

# Measuring Light-dependent Proton Translocation in Isolated Thylakoids

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**Abstract** An improved protocol for measuring light-dependent proton translocation across the membrane of isolated thylakoids is described. The method uses a pH electrode attached to data acquisition software to measure the pH increase in the bathing solution as protons are pumped from the solution to the internal compartments of the thylakoid vesicles. Up to the point of light saturation, the magnitude of proton movement depends on irradiance level. Proton translocation is inhibited in a concentration-dependent manner in the presence of dichlorophenyl dimethyl urea (DCMU). In the presence of gramicidin D, a proton gradient across the membrane can be neither established nor maintained. Depending on its redox state, dichlorophenol indophenol (DCPIP) can act as an electron donor or acceptor to or from different components of the thylakoid membrane. Studies using oxidized or reduced DCPIP with or without a high concentration of DCMU demonstrate that both linear and cyclic electron flow both result in light-dependent proton translocation. Proton translocation is a fundamental process used by most types of organisms to create a transmembrane proton gradient that provides the energy for ATP synthesis. The value of the described method is that students can *directly* measure the disappearance of protons from the thylakoid suspension solution and, by adding DCMU and oxidized or reduced DCPIP, demonstrate the coupling of electron transport to proton translocation in the thylakoid membrane.

**Keywords** Isolated thylakoids, Proton translocation, Light, Dichlorophenol indophenol (DCPIP), Dichlorophenyl dimethyl urea (DCMU), Gramicidin D, pH change

## 1. Introduction

I teach a section of a second year undergraduate laboratory course called Scientific Methods in Biology. We deal with experimental design, instrumentation, the evaluation of experimental data and scientific writing. Because the course is concerned with methods, the experimental systems we use are of secondary importance. However, the measurement of light-dependent rates of electron transport through the thylakoid membranes of isolated chloroplasts has proved to be a system that can be used to address a wide variety of experimental questions. In class, many experimental variables have been tested which depend on measuring rates of photoreduction of DCPIP that intercepts electrons from the plastoquinones in the thylakoid membrane and, consequently, becomes reduced.

Although measuring rates of photoreduction of DCPIP has generated many robust, inexpensive exercises for biology laboratory education, there are limitations to this assay method. For example, it can only be used to measure linear electron flow and then only from the oxidation of water to

the plastoquinones from which the DCPIP accepts electrons.

Also, the method can only be used to infer the effect of different uncoupling reagents such as ammonium ion or gramicidin D on proton translocation because only the rate at which DCPIP accepts electrons from the plastoquinones is being measured. In the presence of either ammonium ion or gramicidin D, at light saturation, rates of photoreduction of DCPIP are increased by the same amount [1]. Although we have used an oxygen electrode [2] and fluorescence measurements [3] to corroborate some of the results using DCPIP reduction, these methods either require too much skill or are too expensive to be of much value in most undergraduate classes. As a consequence, I looked for other ways of measuring rates of light dependent activity in the thylakoid membrane and found a promising method in a widely-used biochemistry laboratory text book [4] and a paper cited in the book [5]. The method measures light-dependent changes in the pH of a solution in which isolated chloroplasts are suspended. The change in pH results from the translocation of protons from the suspension solution into the internal compartment of the thylakoids. My paper is based on my experience using this method to measure pH change and includes modifications to the experimental procedure, modifications of the chloroplast isolation and suspension solutions and ways to generate much more data from a single preparation than those

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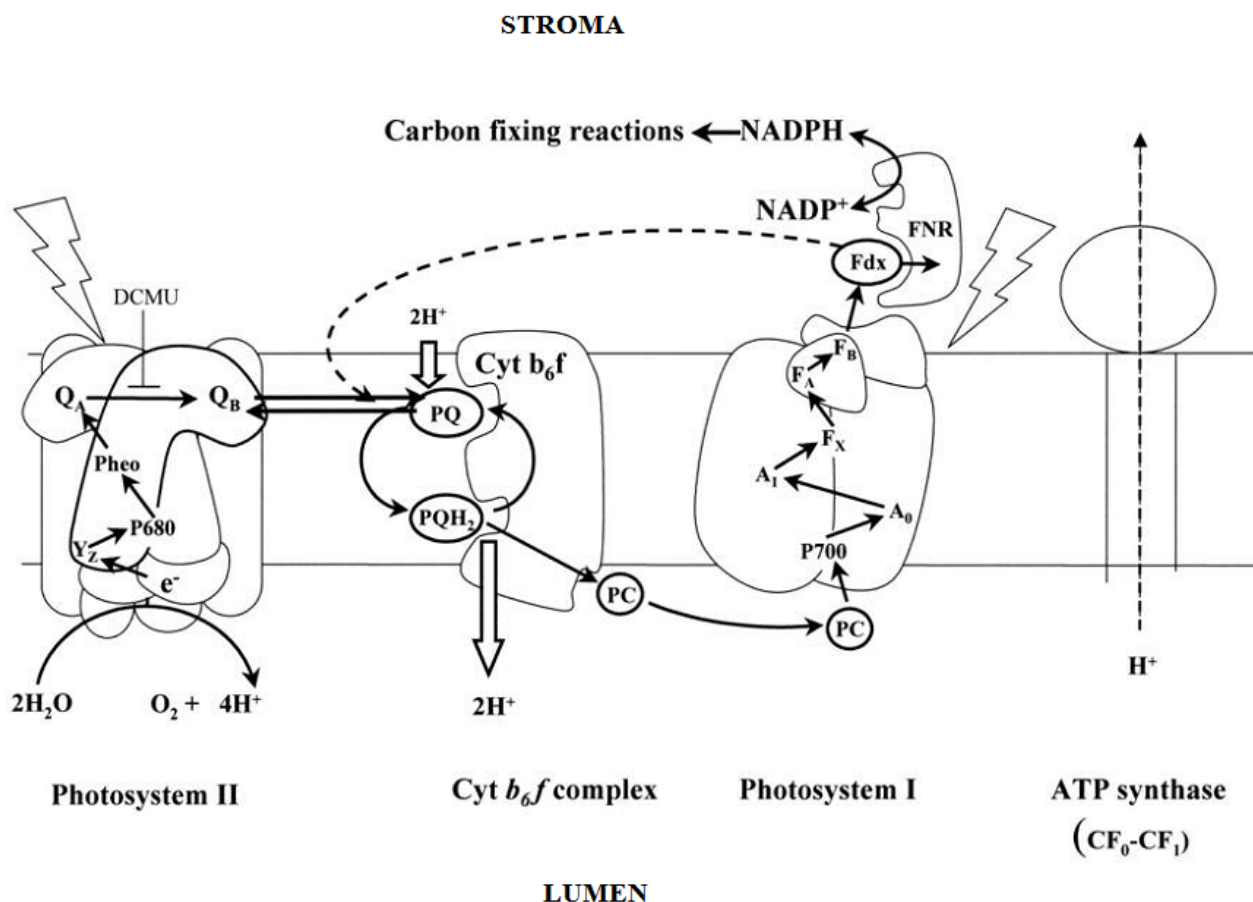
described in the source publications [4, 5]. Variants of the procedure are still used in research laboratories [6, 7, 8, 9].

The value of the described method is that students can *directly* measure proton translocation, a fundamentally important process used by most types of organisms to generate ATP. It is an excellent introduction to both chemiosmosis and light dependent activities in the chloroplast and can, of course, lead to discussion of mitochondrial function. As a bonus, students can monitor these activities on a familiar interface; the computer monitor.

Figure 1 illustrates both linear and cyclic electron flow in the thylakoid membrane. In linear electron flow, energy derived from photons absorbed by pigments in the antenna complexes is used to oxidize water at the oxygen evolving complex of photosystem II (PS II). The resulting electrons pass through a series of electron acceptors in PS II, then to plastoquinones in the membrane, to the cytochrome  $b_6f$

complex, to plastocyanin, to photosystem I (PS I), to ferredoxin (Fdx) and on to the final electron acceptor,  $\text{NADP}^+$ .

Plastoquinones require both protons and electrons to become reduced. Consequently, when plastoquinones accept electrons from PS II they also accept protons from the stromal compartment of the chloroplast. When they become reoxidized by passing electrons to cytochrome  $b_6f$ , they liberate protons on the luminal side of the membrane. This process “pumps” protons across the membrane producing a proton gradient that provides energy for ATP synthesis (photophosphorylation). Since protons are pumped from the outside to the inside of the thylakoids, the pH of the solution in which isolated thylakoids are suspended increases when they are exposed to light. In the method described below, the thylakoid suspension solution is a proxy for the chloroplast stromal compartment.



**Figure 1.** Linear and cyclic electron flow in the thylakoid membrane. In linear electron flow (unbroken arrows) energy from absorbed photons is used to oxidise water on the luminal face of photosystem II (PS II). Electrons generated by this process pass through a series of electron carriers in PS II and then to the oxidized plastoquinones (PQ) that diffuse within the membrane. As PQ becomes reduced ( $\text{PQH}_2$ ) it also accepts protons from the stroma. When  $\text{PQH}_2$  becomes oxidized, by donating electrons to cytochrome  $b_6f$  (cyt  $b_6f$ ) complex it liberates protons into the lumen. The electrons are then passed in turn to plastocyanin (PC), to photosystem I (PS I), to ferredoxin (Fdx) and ultimately participate in the reduction of  $\text{NADP}^+$ , a reaction catalyzed by the FNR (ferredoxin:NADP<sup>+</sup> reductase). In cyclic electron flow, energy from absorbed photons causes the oxidation of the reaction centre (P700) in PS I. The resulting electrons then flow sequentially to Fdx, to PQ (unbroken arrow), to cyt  $b_6f$ , to PC then back to PS I. Proton translocation from the stroma to the lumen is indicated by open arrows. DCMU blocks electron transfer from  $Q_A$  in PS II to PQ in the membrane. (Figure modified from a template provided by Dr. Alex Ivanov)

## 2. Materials and Method

### 2.1. Equipment

I have been using a pH sensor (S165 pH Sensor) and Logger Pro 3 software (Qubit Systems, Kingston, Ontario, Canada) with a computer. An alternative is a pH probe attached to a pH meter with a chart recorder.

Magnetic stirrer with small spin bar.

Light source (I use a 100 watt incandescent lamp).

Timer (if you are not using data acquisition software).

Shallow glass dish containing water to control temperature of reaction container.

Thermometer.

Small reaction container (I use a 20 mL glass beaker. Any small glass container that can accommodate the pH probe will suffice).

Spectrophotometer and cuvettes to measure absorbance at 652 nm.

Domestic blender.

Clinical centrifuge.

Variable volume mechanical pipettes (10 – 100  $\mu$ L range) with tips.

Pasteur pipettes with pipette bulbs.

Cheesecloth, Parafilm®.

(A Li-Cor LI-189 quantum/radiometer/photometer attached to an LI-190 SA quantum sensor is useful but not essential).

### 2.2. Stock Solutions

Some of the following reagents present potential hazards. The MSDS sheets should be read before handling to identify special handling and disposal requirements.

Thylakoid isolation buffer: 20 mM tricine buffer containing 0.4 M sorbitol, 10 mM NaCl, 2.5 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and 1.25 mM  $\text{MnCl}_2$  adjusted to pH 7.8 with saturated aqueous NaOH.

Thylakoid suspension solution: 0.4 M sorbitol, 20 mM KCl, 5 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and 0.25 mM  $\text{MnCl}_2$ .

80% (v/v) aqueous acetone.

6.0 mM 2, 6-dichlorophenol indophenol (DCPIP), (BDH Chemicals, Poole, United Kingdom) in 95% ethanol. (The DCPIP in this blue solution is predominantly in the oxidized form).

Reduced DCPIP (a mixture of 100  $\mu$ L of 6.0 mM DCPIP and 20  $\mu$ L of 0.05 M ascorbic acid prepared freshly before each experimental use). This mixture rapidly loses its blue colour as the DCPIP is reduced by the ascorbic acid. It becomes very pale yellow.

1.0 mM 3-(3, 4- dichlorophenyl)-1, 1-dimethyl urea (DCMU), (Sigma, St. Louis, MO) dissolved in methanol.

100  $\mu$ M gramicidin D (Sigma), (Molecular weight, not shown on the container, is ~ 2000) dissolved in dimethyl sulfoxide (DMSO).

0.05 M ascorbic acid (Sigma).

### 2.3. Thylakoid Isolation

Weigh ~30 g of spinach leaves from which the petioles and major veins have been removed. Cut the leaves into smaller fragments and place them in a blender cup that has been stored in the freezer. Add 100 mL of cold thylakoid isolation buffer and blend the leaves with three 5 second bursts at full speed. Filter the blended mixture (called a brei) through 4 layers of cheesecloth into a beaker on ice. Transfer six 10-15 mL aliquots into 15 mL capacity centrifuge tubes. Centrifuge for 5 - 6 minutes at 1300 x g. Discard the supernatant using a Pasteur pipette and add 2.0-2.5 mL of ice-cold isolation buffer to each pellet. Resuspend the clearly visible, green pellets by back and forth rotation with a camel hair paintbrush and pool them in a single tube. Store on ice. The thylakoid isolation protocol is extremely reliable. Apart from keeping the solutions on ice, no additional precautions are required.

I have previously referred to the isolated organelles as chloroplasts. However, we have demonstrated that chloroplasts isolated using this method are 100% broken, i.e. the outer envelope is ruptured [2], so they are more precisely called isolated thylakoids.

### 2.4. Estimation of the Chlorophyll Concentration in the Thylakoid Suspension

Add 50  $\mu$ L of thylakoid suspension to 5.0 mL of 80% acetone. Cover the tube with Parafilm® and shake to dissolve the chlorophyll. Centrifuge at 1300 x g for 3 minutes. Read the absorbance of the supernatant at 652 nm ( $A_{652}$ ). The chlorophyll concentration, in mg chlorophyll/mL of chloroplast preparation, is calculated using equation 1 from reference [10].

$$\text{mg/mL} = A_{652} \times 100 / 34.5 \text{ mL mg}^{-1} \text{ cm}^{-1} \quad (1)$$

(34.5 mL  $\text{mg}^{-1} \text{ cm}^{-1}$ , a proportionality constant, is the absorption coefficient for chlorophylls dissolved in 80% (v/v) acetone at 652 nm [10]).

### 2.5. Equipment Arrangement

Diagrams of the equipment set-up have been published elsewhere [4, 5]. In my work, a 2 cm thick piece of polystyrene board was taped to the top surface of the magnetic stirrer to reduce heat-transfer from the stirrer to the assay medium. A shallow, glass dish (9 cm in diameter, 5 cm deep in this work) containing water at room temperature was placed on the polystyrene board. The water temperature was measured with a thermometer and, because the lamp generates heat, ice was added periodically to maintain the water at room temperature. The container, holding 10 mL of the thylakoids suspended in assay medium, was placed at the center of the glass dish and the water level in the dish was brought up to the level of the surface of the assay medium. After rinsing and blotting the pH probe, the tip was immersed in the thylakoid suspension. The magnetic stirrer

was used to stir the reaction mixture vigorously. At this point, the pH rises quite rapidly. Wait for 3 to 5 minutes until the pH is stable before recording any activity. The light source was placed at the chosen distance from the reaction mixture.

## 2.6. Assay Protocol

Assays are performed using 10 mL of thylakoid suspension solution in which isolated thylakoids are suspended to a concentration of 300  $\mu\text{g}$  of chlorophyll/mL. To make this, transfer a volume (typically 4-6 mL) of thylakoid preparation that contains 3 mg of chlorophyll to a conical tube. Centrifuge at 1300 x g for 5 minutes to pellet the thylakoids. Discard the supernatant using a Pasteur pipette and resuspend the pellet up to 10 mL with ice-cold thylakoid suspension solution (This step is to dilute the isolation buffer out of the assay mixture). Centrifuge at 1300 x g for 5 minutes to pellet the thylakoids. Discard the supernatant and resuspend the pellet up to 10 mL with thylakoid suspension solution at room temperature. At this stage, the thylakoid suspension is ready for assays which investigate the 4 types of treatment described below. During the course of this work room temperature was  $21^\circ \pm 1^\circ\text{C}$ . Transfer the suspension to the reaction container and add the stir bar.

## 2.7. Data Analysis

The data can be copied from the Logger Pro software and pasted into a graphing program. After printing the experimental traces, the change in pH in response to any treatment is determined by measuring the vertical height of the peak and converting it to a pH value by reference to the scale on the y axis. The scale on the y axis can be exaggerated for measuring smaller peaks.

## 2.8. Student Preparation

To obtain maximum heuristic benefit from the experimental work described below, students should prepare by reading about light-dependent events in photosynthesis from any recent cell biology or biochemistry textbook. Even the grade 12 Biology students that I have worked with understand the concepts well but I start the class with a review using electron micrograph images of leaf structure, chloroplast structure, thylakoid membranes and the diagram included here as figure 1. By reference to the diagram, I also introduce the location and manner in which the reagents used interact with the components of the thylakoid membrane. With this information, and in discussion with their peers and instructors, students can make predictions as to how the treatments they chose to try will affect proton translocation. Alternatively, instructors may, of course, prefer to use the exercises presented here in a more prescriptive fashion.

## 2.9. Experimental Design

The magnitude of the light-dependent pH change appears to depend on the time that the thylakoids have been kept in darkness. To compensate for this, I have routinely started

each experimental trace by turning on the data acquisition software, leaving the reaction in the dark for 30 seconds, turning on the lamp for 60 seconds, turning off the lamp and waiting for the signal to stabilize before proceeding with the experiment. I have operationally-defined stability as when the pH remains constant, to two decimal places, for 30 seconds before, during or after a treatment. I have used two ways to time experiments:

(1) Following a pre-determined timing protocol for turning the lamp on and off (e.g. light on for 60 seconds followed by light off for 60 seconds and so on). This has been useful for some comparisons, for example, measuring the response to different levels of irradiance.

(2) Making decisions as to when to turn the lamp on or off based on the observed response. This is useful if you choose to observe the full magnitude of the response, for example, to determine whether a plateau in response has been reached or maintained.

Preliminary experiments were undertaken using an isolation buffer and chloroplast suspension solution as previously described [4]. However, I have isolated thylakoids hundreds of times and because I have confidence in the solutions I have used before, I modified these solutions for use in the pH assay. Changes include the substitution of sorbitol for sucrose and the addition of  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  to the isolation buffer and thylakoid suspension solution. Thylakoids show minimal loss of activity over a number of hours when stored on ice in this isolation buffer and the magnitude of pH change is greater in the modified suspension solution. Other modifications to the assay include not adjusting the pH of the thylakoid suspension solution into a pH range from 6.0 – 6.2 [4, 5] before running the assays. Addition of acid or base to adjust the pH to a target value is (a) very difficult, and (b) adds varying concentration of ions to the suspension solution thereby adding an unnecessary experimental variable. Instead, if you wait for 3 – 5 min after the thylakoids are added to the solution, it comes to equilibrium at  $\sim \text{pH } 7.0$ . Moreover, since the assay runs well at room temperature, I did not attempt to reduce the reaction temperature to, and maintain it at,  $10^\circ\text{C}$  [4]. Also, if the same concentration of chlorophyll is used in each assay, it is easier to compare data from one day to the next. The final modification is one of experimental design. In both resource publications [4, 5], experiments were designed to measure only one response for each aliquot of thylakoid preparation. By thinking about the sequence in which treatments are administered, much more data can be collected during each trial.

The time required to perform each assay shown below, including the time needed to prepare the thylakoid suspension and measure its chlorophyll concentration, is included in parentheses at the end of each protocol. If the thylakoid suspension is prepared for the students prior to class, subtract  $\sim 30$  minutes.

Here I describe experiments dealing with 4 types of treatment: (i) Response to irradiance level, (ii) response to different concentrations of DCMU, (iii) response to

gramicidin D and (iv) response to the presence of oxidized or reduced DCPIP. All have been repeated in a variety of ways. These are examples.

#### 2.9.1. Response to Irradiance Level

All data can be collected using a single aliquot of thylakoid suspension. The irradiance is changed by placing the light source at different distances from the suspension. In the example shown here the reaction was run at 33.9, 28.2, 22.9, 17.9, 12.3 and 10 cm from the light source. These distances corresponded to photon fluence rates, measured with a quantum sensor, of 10, 20, 30, 50, 100 and 150  $\mu\text{moles of photons}/\text{m}^2/\text{s}$  respectively. After the routine 30 seconds of darkness followed by 60 seconds of light and 60 seconds of darkness the light was turned on and off every 60 seconds. Four peaks were recorded at each photon fluence rate. The lamp was moved to a new distance from the sample during the last dark phase of each of the four light/dark cycles. Because the proton translocation slowly declines over time after continuous exposure to light/dark cycles, the experiment was run from lowest to highest photon fluence rates to avoid bias.

(Time required: 1.5 – 2.0 hours).

#### 2.9.2. Response to DCMU Concentration

After data collection was started, the light was turned on at 30 seconds, off at 90 seconds and, at 150 seconds, 20  $\mu\text{L}$  of 250  $\mu\text{M}$  DCMU was added. Thereafter, the following sequence of events was repeated 14 times: light on after 60 seconds, light off after an additional 60 seconds, addition of 20  $\mu\text{L}$  of 250  $\mu\text{M}$  DCMU after an additional 60 seconds. Since the stock DCMU is dissolved in methanol, the experiment was repeated with a fresh aliquot of the same thylakoid preparation adding 20  $\mu\text{L}$  of methanol (control) in place of the 20  $\mu\text{L}$  of 250  $\mu\text{M}$  DCMU. (Time required: 2.0-2.5 hours).

#### 2.9.3. Response to Gramicidin D

After the start of data collection, the light was turned on at 30 seconds and off at 180 seconds after a stable plateau in response was reached. The light was turned on when the pH value reached a stable valley at 350 seconds and off when a stable plateau was attained at 500 seconds. The light was turned on again when the pH value reached a stable valley at 620 seconds and 100  $\mu\text{L}$  of 100  $\mu\text{M}$  gramicidin D was added at 770 seconds. The light was then turned off at 900 seconds. The experiment was repeated adding 100  $\mu\text{L}$  of DMSO (control) instead of 100  $\mu\text{L}$  of 100  $\mu\text{M}$  gramicidin D at 770 seconds. (Time required:  $\sim$  1.0 hour).

#### 2.9.4. Response to DCPIP

The timing for this experiment was determined empirically for the first recorded trace and then repeated for the following three traces. After the start of data collection,

the light was turned on at 30 seconds, off at 90 seconds, on at 300 seconds, off at 450 seconds, on at 750 seconds, off at 1000 seconds, on at 1170 seconds, off at 1270 seconds, on at 1420 seconds, off at 1520 seconds, on at 1720 seconds, off at 1820 seconds and on at 2020 seconds. In the first trial, 50  $\mu\text{L}$  of 6.0 mM oxidized DCPIP was added at 630 seconds. In the second trial, 60  $\mu\text{L}$  of reduced DCPIP (see stock solutions) was added at 630 seconds. In both of trials three and four, 80  $\mu\text{L}$  of 1.0 mM DCMU was added at 250 seconds. In the former, 50  $\mu\text{L}$  of 6.0 mM oxidized DCPIP was added at 630 seconds and in the latter, 60  $\mu\text{L}$  of reduced DCPIP was added at 630 seconds. (The last four assays will not fit into a 3 hour class. If the thylakoid suspension is prepared before class, the first two assays can be run in one class and the second two assays can be run in a later class).

### 3. Results and Discussion

#### 3.1. Response to Irradiance Level

Figure 2 shows the complete experimental trace for thylakoids exposed to four 60 second light/60 second dark cycles at 10, 20, 30, 50, 100 and 150  $\mu\text{moles of photons}/\text{m}^2/\text{s}$  respectively. To make the measurements, the segment of the graph at each irradiance level was printed separately with an exaggerated y axis scale to improve accuracy. The data are summarized in figure 3 which shows the average change in pH ( $\Delta$  pH) of the four peaks at each irradiance level. No statistics were applied to these averages because the measurements are pseudoreplicates [11]. The reaction is clearly light-dependent and shows evidence of reaching light-saturation at the higher photon fluence rates. The experiment was run from the lowest to highest photon fluence rates to avoid introducing bias caused by the fact that proton translocation slowly declines over time after continuous exposure to light/dark cycles. Interestingly, this decline in response to continuous light on/light off cycles does not appear to result from deterioration of the thylakoid preparation. As an example, aliquots from a single thylakoid preparation that were exposed to light for 1 minute after 1, 2, 3, 4 and 10 minutes in the dark generated pH increases of 0.107, 0.125, 0.131, 0.126 and 0.116 respectively. This demonstrates no evidence of deterioration over time.

#### 3.2. Response to DCMU

The decline in proton translocation in the presence of increasing DCMU concentration is illustrated in figure 4. DCMU blocks the efflux of electrons from PS II (figure 1) [11]. As a consequence, the plastoquinone pool in the thylakoid membrane does not become reduced by accepting electrons from PS II so no protons are translocated into the thylakoid lumen. Since one DCMU molecule blocks the efflux of electrons from one PS II unit, the concentration-dependence of the response is expected. In

this case, proton translocation is reduced to  $\sim 50\%$  of that seen in methanol controls at a DCMU concentration of  $\sim 4.0$   $\mu\text{mol/liter}$ .

### 3.3. Response to Gramicidin D

The expected response to the addition of  $1.0$   $\mu\text{M}$  gramicidin D is illustrated in figure 5. Gramicidin D inserts into the thylakoid membrane creating an aqueous channel through which protons can diffuse [13]. Consequently, when gramicidin D is added after the light-dependent proton gradient has been established, the protons diffuse rapidly back to the thylakoid suspension solution and return it to the pH level that would be expected if the light had been turned off. Since the gramicidin D is dissolved in DMSO, the experiment was repeated but only DMSO (control) was added at 770 seconds. No response was observed (not shown). There is one surprise towards the end of the trace shown in figure 5. After adding gramicidin D at 770 seconds, the light was turned off at 900 seconds and on again at 950 seconds. A small increase in the pH of the solution is seen in response to darkness and the pH declines when the light is turned on. Although we would expect to see no response to light and darkness, what we in fact see is the reverse of the response to light and darkness typical of untreated thylakoids. I have repeated this several times, the response is always the same with  $\Delta$  pH values increasing by  $\sim 0.012$  units when the light is turned off.

### 3.4. Response to Oxidized and Reduced DCPIP

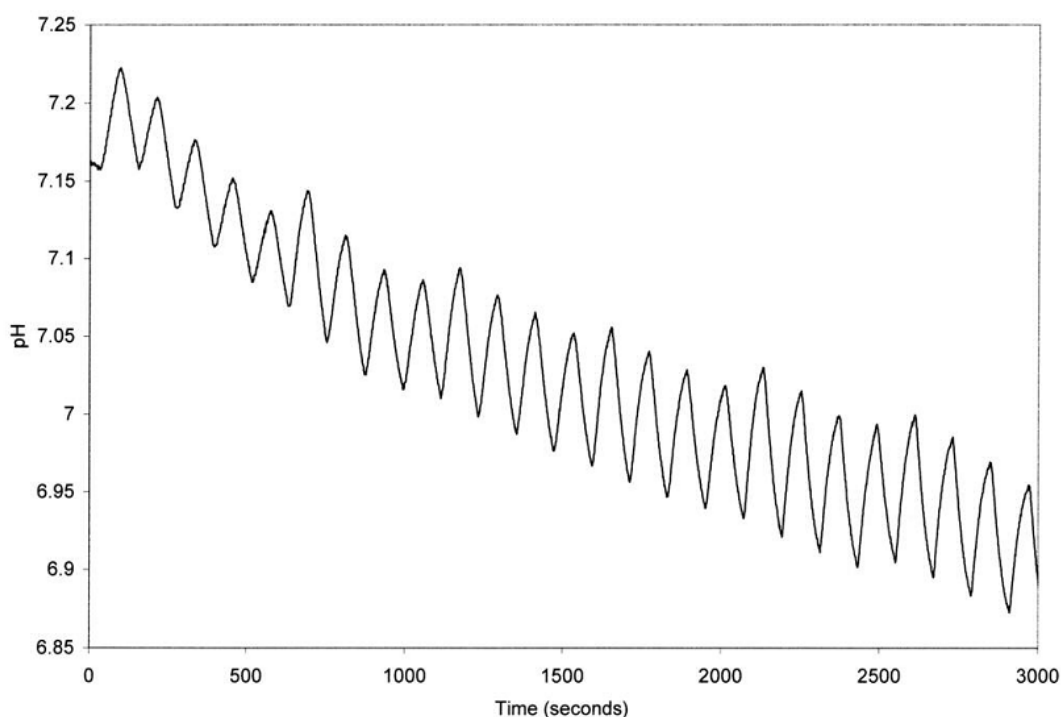
Once students have gained experience with this assay, the following types of experiments could form the basis of an

investigative study involving DCPIP and DCMU. Depending on its redox state, DCPIP can interact with different components of the electron transport chain in the thylakoid membrane [14]. The oxidized form (DCPIP) can accept electrons from the plastoquinones and the reduced form (DCPIPH<sub>2</sub>) can donate electrons to PS I and initiate cyclic electron flow (Figure 1).

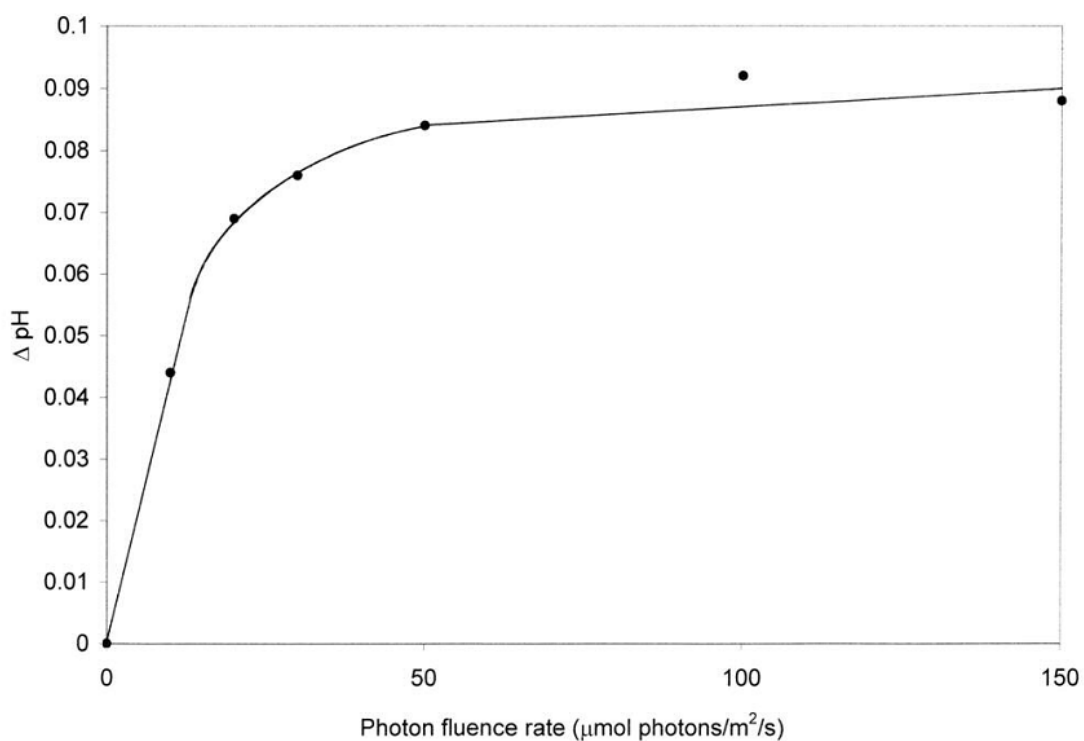
Figure 6 shows the response to the addition of  $30$   $\mu\text{M}$  oxidized DCPIP at 630 seconds followed by four light/dark cycles starting at 750 seconds. Addition of the DCPIP in darkness causes a small increase in pH that reaches a plateau. This rise is not reduced in the presence of  $1$   $\mu\text{M}$  gramicidin D (not shown) so it does not result from proton pumping into the thylakoids. When the light was turned on, a rapid and large increase in pH was seen but subsequent exposures to light resulted in pH increases that rapidly returned to control levels. The increase in proton translocation depends on two mechanisms that occur simultaneously.

(i) In the presence of light, oxidized DCPIP rapidly accepts electrons from the plastoquinone pool and is consequently reduced. By keeping the plastoquinone pool oxidized, the DCPIP increases the rate of transfer of electrons from PS II to DCPIP and thereby enhances the rate of proton translocation across the membrane.

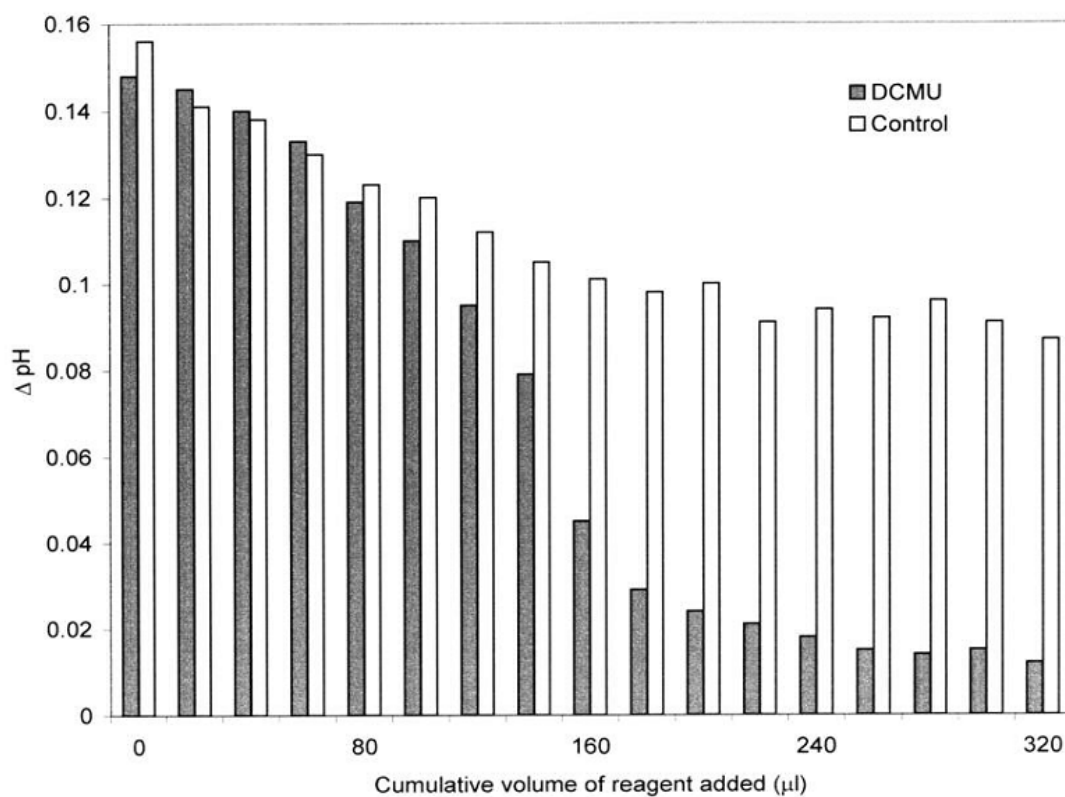
(ii) When DCPIP is reduced by accepting electrons from the plastoquinones, it may then donate electrons to PS I and initiate a cyclic flow of electrons from PS I to ferredoxin to the plastoquinones and back to PS I (Figure 1), again increasing the rate of proton translocation across the membrane.



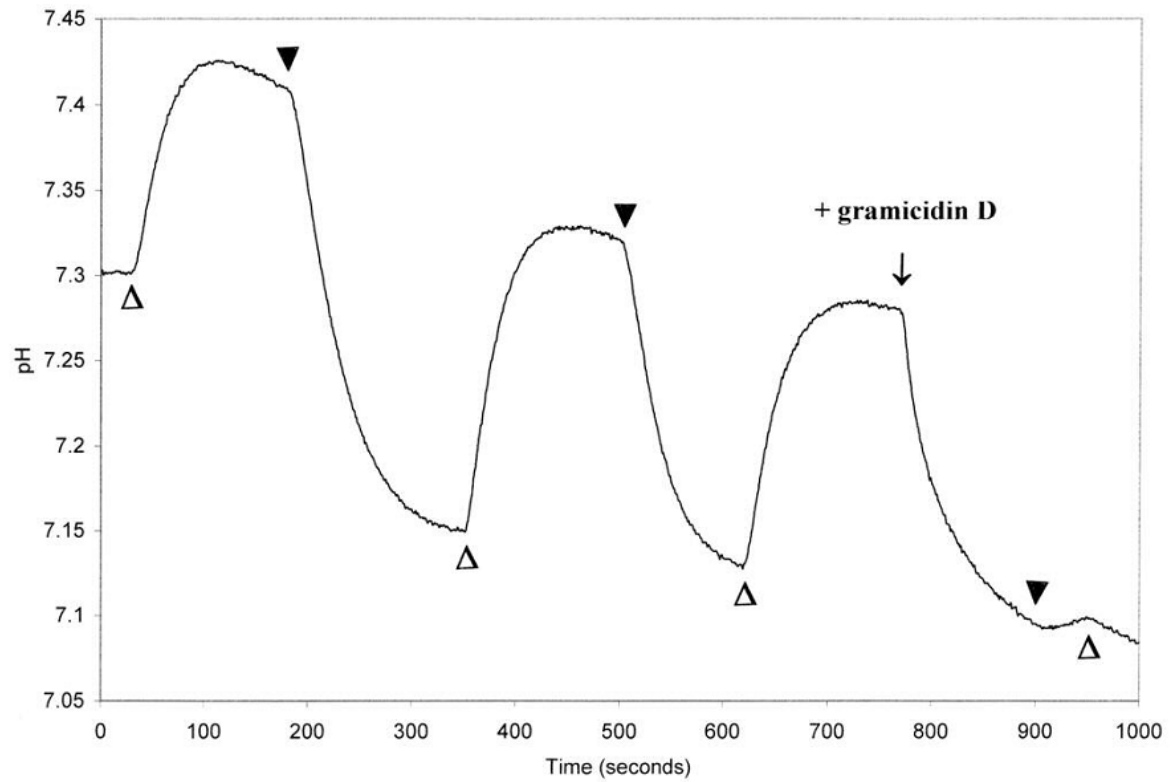
**Figure 2.** pH changes in response to light/dark cycles (60 seconds each) at different photon fluence rates. The first 5 cycles were at  $10$   $\mu\text{mol photons/m}^2/\text{s}$ , followed by 4 cycles each at  $20$ ,  $30$ ,  $50$ ,  $100$  and  $150$   $\mu\text{mol photons/m}^2/\text{s}$



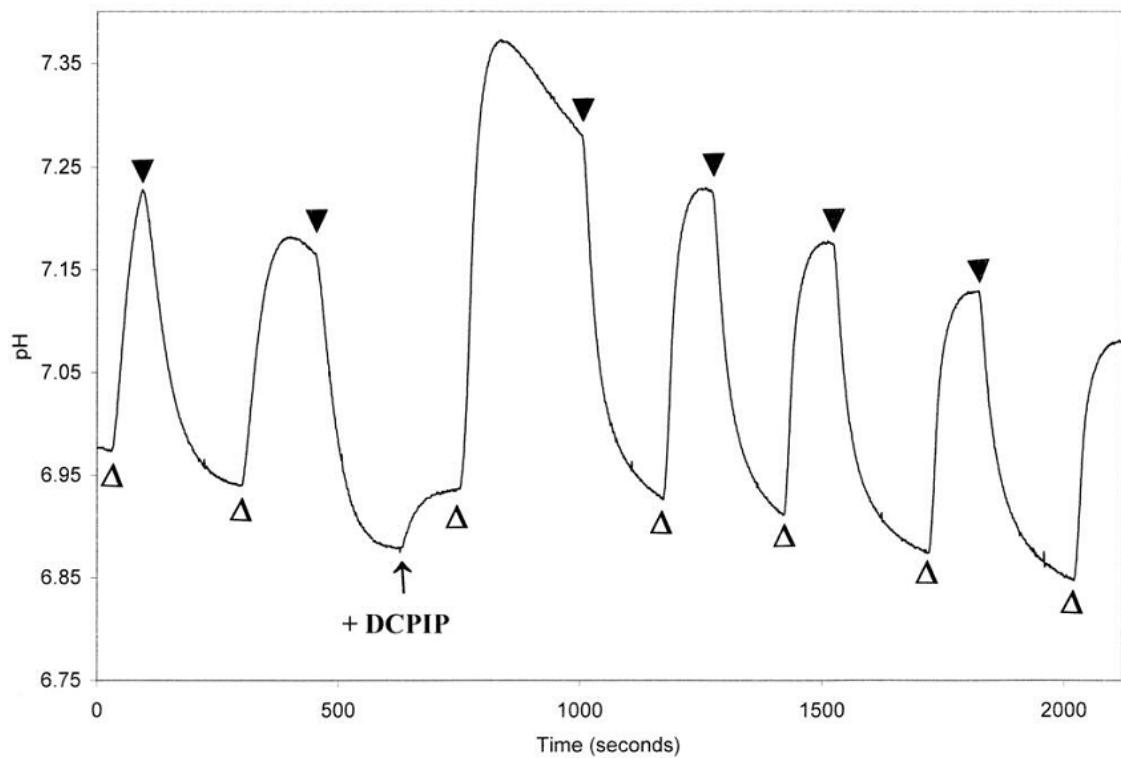
**Figure 3.** The average ( $n = 4$ ) increase in pH ( $\Delta \text{pH}$ ) after the light was turned on at different photon fluence rates (summary of data in figure 2). The  $\Delta \text{pH}$  increases by large increments as the photon fluence rate is increased at low light levels but shows light-saturation at higher light levels



**Figure 4.** The increase in pH ( $\Delta \text{pH}$ ) after the light was turned on. Between each light/dark cycle,  $20 \mu\text{L}$  of  $250 \mu\text{M}$  DCMU dissolved in methanol was added (grey bars). Using a separate aliquot of the same thylakoid preparation, the experiment was repeated but  $20 \mu\text{L}$  of methanol was added instead of DCMU dissolved in methanol (control, white bars)

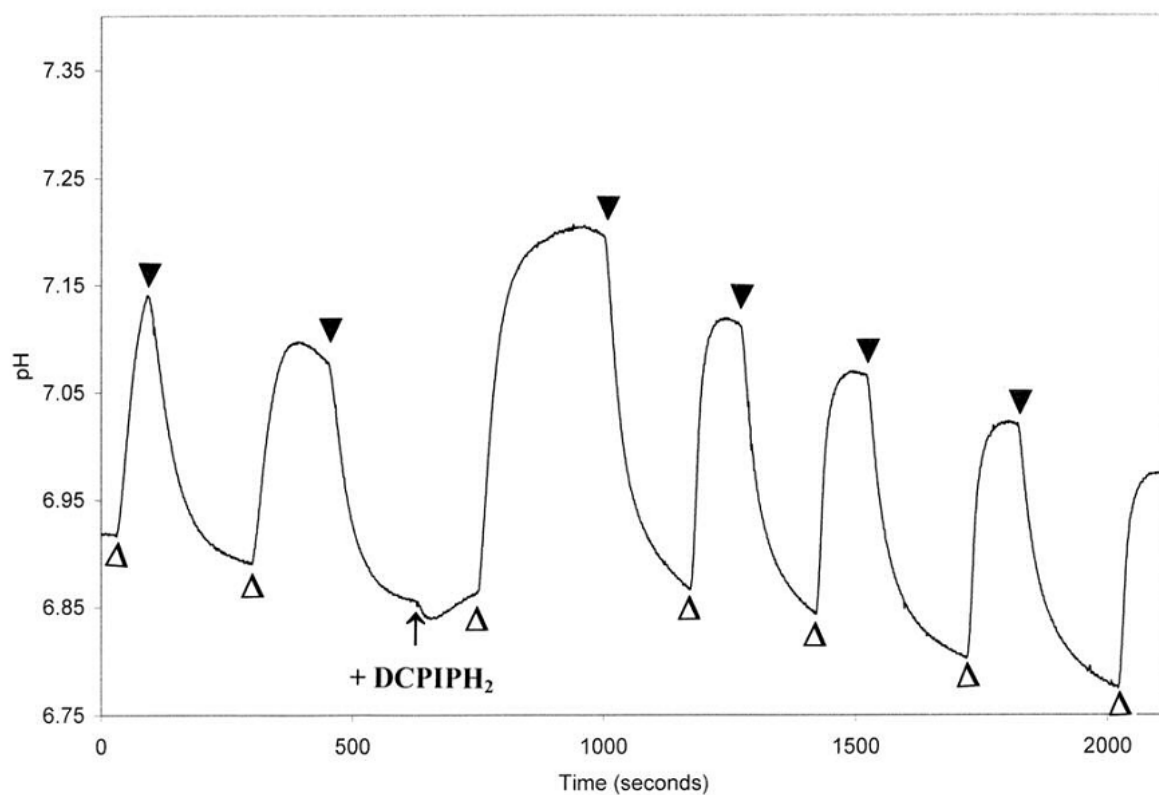


**Figure 5.** The changes in pH during three cycles of light on (open triangles) and light off (closed triangles). Gramicidin D, added during the third period of illumination, reduced the pH to a level expected if the light had been turned off

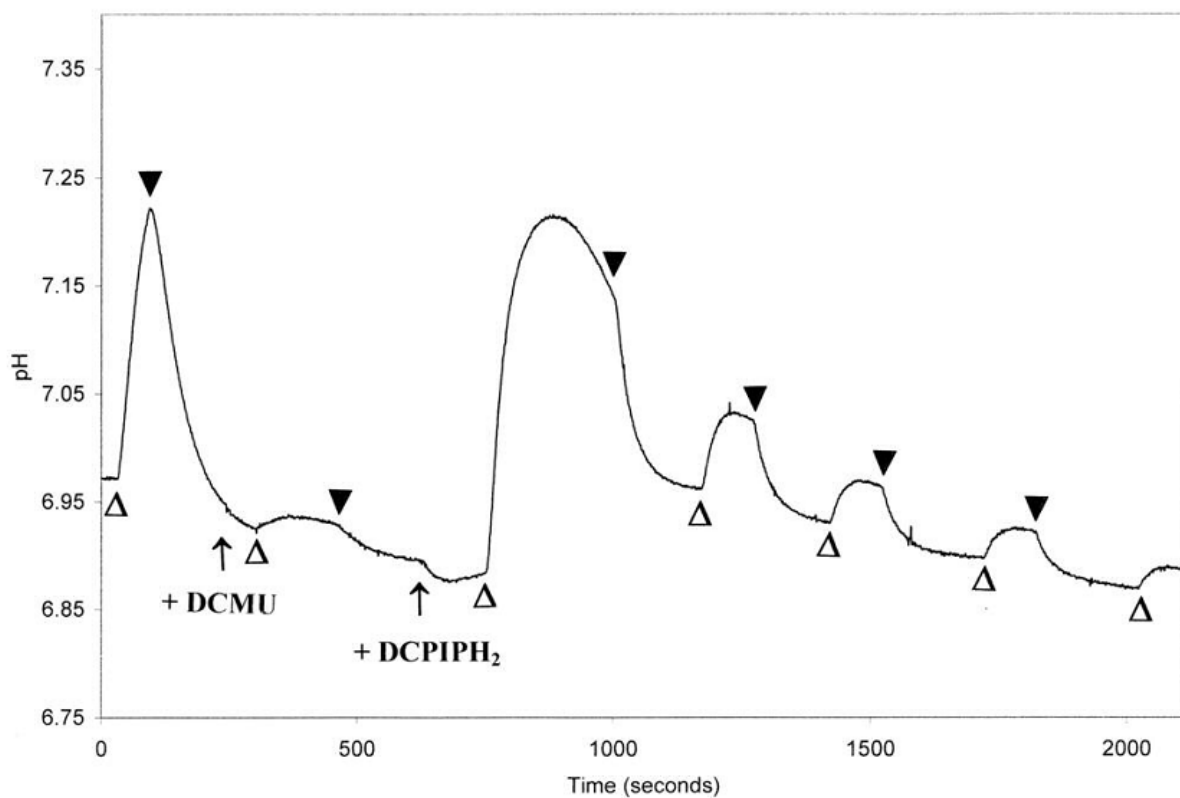


**Figure 6.** The change in pH in response to light on (open triangles) in the absence and presence of oxidised DCPIP. DCPIP enhances the  $\Delta$  pH in response to light but the change rapidly declines to control levels during subsequent exposure to light (closed triangles indicate light off)

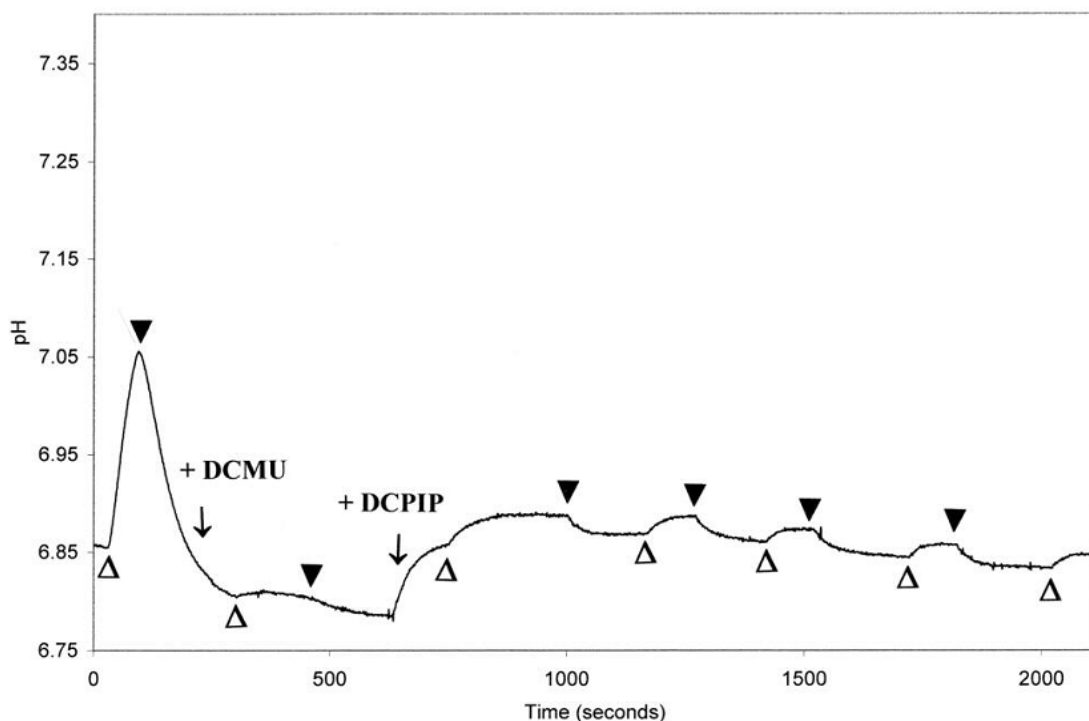




**Figure 7.** The change in pH in response to light on (open triangles) in the absence and presence of reduced DCPIP (DCPIPH<sub>2</sub>). DCPIPH<sub>2</sub> enhances the  $\Delta$  pH in response to light but the change rapidly declines to control levels during subsequent exposure to light (closed triangles indicate light off)



**Figure 8.** The change in pH in response to light (open triangles) in the absence and presence of DCMU and in the presence of reduced DCPIP (DCPIPH<sub>2</sub>). DCMU almost completely abolishes the increase in pH in response to light that occurs in its absence. When DCPIPH<sub>2</sub> is added it enhances the pH increase in response to light but the response on subsequent light exposure is much diminished (Closed triangles indicate light off)



**Figure 9.** The change in pH in response to light (open triangles) in the absence and presence of DCMU and in the presence of oxidized DCPIP (DCPIP). DCMU almost completely abolishes the increase in pH in response to light that occurs in its absence. When DCPIP is added, the response to light is also minimal and very little response is detected on subsequent light exposure (Closed triangles indicate light off)

Figure 7 shows data from an identical experiment to that shown in figure 6 except that reduced DCPIP (DCPIP added in the presence of ascorbic acid) was added at 630 seconds. Addition of reduced DCPIP in darkness causes a slight decline in pH that quickly stabilizes. When the light was turned on, again a large and rapid increase in the pH was observed. Since reduced DCPIP will not accept electrons from the plastoquinones, it must be donating electrons to PS I and initiating cyclic electron flow. This hypothesis is further supported by data shown in figures 8 and 9 from experiments which were identical to those shown in figures 7 and 6, respectively, except for the addition of DCMU to a concentration of 8  $\mu\text{M}$  at 250 seconds in each case. This concentration of DCMU greatly curtails the efflux of electrons from PS II as is demonstrated by the very small response to turning the light on at 300 seconds in both cases (figures 8 and 9). In the presence of reduced DCPIP, a large increase in the pH was seen when the light was turned on at 750 seconds but subsequent responses to turning on the light were much diminished. This is probably because the concentration of reduced DCPIP drops rapidly as it donates electrons to PS I and it is either not being re-reduced or is being re-reduced at a very low rate by accepting electrons from the DCMU-blocked PS II. The large pH increase starting at 750 seconds must be almost independent of proton pumping by the plastoquinone pool between PS II and cytochrome  $b_6f$  because the DCMU dramatically reduces the flow of electrons from PS II required to reduce the plastoquinones (the magnitude of the peak after DCMU addition is only 5% of that for the peak that preceded DCMU

addition (figure 8)). Finally, in the presence of DCMU and oxidized DCPIP (figure 9), few electrons from PS II are available to reduce the DCPIP so that, in response to light, it will donate few electrons to PS I and, consequently, little electron cycling around PS I will occur and, therefore, only a low rate of proton translocation.

Many reagents have been used to “dissect” the flow of electrons and protons in the thylakoid membrane. Some act as electron donors or acceptors to or from specific complexes in the membrane (e.g. DCPIP) while others more directly affect the capacity to generate a proton gradient (e.g. gramicidin D). I have tried other reagents and treatments (including ammonium ion, phenazine methosulfate, heat treatment plus the addition of diphenyl carbazide) without success. One problem arises from adding reagents that greatly affect the pH of the suspension solution. The solution is, at best, lightly buffered. Although a small amount of buffering capacity may be carried over from the isolation buffer, the suspension solution is still able to come to equilibrium with materials from the thylakoids in suspension. The thylakoid suspension solution, without added thylakoids, has a pH of  $\sim 4.6$ . When thylakoids are added, the pH of the suspension rises quite rapidly over 3 to 5 minutes and settles at  $\sim \text{pH } 7.0$ .

## 4. Conclusions

Although the pH probe and data acquisition software described above may sound expensive, they are not. Firstly, the system requirements for running the data acquisition

software are so minimal that it is possible to use computers that your institution would otherwise discard. Secondly, the Logger Pro software is inexpensive and comes with a site license that permits use on any number of PCs in your department. The modest expense is well justified in terms of student response to using it. It is, frankly, exciting to see the data appear on the screen as it accumulates in real time and to see, even before more detailed analysis, if your hypothesis-based predictions are supported.

I have used this experimental system in both my second year laboratory course and in outreach workshops for grade 12 students and their teachers. Written feedback from the latter groups include: "The (assay) was something new for me and it really captivated my interest", "It was the first time that I have ever enjoyed a biology-related exercise!" and "It most definitely was (a valuable educational experience)! I will never be able to do high school experiments again, they're much too drab now!"

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