromes (Cyts). This transfer of electrons is associated with the production of a transthylakoid proton gradient which may be coupled to the chemiosmotic production of ATP. This process is called non-cyclic photophosphorylation, and requires processing of 2 electrons from 2 P680 molecules for the production of ATP.

Light harvesting complexes are also responsible for transferring energy to a second specialized chlorophyll molecule, called P700, at the heart of Photosystem I (PSI). When P700 becomes excited an electron is raised to a higher energy level and this may be involved in ATP production before it is returned to the oxidized P700. This process is called cyclic photophosphorylation and, like non-cyclic photophosphorylation, requires processing of 2 electrons from 2 P700 molecules. Alternatively, the electrons may reduce ferredoxin (Fd) which is then involved in the reduction of NADP to $NADPH_2$. The $NADPH_2$ may then be used in the reduction of CO_2 to sugar phosphates in the so-called "dark reactions" of photosynthesis as shown in Figure 11.1.

Figure 11.1. Simplified Scheme for the Photosynthetic Light Reactions



If the electron released from P700 is used ultimately in the reduction of CO_2, the P700 molecule is left with a net positive charge. Electrical neutrality is restored by P700 accepting the electron released form P680 in PSII. Of course, the loss of an electron from P680 leaves this molecule with a positive charge and in this case the electron required to restore electrical neutrality is derived from the splitting of water. This reaction also generates the O_2 gas that is released from the leaves of photosynthetic higher plants.

Alternative Pathways of Energy Dissipation

Like any other energy transduction system, conversion of light energy to chemical energy in photosynthesis is far from a perfect process. For various reasons, not all electrons raised to higher

energy levels by irradiance are passed to electron acceptors and these electrons return to their parent chlorophyll molecules without the production of ATP and/or $NADPH_2$. The energy lost during the return to the ground state may be manifested as heat or light. If light is produced, it is emitted at a longer wavelength than the light that stimulated electron release from chlorophyll, and under these circumstances the chlorophyll molecule is said to fluoresce.

Chlorophyll fluorescence usually occurs maximally under conditions of high light when photosynthetic activity is inhibited. Consider, for example, a leaf that is exposed to high light after being maintained in the dark for several hours. During the dark period, several of the enzymes involved in the Calvin Cycle become deactivated and must be re-activated by light before they can operate. Also, the metabolites involved in the Calvin Cycle reactions must reach appropriate levels before CO_2 fixation can occur at the optimal rate. Thus, there is a "Photosynthetic Induction Period" on the transfer of a leaf from dark to light during which CO_2 fixation is very slow.

During the photosynthetic induction period the electron acceptors in the leaf continue to accept electrons from excited chlorophyll molecules but have no way of dissipating the energy from these electrons because the "dark reactions" are not yet active or optimized. As a result, the number of these acceptors which can accept electrons quickly falls to zero, since all reduction sites are occupied. Consequently, the electrons released from chlorophyll must dissipate their energy in another way. This is done partly by fluorescence, in which light is emitted as the electrons return to their initial energy level, and party by dissipation of energy as heat. Therefore, we would expect fluorescence to be high during the photosynthetic induction period, and then decline, as the CO_2 fixation reactions become more active.

Measurement of Fluorescence

Measurement of chlorophyll fluorescence using Qubit Systems' Chlorophyll Fluorescence Package requires a chlorophyll fluorometer with an LED light source to excite fluorescence, a detector to measure this fluorescence, a halogen light source for activating photosynthesis (actinic light), a leaf chamber or leaf clamp for positioning the leaf with respect to the light sources and detector, and a data acquisition system.

To measure fluorescence, the leaf is illuminated with a very weak LED light source (660 nm peak wavelength) that is pulsed 50 times per second (i.e. at a 50 Hz frequency). The duration of each flash is only 4 μseconds. This light induces fluorescence to occur at the same frequency as the pulsed light, but the amount of light supplied to the leaf by the LED is, on its own, insufficient to drive photosynthesis. The chlorophyll fluorescence excited by the LED light is detected by a photodetector that has a long-pass filter so that it only detects wavelengths greater than 700 nm. Also, the signal from the detector is decoded by the fluorometer so that only fluorescence occurring at the same frequency as the pulsed LED light is measured. Thus, fluorescence caused by other light sources, including the actinic light source, is not measured by the system. The measured fluorescence is indicative of total leaf fluorescence, but because only the pulsed fluorescence signal is measured, the signal from the photodetector does not saturate the detection system even under maximal fluorescence conditions. As a result, changes in fluorescence can be measured under a very wide range of incident irradiances from darkness to conditions equivalent to many times full sunlight.

Fluorescence Yield and Photochemical Quantum Yield

When a photon of light excites a chlorophyll molecule a single electron is raised to a higher energy level as described above. This energy may be dissipated by use in photochemical reactions (P), by release of heat (D), or by fluorescence (F). Thus, the probability of each of these energy transduction processes occurring is given by the expression:

$$P + D + F = 1 \qquad \text{Eqn. 1}$$

where P is called the Quantum Yield of Photosynthesis and F is called the Fluorescence Yield. Of these parameters P is of greatest interest to plant scientists since this provides a measurement of the photochemical activity of the leaf. F may be measured quite easily by Qubit Systems' Fluorescence Package, but P and D are very difficult to measure directly. Nonetheless, accurate measurements of P can be derived from measurements of F alone by the following method.

Again, consider a leaf that has been maintained in the dark for a time long enough to require a photosynthetic induction period on re-illumination. If, after this period, the leaf is exposed to an extremely bright flash of light, the chlorophyll molecules within the leaf are excited and pass their excited electrons to the electron acceptors of PSII and PSI. However, because the Calvin cycle reactions are not active, the $NADPH_2$ formed as a result of this photochemistry cannot be used and the pool of NADP available for reduction is very quickly exhausted. As a consequence, within a few microseconds of illumination, the electron carriers in PSI and PSII become unable to pass on their electrons, and they therefore remain in the reduced condition. As a result, electrons released from chlorophyll during continued illumination cannot be used for photochemical reactions. Consequently, during a saturating flash of light following dark-adaptation of a leaf, the quantum yield of photochemistry (P) declines to zero and the Fluorescence yield (F) and Heat dissipation (D) reach maximal values (Fm and Dm). Thus, during this flash of light:

$$Fm + Dm = 1 \qquad \text{Eqn. 2}$$

We can now express heat dissipation in terms of Fluorescence:

$$Dm = 1 - Fm \qquad \text{Eqn. 3}$$

It has been shown experimentally that the relative amounts of fluorescence and heat dissipation that occur during a brief (0.8 second) saturating flash of light are similar to those that occur under normal conditions of irradiance. Therefore:

$$Dm/Fm = D/F \qquad \text{Eqn. 4}$$

By substitution using Eqn. 4 we can derive the expression:

$$D/F = (1 - Fm)/Fm \qquad \text{Eqn. 5}$$

Therefore:

$$D = F(1 - Fm)/Fm \qquad \text{Eqn. 6}$$

We can now substitute the D in Eqn. 1 for the expression in Eqn. 7 thus:

$$P + [F(1 - Fm)/Fm] + F = 1 \qquad \text{Eqn. 7}$$

Therefore:

$$P = 1 - F - F(1 - Fm) \qquad \text{Eqn. 8}$$

Simplifying:

$$P = 1 - F - F(1 - Fm) = (Fm - F)/Fm \quad \text{Eqn. 9}$$

The term (Fm-F)/Fm in Eqn. 10 is usually expressed as Fv/Fm, where Fv is termed variable fluorescence since it represents the difference between maximum fluorescence (Fm) and background, or minimum fluorescence (see below).

Using the correct procedure of dark-adaptation of leaves prior to fluorescence measurements we can measure photochemical quantum yield purely in terms of chlorophyll fluorescence parameters thus:

$$P = Fv/Fm \quad \text{Eqn. 10}$$

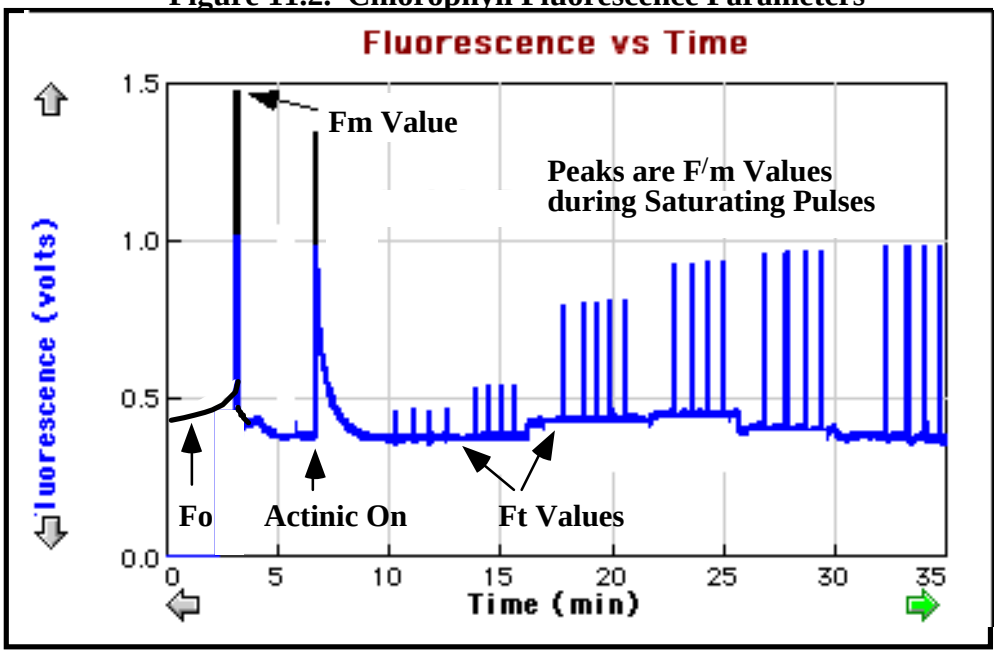
It must be noted that chlorophyll fluorescence, and quantum yield, measured using Qubit Systems' Chlorophyll Fluorescence Package relates only to the activity of PSII, since it has been shown experimentally that only PSII (and not PSI) photochemistry is involved in inhibiting (quenching) the fluorescence measured by this system.

General Procedure for Measurement of Fluorescence Parameters

At the beginning of each experiment the background fluorescence from a dark-adapted leaf is measured when only the LED light is turned on. This value is termed Fo (see Figure 11.2 below). The output from the LED light is insufficient to drive photosynthesis and does not disturb the dark-adapted state.

Next maximum fluorescence yield of the leaf is measured, i.e. the maximum fluorescence signal achieved, when all chlorophyll molecules are saturated with light, and all electron acceptors are fully reduced. This is the Fm value. It is measured after applying a brief (0.8 second) saturating pulse of actinic light to the leaf after a period of dark equilibration. Quantum flux during the saturating pulse is in excess of 5000 µmol quanta/m^2/s. Quantum yield (P = Fv/Fm) of a dark-adapted leaf is calculated as (Fm – Fo)/Fm (see Eqn. 9 above). In healthy leaves this value is usually about 0.8.

Figure 11.2. Chlorophyll Fluorescence Parameters



To measure quantum yield under illuminated conditions the actinic light source is turned on and steady state fluorescence (the Ft value) is measured. A saturating pulse of light is then applied (of the same intensity and duration of that applied to the dark-adapted leaf) to obtain the maximum fluorescence yield under illuminated conditions (the F'_m value). F'_m has a lower value than Fm, and the F'_m value tends to decrease with increasing irradiance. This is because under high light conditions the proportion of photosynthetic electron acceptors that are oxidized at any one time decreases, and heat dissipation with respect to fluorescence increases. The latter is due, in part, to the development of the transthylakoid proton gradient. As a result, the quantum yield of photochemistry, calculated as $(F'_m - Ft)/F'_m$ (see Eqn. 9) is reduced under high light conditions.

Measurement of Electron Transport Rate

The relative quantum yield of photochemistry (P) can be used to estimate the rate of photosynthetic electron transport in the leaf provided that the photon flux incident on the leaf is known. Since 1 µmol of photons causes the excitation of 1 µmol of electrons from chlorophyll, and P represents the proportion of these electrons that are used in photochemistry, the electron transport rate (ETR) is related to product of P and the photon flux density of photosynthetically active radiation at the leaf surface (PAR). However, not all the light incident on a leaf is absorbed by chlorophyll molecules, since some is transmitted through the leaf and some is reflected. About 84% of incident light is absorbed by chlorophyll molecules in the average leaf, with 50% of the photons activating chlorophyll molecules associated with PSII and 50% activating PSI. Therefore, an estimate of the electron transport rate can be derived from the following:

$$ETR = P \times PAR \times 0.42 \qquad \text{Eqn. 11}$$

PAR is measured by a light sensor that is supplied with the fluorescence package.

In this experiment you will investigate the relationship between photosynthetic rate, irradiance, the quantum yield of photosynthesis and electron transport rate through Photosystem II as measured by chlorophyll fluorescence.

Experimental Procedure

Running Logger Pro Data Acquisition Software

Data from the fluorescence system and the CO_2 analysis system will be recorded via a computer interface using Logger Pro data acquisition software. The software is available for Macintosh or PC computers. The following instructions are for Macintosh users, though few modifications are required to operate the software with a PC.

- Click on the Logger Pro icon on the computer desktop.
- The default port for the Universal Lab Interface (ULI) is COM 2. If the ULI is plugged into any other port a message will appear stating that the ULI cannot be found on COM 2. You may then click on COM 1, if this is where the ULI is connected, or click on 'Scan Port'.
- When you receive a message stating 'ULI2 Rev 1.00', click on OK.
- When Logger Pro loads, it will call up a default file which may not be the file you require. To load the desired file click on 'FILE' and then 'PREFERENCES'.
- A window will appear showing the current experiment folder. If this does not read : c:\VernierSoftware\LoggerPro\Fluor$CO_2$, click on 'MODIFY' and then scroll down the

directory options until you reach 'Vernier Software'.
- Click on Vernier software, and then highlight the FluorCO_2 file.
- Click on 'OK' and the window will change to show the experiment folder you have selected. Click on OK if this is correct.

Select 'FILE' from the main menu, and then 'OPEN'. Click on FluorCO_2 and the file will load.

Calibration of the CO_2 Analyzer

(1) When the FluorCO_2 Set-up file is loaded, 3 graphs will appear on the screen showing CO_2 concentration, fluorescence, and irradiance. Click on EXPERIMENT from the main menu, and select 'Calibrate'.

(2) Click on 'Sensor Set-up' at the top of the dialogue box and then on the DIN 1 icon. 'CO_2 Analyzer' should appear in the Sensor box. Select the CO_2_500 file in the Calibration box. **Ensure that you set the range switch on the IRGA to the 0 - 500 ppm calibration range.**

(3) Select 'Calibrate' at the top of the dialogue box, and click on the DIN 1 icon so that 'CO_2 Analyzer' appears in the Sensor dialog box. Click on 'Perform Now'. A box labeled 'Reading 1' will appear showing the current voltage output from the IRGA (Input 1) and the CO_2 concentration this corresponds to (Value 1).

(4) Attach the outlet of the pump to the inlet of the flow meter. Attach the outlet of the flow meter to the inlet of the soda lime column. Attach the outlet of the soda lime column to the inlet of the magnesium perchlorate drying column and the outlet of this column to the inlet of the IRGA. You now have a system for flushing dry CO_2-free air through the IRGA to establish a zero reading.

(5) Turn on the pump and flush CO_2-free air through the IRGA at 300 mL/min. Observe the voltage change on the computer screen and wait until this declines to a steady reading. At zero CO_2, this reading should be approximately 0.8 volts (a value set by Qubit Systems during manufacture).

(6) Using a fine screw driver, adjust the **fine** zero control on the IRGA so that the digital display reads zero CO_2. The fine zero control is located beneath the detachable plastic cover on the front of the IRGA. Note that the IRGA will take several seconds to respond fully to adjustments of the zero potentiometer, so wait for a stable response between incremental adjustments.

(7) When a stable zero reading is displayed, type 0 against 'Value 1' and then click on 'Keep'. A box will appear labeled 'Reading 2' showing the current voltage output of the IRGA (Input 1) and a CO_2 concentration (Value 2).

(8) Attach a gas bag containing a known concentration of CO_2 to the inlet of the pump. Pump this gas through the IRGA at 300 mL/min and wait until the voltage output stabilizes.

(9) Adjust the span control of the IRGA until the digital display reads the CO_2 concentration in the gas stream. Enter this ppm value in the 'Value 2' box on the screen. Click on 'Keep'.

(10) The screen will revert to the original calibration dialog box. Click on 'Save'. Type CO_2_500 into the DIN 1 box. You will be asked if you wish to over-write the current corresponding file. Choose YES.

(11) Record the slope and intercept details of your calibration by selecting DETAILS at the top of the screen. Write down the slope and intercept values in a note-book. If your computer malfunctions you may type these values into Logger Pro without repeating the IRGA calibration.

(12) Click on OK. The IRGA is now calibrated and the value on the IRGA digital display should match that shown numerically and graphically in Logger Pro.

If considerable adjustment of the span control was required to match the IRGA display to the CO_2 concentration of the calibration gas, it is possible that you may have to re-zero the IRGA after adjustment of the span. To do this, re-attach the soda-lime column to the input of the IRGA and pump CO_2-free air through the instrument. If the IRGA does not read zero, repeat the calibration procedure in Logger Pro as described above. You should not need to adjust zero and span more than twice to obtain a satisfactory calibration.

When calibration is complete, select "File" from the Menu and then "Save as". Save your file with an appropriate file name. The same calibration will be loaded each time you select the set-up file, and you may re-calibrate when necessary by repeating the procedure outlined above.

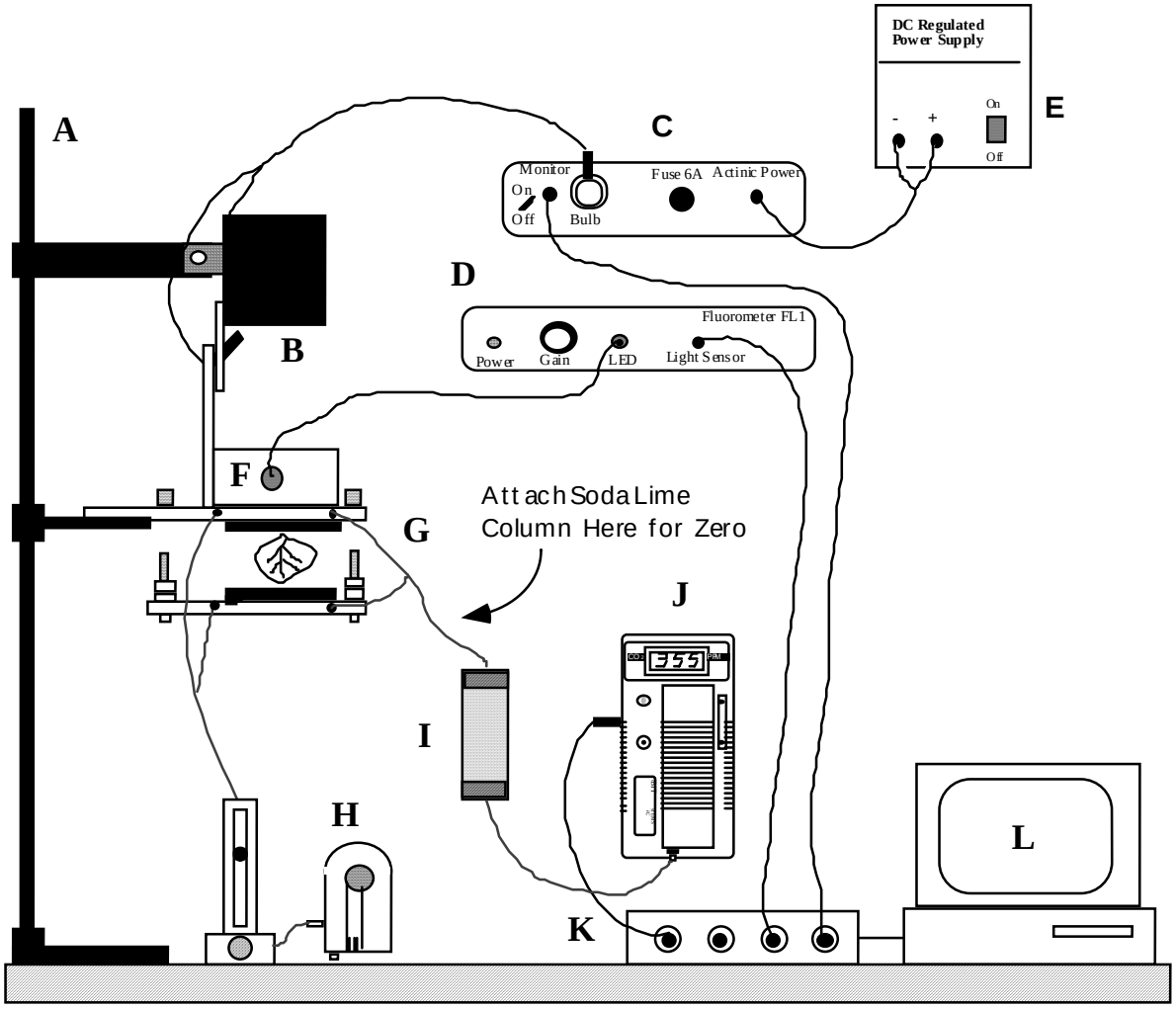
Note that your Logger Pro calibration is valid only for CO_2 range selected in your set-up file (0 - 500 or 0 - 2000 ppm). If you calibrate at one range, and then switch to the other range during an experiment, the digital display on the IRGA will provide a correct CO_2 reading, but the value shown on the computer screen and saved in Logger Pro will be incorrect.

Set Up of Chlorophyll Fluorescence System

Refer to Figure 11.3 and configure the chlorophyll fluorescence system as follows:

(1) Attach the lab stand to the lab bench using the clamp and thumb screws.
(2) Attach the leaf chamber to the lab stand at a height suitable for the plant you will be using. Slide the aluminum bracket attached to the actinic light support down the stand until base of the aluminum bracket touches the upper surface of the leaf chamber.
(3) Place the brass fitting holding the LED chlorophyll excitation light into the smaller of the two holes in the aluminum bracket and secure it in place with the nylon screw. Plug the cable from the LED light into the socket labeled "LED" on the front of the chlorophyll fluorometer.
(4) Place the brass fitting holding the chlorophyll fluorescence detector in to the larger of the two holes in the aluminum bracket, and plug the cable from the detector into the socket labeled "Light Sensor" on the chlorophyll fluorometer.
(5) Attach the ground plug on the green cable to the green socket on the back of the fluorometer and attach the alligator clip to any point on the aluminum bracket.
(6) Attach the plug on the cable from the actinic light and light sensor to the socket labeled "Bulb" on the rear of the actinic light control box.
(7) Attach the plug on the FL1 DC power supply to socket labeled 12 V Power on the rear of the fluorometer.

Figure 11.3. Equipment Set-Up



A = Lab Stand with Mounting Brackets
B = Actinic Halogen Light Source and Sensor
C = Actinic Light Control Box
D = Fluorescence Control Box
E = Power Supply
F = Measurement Light and Detector Fixture
G = Flow-Through Leaf Chamber
H = Gas Pump and Flow Meter
I = Magnesium Perchlorate Drying Column
J = Infra Red CO_2 Analyzer
K = 4 Channel Computer Interface
L = PC or Macintosh Computer

(8) Attach the red and black banana plugs on the power supply cable to the positive and negative sockets of the large DC power supply and attach the plug on the other end of the cable to the socket labeled "Actinic Power" on the rear of the actinic light control box. Plug the power supply into the main socket and turn it on.

(9) Attach the 5 pin DIN plug on the cable from the rear of the fluorometer to the DIN 3 socket on the Universal Lab Interface.

(10) Attach the 5 pin DIN plug on the cable from the rear of the actinic light control box to the DIN 4 socket on the Universal Lab Interface.

(11) Before placing a leaf in the leaf chamber or leaf clamp ensure that the potentiometers labeled "Actinic" and "Auto Flash" on the light control box are turned fully counter-clockwise and that the power switch is set to the 'Off' position. It is also advisable to turn the Gain dial on the fluorometer fully counterclockwise before powering the unit. This minimizes irradiance reaching the leaf from the LED measurement light.

Set-Up of Gas Exchange System

Refer to Figure 11.3 when following the instructions below:

(1) If this has not been done already, fill a gas bag with laboratory air by attaching the bag to the inlet of the pump and pumping air into the bag at maximum flow rate. Seal the bag with the clip on the tubing.

(2) Your experiment should take approximately 60 minutes to complete. If the time axis on the computer display shows a maximum value different from 60 minutes, adjust this by clicking on the maximum value displayed and typing in an appropriate value.

If your experiment takes longer than 60 minutes, click on DATA in the main menu when the first 60 minutes period has elapsed, and then select 'Store Latest Run'. Restart data collection by clicking on the COLLECT icon in the main menu. The second part of your data will appear as a bold trace on the computer screen, and the initial data will show as a fainter trace. The two data sets will be saved in different data tables in the same file when you save your data according to point 16 below .

(3) With Logger Pro running attach the gas bag to the inlet of the pump, and attach the outlet of the pump to the inlet of the flow meter. Attach the outlet of the flow meter to the inlet of the magnesium perchlorate drying column, and the outlet of this column to the inlet of the IRGA. If the IRGA is calibrated correctly, the stable CO_2 concentration shown on the digital display will match that shown numerically on the computer screen underneath the graph.

(4) Record the CO_2 concentration in the gas bag in Table 11.1. This is your "reference CO_2" concentration.

(5) To observe CO_2 consumption, or evolution from the leaf, you will need to set the Logger Pro display so that the y axis of the graph has a range of approximately 130 ppm CO_2, including

values up to 30 ppm above the CO_2 concentration in the gas bag, and up to 100 ppm below the values in the gas bag. For example, if the bag contains 350 ppm CO_2, adjust the y axis so that the upper limit is 380 ppm CO_2 and the lower limit is 250 ppm CO_2.

(6) If the y axis requires adjustment, click, in turn, on the current maximum and minimum values on the y axis and enter new values. Press Enter. If your trace goes off screen at any time during a run, you may use the slider control at the right side of the graph to alter the range of the y axis. Alternatively, you may select VIEW from the main menu, and then 'Autoscale' to bring your trace back on screen.

(7) The leaf chamber has four gas ports grouped in pairs. Each pair consists of a port on the upper surface of the chamber and a port on the lower surface. One pair of ports distributes gas to the upper and lower surface of the leaf through an inlet manifold, and the other pair collects gas that has passed across the leaf surface. Either pair may be used for the gas inlet or the gas outlet. Both pairs of ports are attached to vinyl tubing joined at a Y-piece and terminating in a plastic connector. Attach the outlet of the flow meter to one pair of ports. Attach the other pair of ports to the inlet of the drying column. Check your gas exchange system. It should consist of a gas bag attached to the inlet of the pump; the outlet of the pump attached to the inlet of the flow meter; the outlet of the flow meter attached to the inlet of the leaf chamber; the outlet of the leaf chamber attached to the inlet of the drying column; and the outlet of the drying column attached to the inlet of the IRGA. The outlet of the IRGA vents to atmosphere.

(8) With Logger Pro running, turn the Gain control on the Fluorometer to midway through its range. The fluorescence value should read close to zero. If it reads 0.00 you must increase the value to a positive number (e.g. 0.05) by turning the zero control on the rear of the fluorometer. This sets a readable offset zero value that is subtracted from the experimental fluorescence values. Record the offset zero value in Table 11.1.

(9) Place a leaf in the leaf chamber, and tighten the nuts to produce a seal. The fluorescence signal will increase as the LED activation light stimulates chlorophyll fluorescence. This is your Fo value, and represents background fluorescence of the dark adapted leaf. You will record this value AFTER you have completed the experiment.

(10) Press the Flash button on the front of the light control box and observe the increase in the fluorescence signal during the flash, and the decline in the signal after the flash. The peak fluorescence value is your Fm value, which will be recorded at the end of the experiment.

(11) Turn the Actinic control dial on the Actinic Light Control Box clockwise to maximum. Make a note of the light level in Table 11.1. The fluorescence signal should increase and then oscillate before declining to a steady level. The shape of the response is called a Kautsky Curve, and it characterizes changes in chlorophyll fluorescence as photosynthesis is induced. You will also observe that a slow decline in CO_2 concentration of the gas entering the IRGA occurs as the actinic light is turned on. This represents photosynthetic CO_2 uptake by the leaf during the induction period.

(12) When the CO_2 signal has stabilized record the value in Table 11.1. Press the saturating flash button on the light control box and observe the F'_m value. You will record this value later.

(13) Reduce light output to approximately 80% of the initial output. This can be achieved by observing the response of the light sensor display as the Actinic dial on the light control box is adjusted.

(14) Observe the change in the CO_2 concentration of the analysis gas stream, and wait until this has reached a steady state. Record the new reading and the irradiance value in Table 11.1. Press the saturating flash button on the light control box and observe the $F^{/}m$ value.

(15) Reduce the output of the light source to 60% of initial, and repeat step 12. Repeat this procedure until you have measured the photosynthetic rate at a number of irradiances equal to 100, 80, 60, 40, 20 and 10% of the initial irradiance. At each stage, press the Flash Button to observe changes in $F^{/}m$. Finally turn off the light completely and note that the CO_2 concentration in the analysis gas increases above that in the reference gas. Record this value CO_2 concentration when it reaches a steady value.

(16) After you have made all your measurements, stop the experiment by clicking on the "Stop" button. Save your data by clicking on "File" in the menu, and selecting "Save as....". Give your data an appropriate file name, and save it to disk, or in the subdirectory allocated for it by your laboratory instructor.

Calculation of CO_2 Exchange Rate

Measurements of photosynthetic, photorespiratory, and respiratory rates in leaves are usually expressed as rates of CO_2 exchange per unit time per unit leaf area. The units most commonly used are µmoles of CO_2 per m^2 per second. To express your data in these units use the following procedure:

(1) Calculate the difference between the CO_2 concentration in the reference and analysis gases and record this under δCO_2 in Table 11.1. For example, if an experiment was conducted in air of 350 ppm CO_2, at a flow rate of 500 mL/min, the depletion of CO_2 due to leaf uptake in photosynthesis at high light may result in an analysis gas CO_2 concentration of 310 ppm. The difference between the reference and sample gas streams (δCO_2) in this example would be 40 ppm.

(2) Convert the δCO_2 value from ppm into µmoles per liter thus:

$$\delta CO_2 / 22.413 ([T+C]/T)$$

where C is the temperature in ˚C and T is the absolute temperature (273K).
At a temperature of 20˚C, and a δCO_2 of 40 ppm, the δCO_2 would be equivalent to 1.66 µmol CO_2 per liter.

(3) Multiply the δCO_2 value by the flow rate (in liters per second) used in your experiment to obtain a CO_2 exchange rate per second. A flow rate of 500 mL/min is equivalent to 0.0083 L/s. So the CO_2 exchange rate in our example would be 0.014 µmol/s.

(4) Express your CO_2 exchange rate on a leaf area basis by dividing the CO_2 exchange rate per second by the leaf area in m^2. If the leaf completely fills the chamber, the area used in the calculation would be 9 cm^2, equivalent to 0.0009m^2. The photosynthetic rate in our example would therefore be 15.6 µmol $CO_2/m^2/s$ which is a reasonable rate for a C3 species under ambient conditions.

(5) Calculate the CO_2 exchange rates for each irradiance and for the leaf in the dark. Enter the rates in Table 11.1.

Analysis of Fluorescence Data

- Open the file containing your data. Your data will appear on the screen exactly as it appeared when you saved it at the end of the experiment.
- Click on the Fluorescence graph and then select VIEW from the main menu. Click on GRAPH LAYOUT, select ONE PANE and then click on OK. The fluorescence graph will now fill the entire screen making data analysis easier.
- Place the cursor to the left and just above the part of the trace showing your Fm value in the dark adapted leaf. Click and hold on the mouse as you drag the cursor across your data so that a black box appears around the Fo and Fm values collected at the beginning of your experiment.
- Select VIEW and ZOOM IN. The data in the black box will now fill the entire screen.
- Select ANALYZE and then EXAMINE. A vertical line will appear on your graph which can be moved along the data points on the graph by moving the mouse. A box will also appear on each graph showing data values and time values. As you move the vertical line on the graph, the numerical display in the box will change to show you the exact data values and time value at the point on the graph where the line is situated. If the box obscures any part of the trace click on it and hold, then drag with the mouse to place the box in a convenient location.
- Scroll the cursor across your fluorescence data and identify the values of Fo and Fm in the dark adapted leaf. Record these values in Table 11.1. Calculate Quantum Yield in the dark adapted leaf as (Fm- Fo)/Fm. Record this value in Table 11.1.
- Repeat the above procedure to obtain measurements of Ft and F'_m at each irradiance. At each irradiance calculate Quantum Yield as (F'_m-Ft)/F'_m. Record these values in Table 11.1.

Results and Discussion
Table 11.1

Reference CO_2 Conc. = _____ ppm
Leaf Area = _____ cm^2

Irradiance (μmol quanta/m^2/s)	Sample CO_2 (ppm)	δCO_2 (ppm)	Psetic Rate (μmol CO_2/m^2/s)	Fo or Ft Value	Fm or F'_m Value	Quantum Yield
ZERO						

When you have calculated rates of photosynthesis at each light intensity used in your experiment, present your data as a graph with photosynthesis plotted on the *y* axis and light intensity on the *x* axis. On the same graph, plot values of Quantum Yield on a second y axis. Calculate the rate of electron transport through Photosystem II and plot this on your graph also.

A photosynthetic light response curve for a generalized leaf is shown in Figure 11.4. Note that at low irradiance, photosynthetic rate increases steeply as light is increased. This is because at these irradiances the rate of photosynthesis is limited by the rate of the light reactions. Insufficient photons are being supplied to the leaf to produce the ATP and reductant required to sustain maximum photosynthetic rates. At higher irradiances there is less of an increase in photosynthetic rate per unit increase in light intensity, and eventually photosynthesis reaches *light saturation* at the highest irradiance used in the experiment. Under these conditions, the light reactions of photosynthesis are maximized, and photosynthetic rate is limited either by the supply of CO_2 to supply the photosynthetic dark reactions, or by the turnover rate of the photosynthetic enzymes.

The photosynthetic light response curve of a particular plant is influenced by many factors, and a study of the components of the curve can tell us a great deal about the physiology and ecophysiology of the plant.

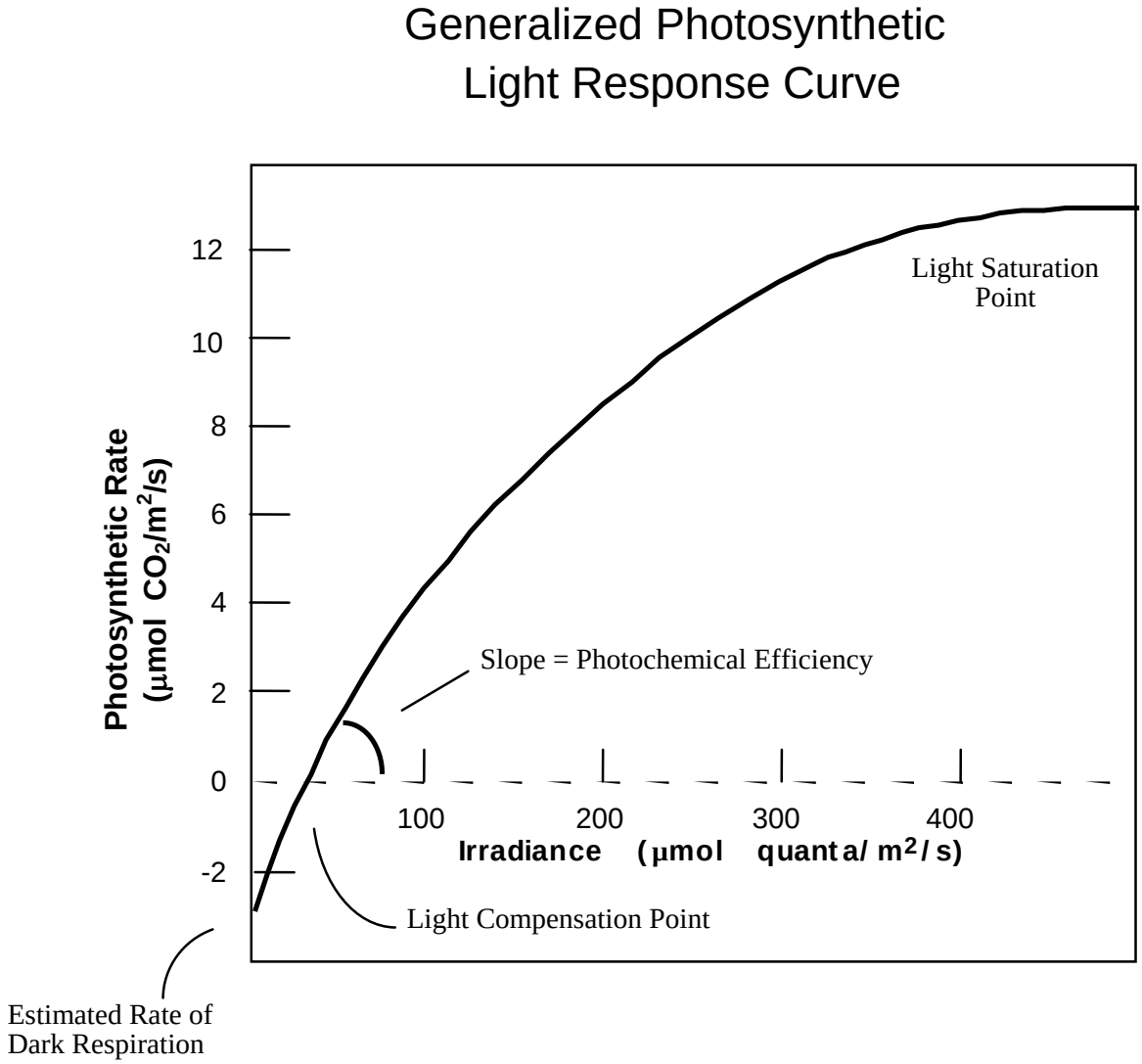
Important aspects of the light response curve include:

(i) ***The Light Compensation Point.*** Extrapolate the linear portion of the light response curve to intercept the *x* axis at the point where photosynthetic rate is zero. The light intensity at this point is called the light compensation point, and it represents the light intensity at which O_2 production in photosynthesis is balanced by O_2 consumption in respiration.

(ii) ***The Rate of Dark Respiration***. If the linear part of the light response curve is extrapolated to intercept the *y* axis at zero light intensity, the negative rate of photosynthesis at this point gives an estimate of the "dark" respiration rate.

(iii) ***Photochemical Efficiency.*** Photochemical efficiency may be defined as the increase in photosynthetic rate achieved per unit increase in light absorbed by the leaf. In your experiment, you did not measure light absorbance by the leaf, but only the amount of light transmitted through the leaf. However, a qualitative measurement of photochemical efficiency may still be made by calculating the initial slope of the light response curve.

Figure 11.4



(iv) ***The Light Saturation Point of Photosynthesis.*** The light intensity beyond which the light response curve plateaus is called the light saturation point of photosynthesis. At this point increases in light intensity do not cause increases in photosynthetic rate, so other factors apart from the supply of light must be limiting the photosynthetic process. These factors include:

(a) The supply of CO_2 to the leaf.

(b) The ability of the leaf to transduce the light energy supplied into chemical energy for photosynthesis (dependent on the photophosphorylation capacity of the leaf).

(c) The capacity of the leaf to use energy from photophosphorylation to fix CO_2 (dependent on the amount, and turn-over rate, of enzymes involved in the "dark reactions" of photosynthesis).

Further Reading

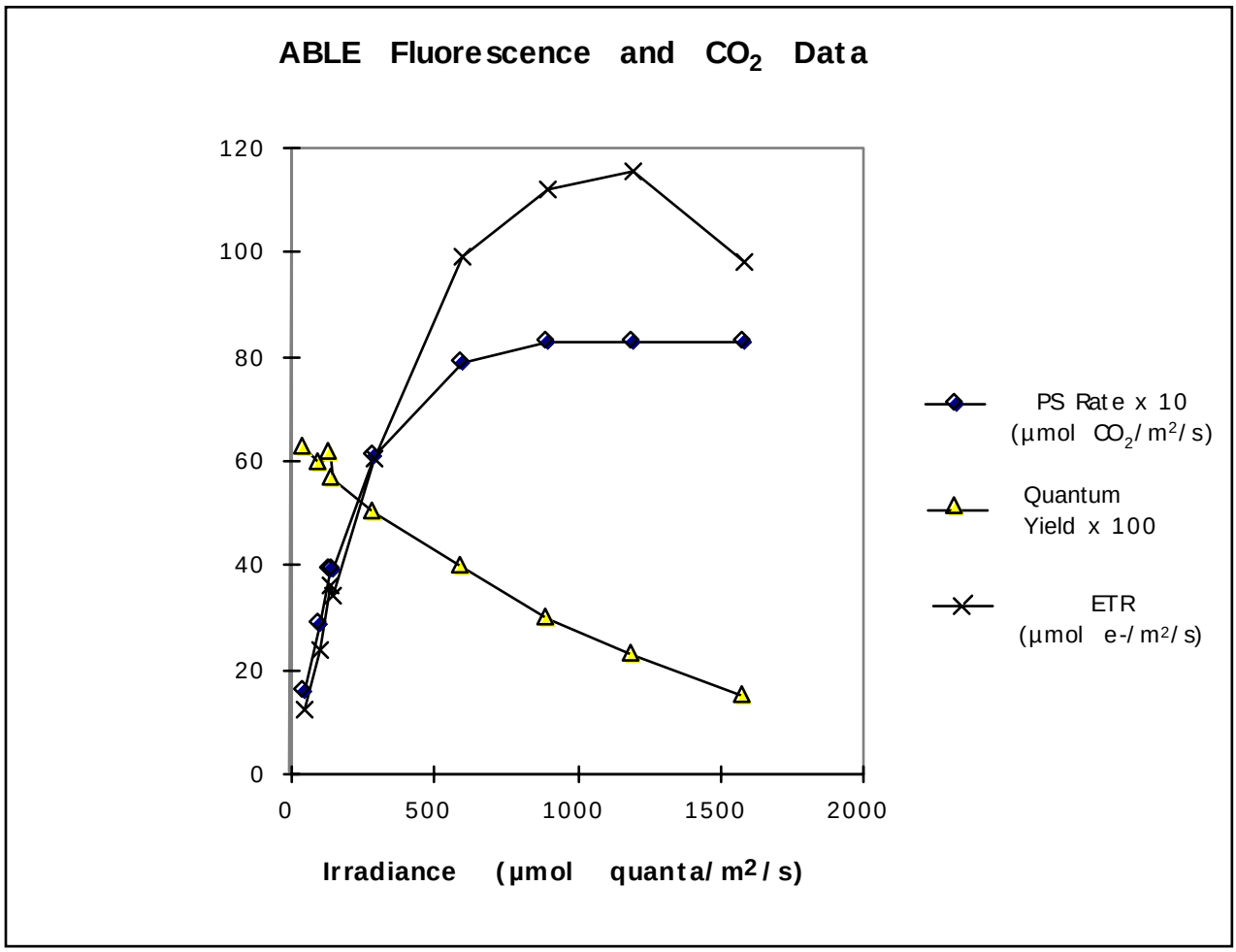
Bolhar-Nordenkampf H.R. and Oquist G. (1993) Chlorophyll fluorescence as a tool in photosynthesis research. Pages 193 – 206 *in* Photosynthesis and Production in a Changing Environment (D.O Hall, J.M.O. Scurlock, H.R. Bolhar-Nordenkampf, R.C. Leegood and S.P. Long, Editors) Chapman and Hall, London, 464 pages, ISBN 0 412 42900 4 (HB).

Walker, D. (1992) Fluorescence (Chapter 2) Pages 36 – 42. *in* Energy, Plants and Man 2nd edition. Packard Publishing Ltd. Chichester, UK. 277 Pages, ISBN 1 870232 05 4.

Genty, B.J., J-M Briantais and N.R. Baker (1990) The relationship between the quantum yield of phostosynthetic electron transport and photochemical quenching of chlorophyll fluorescence. Biochim. Biophys. Acta 990, 87-92.

APPENDIX A

Figure 11.5 Typical Experimental Data



Data collected and graphed by Dr. Wendie Johnston of Pasadena Community College during the ABLE workshop in Nebraska June 3, 1999.

APPENDIX B

Questions and Answers

The following are a selection of questions presented to the students after completion of the laboratory which they are expected to answer as part of the laboratory write-up. The questions are designed to help the students analyze their data , and to criticize experimental design. Example answers are provided to lab Instructors.

Q1. Criticize this method for estimating dark respiration, and compare the results you obtained by measuring dark respiration directly in your experiment.

A1. The method for measuring dark respiration assumes that the initial part of the photosynthetic light response curve remains almost linear when net photosynthesis becomes negative at low light levels. If respiration is stimulated in the dark this may not be true. The rate of respiration in the dark is not necessarily the same as occurs in the light. A major function of respiration is to supply ATP for metabolic reactions in the leaf, but since cyclic photophosphorylation can supply this ATP in the light, respiratory ATP is in less demand. However, respiration also functions to provide carbon skeletons for numerous metabolic pathways in the leaf, and since these pathways occur both in the light and dark, respiration must continue in the light albeit, perhaps, at a lower rate.

Q2. Discuss how you would change the design of the experiment to make more accurate measurements of photochemical efficiency.

A2. This challenges students to evaluate the concept of measuring photochemical efficiency using incident irradiance as a measured parameter. This parameter does not allow an accurate measurement of photochemical efficiency because the light energy that is actually **absorbed** by the leaf, rather than merely the light energy incident on the leaf surface, should be measured. A more involved technique for measuring the amount of light absorbed by the leaf would involve using a light sensor to measure light incident on the leaf and the light transmitted through the leaf. The difference between these two measurements would provide a rough estimate of absorbed light. However, these measurements do not take into account the amount of light that is reflected by the leaf. Accurate measurements of photochemical efficiency (and quantum yield) require sophisticated instruments such as an Ulbricht sphere which accounts for both reflection and absorption of the light supplied to the leaf.

Q3. Did you measure the light saturation point in your experiment? If not, why do you think the light saturation point was not reached? If the light saturation point was reached, do you think that the CO_2 supply was the major factor limiting photosynthesis at this point? How would you test this?

A3. Depending on the plant used, and the conditions under which it was grown, students may, or may not, be able to measure the light saturation point in their experiment. If a C3 leaf was used in the experiment, it is likely that the light saturation point could be reached since

photosynthetic rate at ambient CO_2 would not be CO_2 saturated. C4 plants have a higher light saturation point because their CO_2 concentrating mechanism saturates Rubisco with CO_2 even at ambient external concentrations.

Students may test whether or not CO_2 is limiting photosynthesis at high light by increasing the CO_2 concentration in the reference gas, and measuring whether or not this results in a higher rate of photosynthesis. However, the students should be aware that it is important to measure the CO_2 concentration within the leaf during such experiments, rather than that in the atmosphere, since stomatal closure, and high rates of photosynthesis, at high atmospheric CO_2 can reduce internal CO_2 concentrations to levels significantly below those external to the leaf.

Q4. Explain the change in photochemical quantum yield between the dark-adapted state and the stable value after illumination.

A4. In the dark-adapted leaf quantum should be approximately 0.8, whereas the quantum yield in the illuminated leaf at steady state should be significantly lower. After dark adaptation, all photosynthetic electron acceptors are fully oxidized and available for photochemical energy transduction so quantum yield is maximized. After illumination, a proportion of the electron acceptors will be reduced at any one time and therefore not available to accept electrons from chlorophyll. Also, development of the transthylakoid proton gradient during illumination increases the amount of energy that is dissipated as heat from the chloroplasts. Both of these factors contribute to a decline in quantum yield as irradiance is increased.

Q5. Describe and explain the changes that occur in fluorescence yield (Ft) and quantum yield after transition from darkness to light (Kautsky Effect).

A5. Within seconds of the actinic light being turned on the fluorescence yield increases. It does not, however, reach the maximum value obtained during the saturating pulse after dark-adaptation (Fm). This increase in fluorescence yield represents reduction of the electron acceptors associated with photosystem II (e.g. QA) though full reduction of these acceptors may not be attained. Full reduction only occurs with the imposition of saturating light pulses to obtain $F'm$ values. The $F'm$ values decline rapidly after the saturating pulse because electrons may be passed to PSI as photosynthesis is activated. Ft declines from its maximum value usually within 1-2 seconds of illumination, and then begins a slower decline over several minutes. A corresponding decline in $F'm$ is also seen. The decline in Ft is due to activation of photosynthesis and the opening of more electron acceptors. The decline in $F'm$ results from the development of the transthylakoid proton gradient for ATP generation, and has the effect of increasing heat dissipation from the chloroplasts. After several minutes of continuous illumination $F'm$ values begin to increase again. This is linked with Calvin Cycle activity and associated use of the ATP generated by photophosphorylation. The partial relaxation of the transthylakoid potential (due to ATP synthesis) reduces heat loss from the chloroplasts.

Q6. Describe and explain the relationship between quantum yield and irradiance.

A6. Quantum yield declines as irradiance increases. This is because quantum yield is an index of the efficiency of light energy conversion into chemical energy. This efficiency will be greatest when PSII electron acceptors are fully oxidized and are therefore able to accept electrons from excited chlorophyll molecules. The proportion of oxidized acceptors is greatest under low light conditions because as irradiance increases, and electron release from chlorophyll molecules increases in parallel, the number of PSII electron acceptors that are reduced will increase as a consequence. At the highest light intensities, the rate of reductant supply from photochemistry exceeds reductant use in the Calvin Cycle. As a result, the level of $NADPH_2$ increases and the availability of $NADP^+$ for reduction declines. Since there is insufficient $NADP^+$ to accept electrons from the photosystems, the electron acceptors become fully reduced and quantum yield falls to its minimum value.

Q7. Describe and explain the relationship between electron transport rate and irradiance.

A7. Electron transport rate increases in an almost linear fashion with increasing irradiance because more chlorophyll molecules are excited by the greater number of photons impinging on the leaf and provide more electrons to the electron carriers in the photosystems. At the highest light levels the relationship curves off to reach a steady maximum rate. This occurs when the chlorophyll molecules are light-saturated and any further increase in photon flux cannot increase the rate of electron release from the chlorophyll molecules. Often, electron transport rate may increase with irradiance to a maximum level and then decline somewhat as irradiance is increased further. This occurs more commonly, and to a greater degree, in shade leaves, and is caused by photoinhibition. This process is imperfectly understood but it is thought that excess excitation of chlorophyll leads to a stimulation of heat dissipation processes in the chloroplast so that energy transduction by both photochemistry and fluorescence declines.