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**THE STUDY OF PHOTOSYNTHETIC
ENERGY STORAGE IN AQUATIC PLANTS USING
PHOTOTHERMAL DEFLECTION SPECTROSCOPY**

by

Charles E. Hall, B.Sc (Hons.)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

Master of Science

Department of Biology

Carleton University

Ottawa, Ontario

August, 1995

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ENERGY STORAGE IN AQUATIC PLANTS USING
PHOTOTHERMAL DEFLECTION SPECTROSCOPY"**

submitted by Charles E. Hall, B.Sc.
in partial fulfillment of the requirements for
the degree of Master of Science

A handwritten signature in dark ink, appearing to read "John Sackin", is written over a horizontal line.

Thesis Supervisor

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ABSTRACT

A photothermal deflection spectrometer was constructed to measure the energy storage efficiency (ES) of photosynthetic tissues. This apparatus was used to study ES in four aquatic plants: *Echinodorus paniculatus* (Micheli), *Bacopa monnieri* (L.) Wettst., *Micranthemum umbrosum* (Walt.) Blake and *Vallisneria americana* Michx. *Vallisneria americana* leaves were chosen for more extensive studies to determine the factors influencing ES. The leaves of this plant were chosen because of their simple morphology and large ES.

ES was found to reflect activity of the electron transport chain as it could be effectively inhibited by the addition of atrazine. ES was also influenced by the openness of the reaction centres whose states were altered by varying the light intensity and CO₂ supply.

Chloroplast movement was found to cause a significant increase in ES after *V. americana* leaf sections were exposed to bright light for 40 minutes. These experiments suggest that chloroplast movement acts to optimize the utilization of light energy when exposed to bright light which is insufficient to cause photoinhibition.

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List of Abbreviations

APW- artificial pond water
Atrazine- (2-chloro-4-ethylamino-6-(isopropylamine)-s-triazine
CB- cytocholasin B
DIC- dissolved inorganic carbon
DCMU- (3-(3,4 dichlorophenyl)-1,1-dimethyl urea)
DMSO- dimethyl sulfoxide
ES- energy storage
He- helium
Ne- neon
MOPS- 3-[N-Morpholino]propanesulfonic acid
HEPES- (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
PD-Photothermal deflection
PDS-Photothermal deflection spectroscopy

CHAPTER ONE
GENERAL INTRODUCTION

Photothermal deflection spectroscopy (PDS) is a technique which measures the heat released by a sample after it has been illuminated with light (Jackson *et al.*, 1981). This method is also called the "mirage" effect due to the similarities between this method and the formation of mirages. A mirage occurs when land seen at a distance absorbs solar energy on a sunny day. The absorbed light energy heats the air above the land which causes light passing above the land to be refracted to the eyes of an observer. Creation of warm turbulent air above the ground causes a non-uniform refraction of the light and to the observer it appears that there is a body of water ahead. PDS is a technique which measures the angular deflection of a light beam passing through the heated medium.

After the absorption of light, the release of heat by a substance can give a great deal of information about the substance or the medium which is next to it. The release of heat can give information concerning the absorption coefficient of the substance and/or the quantity of the substance present. This information has been used to determine the absorption coefficient of weakly absorbing substances (Boccaro *et al.*, 1980). In addition this method has been adapted to measure the position of small quantities of materials after various kinds of chromatographic and electrophoretic separation (Morris and Fotiou, 1988).

This technique can also be used to study photoactive samples. These are samples which can use the absorbed light energy to drive a photochemical or photoelectrical process. Such studies have been used to characterize the transport properties of semiconductors (Fournier *et al.*, 1986; Frosio *et al.*, 1986) and more

recently have been applied to the study of photosynthesis. To date this technique has been used briefly to investigate the energy stored as photosynthetic products in leaves of pea and maple (Havaux, 1990; Havaux *et al.*, 1989; Havaux *et al.*, 1990).

This technique is useful in the study of photosynthesis as it is able to measure the light energy which is used in photosynthetic electron transport. This is because photosynthetically active samples will not release all of the absorbed light energy as heat. Some of this energy will be used to drive electron transport. In the chloroplast, light is absorbed by the antenna pigments where the excitation energy is transferred to the reaction centers of the two photosystems. The energy can then be used to reduce an electron acceptor (photochemistry) (Krause and Weis, 1991). The process of photochemistry competes with two other processes which act to deactivate the excited pigments: The pigments can return to their ground state by releasing the energy as heat or by emitting a photon of light (Crippa *et al.*, 1994).

The purpose of this work was to build and characterize an apparatus which is able to measure the heat released by a sample using the mirage effect. This apparatus was then used to determine the photosynthetic characteristics of four aquatic plants: *Bacopa monnieri*, *Vallisneria americana*, *Echinodorus paniculatus*, and *Micranthemum umbrosum*. One of these plants, *V. americana*, was selected for further study to create a model system which could be used for future studies of photosynthesis.

Figure 3 2 Typical PD amplitude trace of an ES measurement from a *I.*
americana leaf section in filtered APW. The modulated light was
added at T=0 min for 6 min. The background light was added after 4
min for a period of 1 min (as indicated by the arrow-up and arrow-
down). The time constant of the lock-in amplifier was 3 s.

Introduction:

Upon the absorption of a photon of light a non-photoactive sample will release all of the absorbed light energy as thermal energy. This thermal energy will diffuse into the absorbing substance as well as into a thin layer of the medium adjacent to the substance. The thermal gradient formed in the adjacent medium will create a corresponding gradient in the refractive index of the medium (Coufal and McClelland, 1988). This refractive index gradient can be probed by measuring the deflection of a beam of light as it passes through the temperature mountain (figure 2.1). This allows one to estimate the heat released by the absorbing substance by measuring the deflection of the probe beam adjacent to it.

The generation and diffusion of thermal energy gradients through a medium and the probing of these gradients with a beam of light has been investigated by Jackson *et al.* (1981) from both a theoretical and experimental standpoint.

The angular deflection (ϕ) of a probe beam by a refractive index gradient formed above an illuminated sample can be described in its simplest terms as:

$$\phi = (l/n) (\partial n/\partial T) (\partial T/\partial x) \quad (1)$$

Where l is the interaction length between the illuminated sample and the probe laser beam, n is the refractive index of the medium adjacent to the sample, T is the temperature, x is the perpendicular distance between the probe beam and the sample and $\partial T/\partial x$ is the temperature gradient (Boccara *et al.*, 1980).

Figure 2.1 Schematic diagram showing the deflection of the probe laser beam by a temperature mountain formed by the illumination of a sample with an excitation beam. This figure shows both the normal and transverse deflection of the laser beam (from Murphy *et. al.*, 1992)

OPTICAL BEAM DEFLECTION

The amplitude (A) of the thermal wave produced by illumination with a modulated light source is proportional to:

$$A \propto \exp (-x/\mu_{TH}) \quad (2)$$

where μ_{TH} is the thermal diffusion length of the medium and is defined as:

$$\mu_{TH} = (D_{TH}/\pi f)^{0.5} \quad (3)$$

by substitution of (3) into (2):

$$A \propto \exp -x[\pi f/D_{TH}]^{0.5} \quad (4)$$

where D_{TH} is the thermal diffusivity of the medium and f is the modulation frequency (Malkin and Canaani, 1994). From this it is predicted that the log of the amplitude of the thermal wave is inversely proportional to the square root of the modulation frequency and to the perpendicular distance of the probe beam from the sample (x).

The purpose of these experiments was to construct an apparatus which is capable of measuring the modulated thermal gradients formed above the surface of an illuminated sample. The apparatus was calibrated using a photoinactive sample (black polyethylene).

Materials and Methods

Figure 2 2 Schematic diagram showing the components of the photothermal deflection spectrometer (A), the sample cell (B), and the position sensor (C). Abbreviations are as described in the text.

Side view of sample cell

The photothermal deflection spectrometer is shown in figure 2.2a. The sample was clamped to a cylindrically shaped perspex holder which was then placed into a perspex cell (figure 2.2b) which had a volume of approximately 20 mL. The cell was then filled with medium filtered through a .45 μm pore filter (Nucleopore). The cell contents were isolated from the atmosphere with a tight fitting clear perspex lid. This cell was constructed to allow the sample to be illuminated from above as well as allowing the probe laser beam to pass over the sample.

The sample was illuminated from above through a trifurcated light guide by 2 light emitting diodes (HLMP-3750, Hewlett-Packard). The light emitting diodes had a peak emission at 635 nm and were modulated at 16 Hz unless otherwise stated. The thermal gradients formed above the sample were probed by a beam of monochromatic light (632.8 nm) emitted from a 2 mW He-Ne laser (Uniphase, model 1103). The laser output was first attenuated by a 665 nm long-wave pass filter (3% transmission @ 632.8 nm) (F_1) and focused above the sample with a 30 mm focal length lens (L_1). The angular displacement of the laser beam was detected with a position sensor (Optikon SD 380-23-21-051) which was shielded from scattered light with a neutral density filter combination (F_2) (3.2 % transmittance at 632.8 nm) and a 632.8 interference filter (F_3). The position sensor consisted of two identical semi-circular photodiodes (A and B) separated by a 0.1mm gap (fig 2.2c). The position sensor was adjusted such that the laser beam fell equally on the 2 halves of the detector (ie $A-B=0$). As the laser position changed the illumination of the two halves of the position sensor varied. The differential signal ($A-B$) was

amplified and then monitored by a lock-in amplifier (Princeton Applied Research, model HR-8) tuned to the modulation frequency of the LEDs. The lock-in amplifier signal was digitized by a 12-bit A/D board (Data Translation, DT2801) and recorded by a 386SX/25 PC computer (Science Technology Centre, Carleton University.) using Asyst (V2.01) software (Macmillan Software Co., New York, NY). The light from a 500 W quartz-tungsten-halogen lamp (Sylvania, 500Q/CL) was filtered by 6.5 cm of water, an infra red reflecting mirror (F4) and neutral density filters (F5) and was being focused (by lenses L_2 and L_3) onto the third arm of the light guide. This non-modulated beam was used to saturate photochemistry when required. All light intensities were measured using a Li-Cor 170 quantum meter (Lambda instruments). The entire apparatus was mounted on a 160 kg steel plate which was supported by four air filled shock absorbers to reduce the influence of external vibrations on the apparatus.

Results:

In the absence of a photothermal gradient, the differential output ($A-B$) of the detector was found to vary linearly with the displacement of the laser beam on the detector. Figure 2.3 shows that as the position of the detector is altered vertically with respect to the position of the laser beam there is a change in the value of ($A-B$) of $80 \text{ mV}/\mu\text{m}$.

A photochemically inert sample was used to determine the effect of the probe

Figure 2 3 The effect of the linear displacement of the probe beam on the differential signal output (A-B) of the detector. The laser position was held constant while the detector was moved vertically with the micromanipulator. The differential signal from the detector was found to vary linearly with the displacement of the laser spot on the detector.

Figure 2.4 Phase (A) and amplitude (B) of the photothermal signal as the distance between the sample and the probe beam is varied. The photothermal signal was created by illuminating a black plastic sample with modulated light (16 Hz) which had an intensity of $110 \mu\text{E m}^{-2} \text{s}^{-1}$. The photothermal signal was measured with the sample submerged in filtered distilled water.

A

B

beam-sample offset on characteristics of the photothermal deflection (PD) signal. Figure 2.4 shows the results from a black polyethylene sample in distilled water which was illuminated with a pump beam having an intensity of $105 \mu\text{E m}^{-2} \text{ s}^{-1}$. These results show that there is a 10 fold reduction in signal amplitude and a 134° increase in the phase lag for each $110 \mu\text{m}$ increase in the distance between the probe beam and the sample.

Discussion:

The sensitivity of the apparatus will ultimately be determined by the sensitivity of the detector to small displacements of the probe beam. The results in figure 2.3 show that there is a change in A-B of 80 mV for each $1 \mu\text{m}$ in offset between the detector and the laser beam. The smallest measurable PD signal which is detectable with this apparatus is approximately $100 \mu\text{V}$ with a modulation frequency of 250 Hz. This corresponds to a change of A-B of $282 \mu\text{V}$ in each cycle. The sample-detector distance is 20 cm therefore the laser is deflected by approximately 1.76×10^{-8} radians in the measurement of this signal.

Detection of these small angular deflections requires that the apparatus be stable and isolated from the environment. It is for this reason that a.) the apparatus is enclosed and b.) it is supported on top of a massive steel plate. The medium bathing the sample is also filtered to remove suspended particles. These precautions produce a very significant reduction in the noise of the system. Without these

precautions useful measurements could not be made.

PD measurements were made in water as the value of $(1/n) (\partial n / \partial T)$ is much larger for water (10^{-4} K^{-1}) than it is for air (10^{-6} K^{-1}) (Lorrain *et al.*, 1990). This is a simple modification which allows measurements to be made with much larger signal to noise ratios.

A linear relationship between the log of the amplitude with the distance between the probe beam and the sample has been predicted and proven experimentally by Jackson *et al.* (1981). In the experiment here, the amplitude of the PD signal decreased logarithmically as the distance between the sample and the probe beam increased. The decrease in the PD amplitude reflected the rapid attenuation of the thermal wave as it propagated away from its source.

This result demonstrates the importance in minimizing the distance between the sample and the probe beam. Careful alignment of the sample with the probe beam will result in an increase in the signal amplitude and hence an increase in the signal to noise ratio of the measurement.

The quality of the sample will be important in determining the minimum obtainable distance between the sample and the probe beam. Irregular samples which have uneven surfaces may increase the minimum distance at which the laser can be brought close to the bulk of the illuminated sample. It is therefore important that the sample be flat and that it be flexible. This allows the sample to be attached securely to the holder without forming wrinkles.

A second method to minimize the sample-probe beam distance is produced

by focusing the probe beam to a tight waist just above the sample. This is important as the angular deflection of the laser will be determined by the mean distance that the probe beam is away from the source of the photothermal signal. Focusing of the laser beam is also important as it subjects the different rays of the beam to approximately the same thermal gradients and thus they experience the same deflection (Lasalle *et al.*, 1988).

The phase angle of the photothermal signal was found to be linearly related to the probe beam-sample offset. The phase angle is a measure of the time lapsed between a reference signal which is used to drive the modulation of the pump beam and the detection of the photothermal signal. An increase in the phase angle reflects a decrease in the phase lag (the time period between the reference signal and the detection of the photothermal signal).

The phase lag between the illumination of a sample and the detection of the thermal gradient that is coming from that sample will be determined by 3 factors. Firstly, electronic sources of the phase lag are produced by the time taken for the modulated signals to travel through the circuitry of the apparatus. This includes the time taken for the signal to travel from the function generator to the LEDs and also from the detector to the lock-in amplifier. The phase lag due to the electronic sources are minor components at low frequencies and are constant at a given frequency. A second source contributing to the phase lag is the time taken for the diffusion of the thermal gradient from the source of the thermal emission to the probe laser beam. The velocity at which this thermal wave travels is constant for a

given medium which means that the time of diffusion will be dependent solely on the distance between the source and the probe laser beam. This effect is seen experimentally in figure 2.4a. A third source of phase lag occurs in photoactive samples. In these samples there may be a delay in the release of heat after light absorption. This is the result of the formation of meta-stable intermediates which subsequently decay yielding heat (Crippa *et al.*, 1994).

CHAPTER THREE
THE MEASUREMENT
OF ENERGY STORAGE IN
AQUATIC PLANTS

Introduction:

When a photosynthetic tissue absorbs light energy there are many fates of the absorbed energy (figure 3.1). The energy may be emitted immediately as heat (prompt heat release); it may form meta-stable intermediates which decay to release heat that is delayed from the initial photoact; it can be released as a photon of light (fluorescence); or the absorbed light energy can be stored in the form of chemical energy as a stable product (Crippa *et al.*, 1994).

The PDS method of heat detection becomes useful in the study of photosynthetic tissue as it allows one to estimate the amount of energy that is stored in the photochemical products of the electron transport chain. PDS can be used to study photosynthesis by comparing the modulated heat release from a photosynthetically active sample to that of a non-active sample with the same thermal properties and absorption coefficient. The latter sample can be simply obtained by illuminating the former with a photochemically saturating non-modulated background light (Malkin *et al.*, 1981). When the sample is illuminated with a background light concurrently with a weak modulated light the amount of modulated light which is used to drive electron transport approaches zero and it is now emitted almost solely as heat (Havaux *et al.*, 1989). The increase in the heat released will be equal to the absorbed modulated light energy which was previously stored as photochemical intermediates (Malkin *et al.*, 1981). This increment in heat release is often termed energy storage (ES) and is described by the following

Figure 3 1 Block diagram showing the main processes in the generation of heat following the absorption of light by a photoactive sample. Heat release can be prompt or can be delayed via the formation of metastable intermediates (adapted from Crippa *et al.*, 1994).

equation:

$$ES = [(Q_m - Q_s) / Q_m] \times 100\% \quad (5)$$

where Q_s is the photothermal amplitude when the sample is illuminated with the modulated light and Q_m is the amplitude when the non-modulated background light is added.

The purpose of this experiment is to describe and test a method in which both Q_m and Q_s can be measured for a photosynthetic sample.

Materials and Methods:

Measurements of energy storage were made with leaf sections in artificial pond water (APW). The APW contained 0.5 mM $MgSO_4$, 0.05 mM NH_4Cl , 1.0 mM $CaCl_2$, 0.1 mM KH_2PO_4 , and 0.3 mM NaH_2PO_4 , 5 mM $NaHCO_3$, and was buffered with 10 mM MOPS (pH 7.0).

Energy storage was measured on leaf sections of the aquatic plant *Vallisneria americana* using the standard method described below. Leaf sections were clamped into the sample holder and immersed in filtered (0.45 μm pore) APW. The sample was adjusted such that the laser passed as close as possible to the surface of the sample. The modulated light was added ($73 \mu E m^{-2} s^{-1}$) and the phase angle was adjusted such that the 90° out of phase (quadrature) amplitude was zero. The phase

angle was then rotated 90° to be in phase for the thermal signal during measurements. The sample was then incubated in the dark for 5 min. The modulated light was then added for 6 min during which the background light ($425 \mu\text{E m}^{-2} \text{s}^{-1}$) was added for 1 min after the fourth min of modulated illumination. The level of Q_s was taken as the average of the amplitude 30 s before the application of the background light. The value of Q_m was taken as the average of the amplitude of the PD signal for the last 30 s of the background light application.

Results:

The use of a strong non-modulated background light as a referencing method is shown in figure 3.2. This figure shows an actual trace of the photothermal amplitude when ES was measured for a leaf section of the aquatic plant *Vallisneria americana*.

Upon the addition of the modulated light ($t=0$ min) the amplitude of the PD signal rose to an initial level and transiently decreased to the steady state level Q_s after approximately 3 min. Application of the background light caused the PD amplitude to rise quickly to the value of Q_m . After the background light was removed the PD amplitude returned to the value of Q_s .

For this leaf section Q_s and Q_m were equal to 2.93 mV and 3.64 mV respectively. ES was calculated to be $[(3.64 - 2.93) / 3.64] * 100 \% = 19.5\%$

When the background light was added in the absence of the modulated light the

Figure 3.2 Typical PD amplitude trace of an ES measurement from a *N. americana* leaf section in filtered APW. The modulated light was added at T=0 min for 6 min. The background light was added after 4 min for a period of 1 min (as indicated by the arrow-up and arrow-down). The time constant of the lock-in amplifier was 3 s

amplitude of the photothermal signal did not deviate from zero (results not shown).

Discussion:

The value of Q_s is a measure of the amount of absorbed modulated light energy which is released promptly after the absorption of light (Malkin *et al.*, 1992). The value of Q_m is determined by the amount of modulated light absorbed by the sample since all of the absorbed light energy will be released as heat. Saturation of photochemistry with the background light causes the maximal conversion of absorbed modulated light energy into heat and fluorescence (Havaux *et al.*, 1990). This gives rise to the maximal photothermal signal (Q_m). By subtraction ES is a measure of the fraction of the absorbed modulated light energy which has not been released within a short time period after the absorption of the modulated light. This energy is referred to as stored energy.

The use of ES as a referencing method is useful because the photosynthetic sample itself becomes its own reference. This means that the reference sample has identical absorption and thermal properties as the photoactive sample. In addition, the effect of the background light is reversible and allows repeated ES measurements on the same sample.

This method is also useful as it removes the variability between samples caused by their positioning with respect to the probe beam. This is because ES is not dependent on the absolute PD amplitude but rather on the ratio of two amplitudes measured for a given sample. This removes any variability from sample to sample with respect to sample positioning and allows them to be compared with one another strictly on the basis of their use of the modulated light.

The PD signal shows an initial high level after the application of the modulated light and then transiently lowers to a steady state level Q_s . This result shows that there is initially a lower proportion of the absorbed modulated light energy being stored as stable photochemical products. The following decrease in the PD signal indicates that there is an increase in the energy being stored. This increase in ES is due to the light activation of photosynthesis. The resulting increase in photochemical activity competes with the thermal dissipation process and hence leads to a lowered thermal emission (Havaux, 1990). This initial low level of ES may reflect the initial closure of PSII centers which occurs after dark adaptation (Buschmann and Kocsányi, 1989).

As stated earlier, absorbed light energy can also be released as a fluorescence photon. The PDS technique is unable to measure the absorbed modulated light energy that is released as fluorescence and in doing so leaves it unaccounted for. This unaccounted energy will not significantly alter the value of ES as the proportion of light energy which is re-emitted as fluorescence is a relatively small component of the total energy budget and therefore it can be neglected without

consequence (Havaux *et al.*, 1989).

CHAPTER FOUR
SELECTION OF A MODEL PLANT
FOR FURTHER STUDIES WITH PDS

Introduction:

The use of PDS in the study of photosynthesis has been very limited to date. This technique has been used to study ES in leaf sections of pea, sugar maple, and durum wheat (Havaux, 1990; Havaux *et al.*, 1989; Havaux *et al.*, 1990). Absorption spectra have been measured of photosynthetic pigments *in vivo* of algae, corn chloroplasts and leaf sections of pea and sugar maple as well as of thin films of chlorophyll (Lorrain *et al.*, 1990). Measurements of photothermal signals were made of whole leaves, both in air and in distilled water. The authors have found that much higher signal to noise ratios were obtained when the samples were tested in distilled water (Lorrain *et al.*, 1990). All other results reported with this technique are reported with samples which were tested in distilled water (Havaux, 1990; Havaux *et al.*, 1989; Havaux *et al.*, 1990).

The measurement of ES in distilled water using leaves taken from plants which have been grown terrestrially is certainly artificial. This is due to the fact that the movement of CO₂ in an aqueous environment is quite different than in air. Many of the morphological adaptations which prevent the loss of water in air may be detrimental to the uptake of CO₂ in aqueous solution. Epidermal cell layers and waxy cuticles will certainly act as diffusional barriers to CO₂ entry into the photosynthetically active cells of the leaf.

The purpose of these experiments was to find a plant whose leaves were more suited for study under these conditions. The search was limited to aquatic plants as this allows water to be used as the physiological medium. Since these

plants were grown in an aquatic environment they are likely to have adaptations which will make them better suited to perform photosynthesis under these conditions. The leaves of four aquatic plants were examined by PDS. The leaves of these plants were also examined microscopically to determine their morphology.

Materials and Methods:

Echinodorus paniculatus (Micheli), *Bacopa monnieri* (L.) Wettst., *Micranthemum umbrosum* (Walt.) Blake and *Vallisneria americana* Michx. were planted in a layer of soil covered with crushed coral and grown in a constantly aerated solution containing 0.5 mM MgSO_4 , 0.05 mM NH_4Cl , 1.0 mM CaCl_2 , 0.1 mM KH_2PO_4 , and 0.3 mM NaH_2PC_4 , which had a pH of 7.0 when measured under growth conditions. The plants were illuminated for 16 h per day under incandescent and fluorescent lamps at a fluence of $55 \mu\text{E m}^{-2} \text{s}^{-1}$. The growth temperature was constant at 23°C .

The increase in the PD amplitude was determined for leaves from each of the four plants upon the addition of a range of background light intensities. These measurements were made in APW with a modulated light intensity of $73 \mu\text{E m}^{-2} \text{s}^{-1}$. From these measurements the values of ES, Q_m , and the background light intensity necessary to reach Q_m were determined for each of the four plants.

Hand cross-sections of each of the four plants were made and stained with

Toluidine blue in benzoate buffer (pH 4.4). These sections were photographed with an Olympus BH photomicroscope under brightfield optics and Kodak T-MAX 100 film.

Results:

The increase in the photothermal signal upon the application of the background light (P) was measured with different background light intensities for three sections of each of the aquatic plants. P was calculated as $[(Q-Q_s)/Q] \times 100\%$ where Q is the PD amplitude when the background light was applied and Q_s is as defined earlier. P is equal to ES when $Q = Q_m$. P was found to show no further increase above a background light intensity of approximately $425 \mu\text{E m}^{-2} \text{s}^{-1}$ for all of the plants tested (fig 4.1). When the background light was $425 \mu\text{E m}^{-2} \text{s}^{-1}$ the mean ES ($n=3$) for each of these plants was: 21.6% for *V. americana*, 17.9% for *E. paniculatus*, 11.1% for *B. monnieri*, and 7.4% for *M. umbrosum* at the modulated light intensity used.

The maximum photothermal amplitude (Q_m) and ES were also measured for each of these plants at this modulated light intensity. Table (4.1) shows Q_m and ES for leaves of each of the plants. *B. monnieri* and *M. umbrosum* both had significantly lower values of Q_m and ES than the other two plants. *V. americana* and *E. paniculatus* gave similar large values of Q_m while *V. americana* had the largest value of ES at the modulated light intensity used.

Figure 4.1 Increase in the photothermal signal (P) as a function of the background light intensity for the adaxial surfaces of *V. americana*, *E. paniculatus*, *M. umbrosum*, and *B. monnieri* leaves. These measurements are the mean and range of three leaf sections measured in APW

Table 4.1 Qm and ES characteristics of leaves from the 4 aquatic plants The values represent the individual results of 3 trials while those in brackets represent the mean. These values were obtained when measuring ES under the standard conditions.

Leaf Source	Qm (mV)	ES (%)
<i>B. monnieri</i>	3.40, 3.78, 3.76: (3.65)	10.2, 10.0, 13.0: (11.1)
<i>M. umbrosum</i>	3.72, 3.27, 2.88: (3.29)	4.6, 6.4, 11.2: (7.4)
<i>E. paniculatus</i>	5.24, 5.66, 6.42: (5.77)	16.0, 18.5, 19.3: (17.9)
<i>V. americana</i>	4.97, 6.20, 4.30: (5.17)	23.0, 21.8, 20.0: (21.6)

Figures 4.2 a-d show hand cross sections of each of the four aquatic plants stained with Toluidine blue (pH 4.4). *B. monnieri* and *M. umbrosum* both have clearly distinguishable palisade (P) and spongy (S) mesophyll cells and upper (UE) and lower (LE) epidermises. *E. paniculatus* is a thin leaf with an upper and lower epidermis with 2 rows of mesophyll cells between them. *V. americana* has an upper and lower epidermis separated by large gas spaces. The epidermises are connected together by cells forming "cross-walls" and by the vascular tissue.

Discussion:

The increase in the PD signal (P) was a function of the background light intensity and showed that $425 \mu\text{E m}^{-2} \text{s}^{-1}$ was saturating for each of the four plants tested. An increase in the background light intensity will progressively cause the closure of the two photosystems causing a larger portion of the modulated light to be released as heat (Havaux, 1990). These experiments were done to ensure that the background light intensity was high enough to saturate photochemistry in each of the different leaves. This allowed the accurate estimation of ES and Qm for each leaf.

There are two main criteria which determine a leaf's usefulness with this technique. In order for a plant to be well suited for this technique it should give rise to large values of Qm and ES at a given modulated light intensity. These characteristics are beneficial as they will give larger signal to noise ratios and allow measurements of ES over a larger range of modulated light intensities.

Figure 4 2 Hand cross-section of a.) *B. monnieri*, b.) *V. americana*, c.) *M. umbrosum*, and d) *E. paniculatus* , stained with Toluidine blue (pH4.4). bar = 100 μ m. Each of these leaves has an upper (UE) and lower (LE) epidermis. Palisade (P) and spongy (S) mesophyll cells are also present in some of these leaves. *V. americana* had the only leaves in which lacunae (L) were observed.

D

C

The value of Q_m for each leaf is primarily determined by the proportion of the modulated light source which is being absorbed by the sample in a region which is detectable by the probe beam (Coufal and McClelland, 1988). Other factors influencing Q_m will be the proximity of the probe beam to the source of the photothermal signal and the attenuation of the thermal gradient by regions of the plant between the source and the probe beam.

ES and Q_m was found to be quite low for *M. umbrosum* and *B. monnieri* (Table 4.1). This may be the result of the more complex morphology of the leaves of these plants. Photographs of leaf cross-sections in figures 4.2 a & c show that *B. monnieri* and *M. umbrosum* have leaf morphologies which are similar to those found in a typical C_3 terrestrial plant such as spinach. These two plants have three main cell types, only two of which may be photosynthetically active. There are two epidermal layers, a palisade mesophyll layer and a spongy mesophyll layer. This type of morphology is commonly seen in plants grown in a terrestrial environment (Esau, 1965) and may indicate that these plants are secondary hydrophytes (plants which primarily grow on land but are able to grow under water).

Terrestrial plants with this type of morphology generally don't have chloroplasts in their epidermal cells (Esau, 1965). As such, the epidermal cells are photosynthetically inactive. This may also be true for these aquatic plants. If so this would mean that the origin of the PD signal lies in the chloroplasts of the palisade mesophyll cells below the epidermis. This would lead to a lower Q_m (due to an increased attenuation of the thermal wave) and possibly to a lower ES due to

an increased diffusional limitation of CO_2 entry into the palisade cells.

The values of Q_m and ES for *E. paniculatus* and *V. americana* are much higher than for the other 2 plants. Their simple cell structure indicate that these plants have adapted more to the aquatic environment. The source of the PD signal is probably the epidermal cells as there are often chloroplasts present in these cells in aquatic plants (Esau, 1965). The presence of chloroplasts in the epidermis would give rise to larger PD signals because of their close proximity to the probe beam. ES may also be expected to be higher as there is less diffusional resistance to CO_2 entry into these cells.

An important observation from these photographs is the absence of leaf hairs. These structures which can be found in terrestrial plants were not present in these plants. This is important as leaf hairs can cause interference with the probe beam leading to increased noise in PD measurements.

Vallisneria americana has been chosen as the plant most suited for further study by this technique. This was due to the high value of Q_m and ES found in sections of their leaves. These features allow the measurement of ES over a wide range of modulated light intensities. *Vallisneria americana* also has a comparatively simple leaf structure which makes interpretation of the PD signal easier.

CHAPTER FIVE
CHARACTERIZATION OF
ES IN *V. americana*

Introduction:

The results in the last chapter indicated that the leaves of *V. americana* were well suited for study using the PDS technique. These leaves were chosen as they gave large values of Qm and ES in addition to having a simple dispersed morphology. Further experiments were done to determine what factors influenced ES in *V. americana* leaves. These factors included the position on the leaf at which it is measured, the pH and the dissolved inorganic carbon concentration of the bathing medium, and the modulated light intensity.

The morphology and ES of *V. americana* leaves were studied to determine whether there was any variation in either parameter with respect to the position on the leaf where they were measured. This will give important information on the homogeneity of these leaves.

To further characterize the photosynthetic activity of *V. americana* leaves, ES was measured as a function of the modulated light intensity. The measurement of ES as a function of the modulated light intensity will allow the estimation of the maximal value of ES (ESm). This method was used to determine ESm in pea leaves also using the PDS technique (Havaux *et al.*, 1989; Havaux *et al.*, 1990).

The effect of the dissolved inorganic carbon (DIC) concentration on ES is important as it will reflect the mechanism by which it enters the leaf. This is important in the aquatic environment because the behaviour of CO₂ in solution is significantly different than in the gaseous environment in which most studies of whole leaf photosynthesis have taken place. The diffusion of CO₂ is 10,000 times

slower in water than it is in air (Sultemeyer *et al.*, 1993). In addition, CO_2 can exist in solution in several different forms. Dissolved CO_2 can become hydrated and ionized (Helder, 1988). These factors will influence the rate at which carbon can be supplied to the leaf in solution.

In an effort to determine the strategy that *V. americana* uses in the acquisition of dissolved inorganic carbon, ES was measured in media which contained different levels of CO_2 and HCO_3^- . The levels of these carbon species were altered by changing the pH.

The objective of these experiments were to: a.) compare ES and leaf morphology along the length of the leaf and b.) to characterize the photosynthetic properties of *V. americana* with respect to light use and carbon uptake.

Materials and Methods:

ES, Qm, and the phase angle were measured under standard conditions at different distances from the tip of the leaf. This was done to determine if there was any significant influence of leaf position on ES and the PD signal. *V. americana* hand cross-sections were also examined at various distances from the tip of the leaf to determine its morphology.

ES was measured at different pump beam intensities for single leaf sections of the abaxial and adaxial surfaces of a *V. americana*. This was used to determine the maximum value of ES (ESm) and the modulated light intensity necessary to

reach the half maximal value (i50). These measurements were made in APW with a background light intensity of $425 \mu\text{E m}^{-2} \text{s}^{-1}$.

To determine the inorganic carbon species taken up by this plant, ES was measured for the adaxial surface of leaf sections when the proportion of dissolved inorganic carbon species were varied. The basic experiment was modified where indicated by changing the pH of the medium to either pH 4.0 (10 mM citrate-phosphate buffer) or pH 8.0 (10 mM HEPES) and also 1.0 mM NaHCO_3 was added instead of 5 mM.

Results:

Vallisneria americana leaves are ribbon like and are 12-15 cm long and 1-1.5 cm wide. Examination of cross-sections of the leaf at different positions have shown that the thickness, number of rows of airspaces and the total airspace width all increase as one moves from the tip to the base of the leaf (Table 5.1). At each position within the leaf, the same basic morphology exists. In all cases there are two single rows of epidermal cells connected by mesophyll cells which create 1 or more gas spaces (lacunae).

Energy storage and the PD signal parameters were measured for *V. americana* at different locations within the leaf to determine if the leaf had uniform characteristics in these regards. Table 5.2 shows the average Qm, phase angle and ES for leaf sections at each position. The values are the means and standard deviations of 6 leaf sections. Leaf position had no significant influence on any of the parameters

Table 5.1 Morphological information describing the characteristics of a *V. americana* leaf. This information was taken from photographs taken from hand cross sections of the leaf at different distances away from the tip of the leaf. The total length of the leaf was 15 cm.

Table 5.2 ES and photothermal signal characteristics as a function of leaf position. Each value represents the mean and standard deviation of 6 leaf sections at each position. ES was measured under the standard conditions.

Table 5.3 Average extrapolated values of i_{50} and ESm for the adaxial and abaxial surfaces of *V. americana*. Values represent the mean and standard deviation of 6 leaf sections for each the adaxial and abaxial surfaces. The adaxial and abaxial values for each parameter are significantly different at the 95% confidence level (Student t-test)

Table 5.1

Distance from tip (cm)	Leaf thickness (μm)	Number of rows of gas spaces	Total lacunar width (μm)
1.5	434	1	322
4.5	700	1	588
7.5	1148	1-2	980
10.5	1470	2	1120

Table 5.2

Distance from leaf tip (cm)	Q_m (mV)	ES (%)	Phase ($^\circ$) angle
3	4.40 ± 0.93	22.0 ± 2.0	256 ± 8
6	3.97 ± 1.50	20.9 ± 2.7	259 ± 12
9	3.51 ± 0.94	21.7 ± 4.4	265 ± 7

Table 5.3

Surface	i_{50} ($\mu\text{E m}^{-2}\text{s}^{-1}$)	ES_m (%)
Adaxial	$132. \pm 31$	$34. \pm 3.$
Abaxial	$53. \pm 13$	$40. \pm 4.$

measured when examined using a 1-way analysis of variance (ANOVA).

ES was measured in the abaxial and the adaxial surface of *V. americana* leaf sections. It was found that the adaxial surface had ES values which were 1.8 ± 0.3 (mean $\pm$ s.d., $n=6$) times larger than those of the abaxial surface when measured for the same leaf section. P was also measured as a function of the background light intensity for the abaxial surface. The background light was also found to saturate P in the abaxial surface at approximately $425 \mu\text{E m}^{-2} \text{s}^{-1}$ as shown in figure 5.1.

The influence of the modulated light intensity on ES was measured for the adaxial and abaxial surfaces of the leaf. ES showed a hyperbolic relationship to the modulated light intensity for both the adaxial and abaxial surfaces. Figure 5.2 shows a semi-reciprocal plot of $1/\text{ES}$ versus modulated light intensity for the abaxial and adaxial surfaces of two different leaf sections. The best fitting straight line was extrapolated to the x-axis for each set of results. The intercept of each of these curves with the y-axis allows the estimation of ES_m , the maximal energy storage efficiency which occurs at zero modulated light intensity. The intercept (i_{50}) of these curves with the x-axis gives the light intensity which will yield the half maximal value of ES. Table 5.3 shows the average extrapolated value of i_{50} and ES_m for the abaxial and adaxial surfaces of *V. americana*. i_{50} was significantly larger (132 vs. $53 \mu\text{E m}^{-2} \text{s}^{-1}$) while the ES_m was significantly lower (34 vs. 40%) for the abaxial surface as compared to the adaxial surface (Student t-test, 95% confidence level).

ES was measured in *V. americana* leaves with the pH of the medium being changed to 4.0, 7.0 or 8.0 in the presence of 1.0 mM NaHCO_3 . A 2-way analysis of

Figure 5.1 Increase in the photothermal amplitude (P) as a function of the background light intensity for the abaxial and adaxial surfaces of *I. americana*. The mean and range are shown for 3 samples of each surface.

Figure 5.2 Semi-reciprocal plot of 1/energy storage at different pump beam intensities for the abaxial and adaxial surfaces of *V. americana* leaf sections. Each curve represents the values for a single leaf section. Measurements were made with the leaves bathed in APW. Solid lines indicate the best fitting straight line through the data set.

variance shows that ES is dependent on pH and leaf position and there is no significant interaction term. Table 5.4 shows that ES increases at all leaf positions as the pH of the medium is decreased from pH 8.0 to pH 4.0. Also the value of ES increased significantly at pH 8.0 when measured further away from the tip of the leaf. At pH 4.0 and 7.0 there was no significant effect of leaf position on ES at the 95% confidence level (Paired Students t-test).

Figure 5.3 shows traces of ES measurements of *V. americana* when the pH of the medium was altered in the presence of 1 mM NaHCO₃. ES at pH 4.0 was unaffected by the position on the leaf that it was measured. The transient was faster and Qs was reached faster at 6 cm than at 2cm from the tip of the leaf. Measurements of ES at pH 8.0 showed a clear effect of leaf position on ES. Sections at 2 cm from the tip gave lower values of ES than did sections at 6 cm from the tip. The transient was also quite small when measured at pH 8.0 compared to pH 4.0.

Discussion:

When ES was measured at different positions within a *V. americana* leaf in APW it was found that there was no significant influence of leaf position on Qm, ES and the phase lag (table 5.2). This is despite a great variation in the thickness of the leaf along its length (table 5.1). These results are important as they give an indication that only the adaxial epidermal cells are contributing to the PD signal.

This probably reflects the differential ability of these two carbon species to enter the epidermal cells. Prins *et al.*, (1980) describe an active HCO_3^- transport mechanism in *Vallisneria spiralis*. These results presented here show that both CO_2 and HCO_3^- are absorbed by the leaf but that CO_2 enters with less resistance than does HCO_3^- .

ES in leaves tested in a medium which contains approximately 1mM of dissolved CO_2 appears to be independent of the position on the leaf that is measured (i.e. pH 4.0 + 1 mM NaHCO_3 or pH 7.0 5 mM NaHCO_3). Only when CO_2 levels are low (created by increasing the pH and/or decreasing the total DIC) does there seem to be any influence of leaf position on ES. This may indicate a differential ability of the different parts of the leaf to use HCO_3^- .

The transients in the PD amplitude also appear to reflect the differential ability of the 2 carbon species to enter the epidermal cells. Leaf sections supplied with CO_2 (i.e. pH 4.0) show a transient decrease in the PD amplitude to the level of Q_s . Leaf sections supplied with HCO_3^- (pH 8.0) show relative higher values of Q_s and no transient. This indicates that the rate of carbon entry into the cells at high pH is limiting the use of the absorbed light energy. These results show that the efficiency of light energy use is regulated by the supply of carbon to the chloroplast. This is similar to the results of Weis and Berry (1987) who found that the rate of PSII photochemistry (and hence the rate of linear electron transport) was regulated by the rate of CO_2 fixation. Krall and Edwards (1992) have also found that the rate of electron flow is tightly linked to CO_2 fixation.

A

pH	ES (%) 2 cm from tip	ES (%) 4 cm from tip	ES (%) 6 cm from tip
4.0	19.4 ± 1.4 (a)	20.3 ± 1.2 (a)	20.6 ± 1.1 (a)
7.0	14.4 ± 2.2 (b)	15.9 ± 1.2 (b)	16.2 ± 1.5 (b)
8.0	9.4 ± 1.2 (c)	11.1 ± 1.1 (d)	13.3 ± 1.0 (c)

B

Source	degrees of freedom	Sum of squares	Mean square	F-value	P-value
pH	2	664.007	332.004	167.785	0.0001
Leaf position	2	56.810	28.405	14.355	0.0001
pH * Leaf position	4	13.794	3.449	1.743	0.1573
Residual	45	89.043	1.979		

Figure 5.3 The effect of pH (A-pH 4.0; B-pH 8.0) and leaf position on the photothermal amplitude when measured with 1 mM NaHCO_3 . Traces were normalized such that the value of Q_m is taken as 1.0. The time constant of the lock-in amplifier was 3 s.

A

B

The results obtained with a black polyethylene sample indicate that as a sample is moved further away from the probe beam, the amplitude decreases exponentially and the phase lag increases (figures 2.4 a & b). Based on this evidence, if the cells of the lower epidermis of *V. americana* were contributing to the PD signal one would expect Q_m to decrease and the phase lag to increase when measured at positions closer to the base of the leaf. Since there is no such trend it appears that the lower epidermis is not making a significant contribution to the PD signal.

The cells directly adjacent to the adaxial epidermis may also make a contribution to the PD signal however it is probably insignificant for several reasons. Firstly these cells are approximately 60 μm further away from the probe beam than are the epidermal cells. This would result in an increased attenuation of the thermal gradient by 70 percent. Secondly there are less than 25% as many cells in this lower layer. Thirdly these cells are subjected to lower light intensities as a result of the shading from the epidermal cells. These three factors make it reasonable to assume that only the adaxial epidermal cells make a significant contribution to the PD signal.

The interpretation of the PD signal therefore becomes greatly simplified due to the simple morphology of *V. americana* leaves. This allows the measurement of the photosynthetic activity of a single layer of cells *in vivo* without detaching them from the rest of the leaf. However measuring ES with these leaves cannot give direct information about the average photosynthetic activity of the leaf. This

information would be important in determining how the overall photosynthetic activity of a leaf is affected by changes in its environment.

The effect of the modulated light intensity on ES has the predicted hyperbolic effect. ES decreases hyperbolically as the light intensity increases for both surfaces of the *V. americana* leaf. The hyperbolic nature of this curve is consistent with the steady state closure of more photosynthetic reaction centers at increasing modulated light intensities (Malkin *et al.*, 1981). As the modulated light intensity increases a smaller proportion of the absorbed light energy will be used to drive photosynthesis resulting in a decrease in ES.

The extrapolated value of ES_m for both the surfaces of the *V. americana* was lower than the 55% reported for pea by Havaux *et al.*, (1990). However these values are similar to those reported by Malkin *et al.* (1992) measured by a related technique (Photoacoustic spectroscopy).

The much lower i_{50} of the abaxial surface compared to the adaxial surface indicates that the adaxial surface is able to use a greater proportion of the absorbed light energy at higher light intensities. This indicates that the chloroplasts in the two epidermal layers have adapted to their local light environment. The abaxial epidermal cells are exposed, on average, to lower light intensities than the adaxial epidermal cells during growth. This adaptation to local light intensity is similar to that seen in the chloroplasts of spinach. Higher rates of electron transport were found in the palisade as compared to the spongy mesophyll cell chloroplasts of spinach described by Terashima and Inoue (1985). They found that this was not due

to an increased number of photosynthetic units but rather to an increase in the intermediate pool sizes.

The determination of ES_m and i_{s0} by extrapolation is a useful method as it does not require the measurement of ES at very low light intensities. Measurement of ES at low light intensities becomes less reliable due to the decreased signal to noise ratio.

The ability of *V. americana* to use various carbon species was examined by measuring ES at different pHs at a constant level of DIC. CO_2 can be dissolved in several forms in solution: CO_2 , HCO_3^- , CO_3^{2-} and H_2CO_3 . The relative amounts of each of these species will be determined by the following equilibrium (Helder, 1988).

the pH of the medium will determine the relative amounts of each species present (figure 5.4). In most physiological conditions, inorganic carbon will be dissolved mostly as CO_2 and HCO_3^- , with the later being favoured above pH 6.35. CO_3^{2-} is in relatively low concentrations until the pH rises above pH 9.0 (Helder, 1988).

The results in Table 5.4 clearly show that the ES measured for a given leaf section is significantly larger when measured at decreasing pH. This shows that ES increases as the CO_2 concentration in the medium increases and as the HCO_3^- concentration decreases. These results show that the epidermal cells are able to use more of the absorbed light energy when CO_2 is present than when HCO_3^- is present.

Figure 5.4 Calculated proportion of each dissolved inorganic carbon species
present as a function of the pH of the solution

This probably reflects the differential ability of these two carbon species to enter the epidermal cells. Prins *et al.*, (1980) describe an active HCO_3^- transport mechanism in *Vallisneria spiralis*. These results presented here show that both CO_2 and HCO_3^- are absorbed by the leaf but that CO_2 enters with less resistance than does HCO_3^- .

ES in leaves tested in a medium which contains approximately 1mM of dissolved CO_2 appears to be independent of the position on the leaf that is measured (i.e. pH 4.0 + 1 mM NaHCO_3 or pH 7.0 5 mM NaHCO_3). Only when CO_2 levels are low (created by increasing the pH and/or decreasing the total DIC) does there seem to be any influence of leaf position on ES. This may indicate a differential ability of the different parts of the leaf to use HCO_3^- .

The transients in the PD amplitude also appear to reflect the differential ability of the 2 carbon species to enter the epidermal cells. Leaf sections supplied with CO_2 (i.e. pH 4.0) show a transient decrease in the PD amplitude to the level of Q_s . Leaf sections supplied with HCO_3^- (pH 8.0) show relative higher values of Q_s and no transient. This indicates that the rate of carbon entry into the cells at high pH is limiting the use of the absorbed light energy. These results show that the efficiency of light energy use is regulated by the supply of carbon to the chloroplast. This is similar to the results of Weis and Berry (1987) who found that the rate of PSII photochemistry (and hence the rate of linear electron transport) was regulated by the rate of CO_2 fixation. Krall and Edwards (1992) have also found that the rate of electron flow is tightly linked to CO_2 fixation.

CHAPTER SIX
THE EFFECT OF ATRAZINE
ON ENERGY STORAGE

Introduction:

The measurement of photosynthetic activity of leaves in an aqueous environment offers the potential to determine the effects of various chemicals on ES simply by adding it to the medium. Inhibitors of the photosynthetic electron transport chain have often been used in the elucidation of the different components of the chain. The objective of the experiments presented in this chapter was to determine if an inhibitor of the electron transport chain added to the external medium could reach its target within the chloroplast. If so, what effect does this inhibitor have on ES?

The contribution of linear electron transport to ES was determined by the addition of atrazine to leaf sections of *V. americana*. This chemical has been found to inhibit the flow of electrons at a point very near the photosystem II primary electron acceptor (Arntzen *et al.*, 1979). Such an inhibition should eliminate the linear flow of electrons from water to NADP⁺.

In the following experiments the time course of atrazine inhibition was monitored by measuring ES at different times after its addition to the medium surrounding the sample. This was done to determine the rate at which it reached its site of inhibition as well as its effect on ES.

Materials and Methods:

The effect of atrazine on ES in *V. americana* leaf sections was determined

by measuring ES in untreated sections and then by measuring ES after the addition of atrazine, ethanol 0.5% (v/v) or no addition to the medium surrounding these sections. Repeated ES measurements were made under the standard conditions with 5 minutes of darkness between each measurement.

When atrazine dissolved in ethanol was added to the bathing medium the final atrazine concentration was 100 μ M while that for ethanol was 0.5% (v/v)

Results:

Vallisneria americana leaves were exposed to 100 μ M atrazine and its effect on ES was determined at various times after application. Figure 6.1 shows that ES is reduced significantly after just 5 minutes of exposure to atrazine. ES is almost completely abolished after 25 minutes of exposure. The same experiment done in the presence of 0.5% ethanol showed no significant change in energy storage as compared to that of the APW control.

The transient usually observed was also reduced or eliminated by the addition of atrazine. The extent to which the transient was reduced was dependent on the time that the sample was exposed to atrazine. Figure 6.2 shows traces of ES measurements in which the sample tested in Trace A was untreated while the sample in Trace B had been exposed to 100 μ M atrazine for 12 minutes. In this figure, the transient decrease in the PD amplitude after the application of the modulated light is completely abolished after 12 minutes of exposure to atrazine.

The decrease in energy storage due to exposure to atrazine was not simply due to an increase in Q_s . Figure 6.3 shows that Q_m fell sharply after five minutes

Figure 6.1 The effect of incubation in atrazine on energy storage in leaf sections of *I. americana* when measured at different times after its application. Leaf sections were either treated with 100 μ M atrazine in ethanol, 0.5% ethanol, or untreated. Energy storage was measured in APW plus the indicated addition. Values are the mean and range of 3 different leaf segments for each treatment.

Figure 6.2 The effects of atrazine on the PD amplitude and ES in a *V. americana* leaf section. Trace A shows the measurement of ES when the leaf sample was tested in APW. Trace B shows the PD signal for the same section 12 minutes after the addition of 100 μ M atrazine

Figure 6.3 The effect of incubation time in atrazine on Q_m and Q_s in *I. americana* leaf sections. Values are the means of 3 different leaf sections. Measurements were made in APW with the addition of 100 μM atrazine.

followed by a more gradual decline. The value of Q_s was found to rise until it almost reached the level of Q_m at which time it followed a similar decline. The decline in Q_m appeared to be unrelated to the presence of atrazine as a proportionately similar decline in the value of Q_m was seen in both of the controls over the same time period (results not shown).

Microscopic examination of the epidermal cells before and after exposure to repeated ES measurements show that the chloroplasts in the epidermal cells rearrange such that they are located at the periphery of the cell instead of being evenly distributed near the upper periclinal walls. Observations made of leaf sections exposed to atrazine but kept in the dark showed no such rearrangement of the chloroplasts.

Discussion:

A block in the electron transport chain at the primary acceptor of PS II would effectively eliminate linear electron flow from water to NADP⁺. This block would also cause PSII to become photochemically saturated at very low light intensities. Therefore any photons of light absorbed by PS II would be remitted as heat and fluorescence. Inhibition of PS II would also result in a shortage of electron donors to PS I (Herbert *et al.*, 1990). Since P700 cannot be reoxidized, a greater proportion of the light energy absorbed will be dissipated as heat since photochemical charge separation at P700 becomes increasingly unlikely.

The effects of atrazine on the electron transport chain described above should result in a decrease in the measured ES when it is added to photosynthetically active samples. This is because the absorbed modulated light energy which was being used to drive electrons through the electron transport chain is now being released as heat.

Inhibition of ES with atrazine strongly suggests that photosynthetic electron transport is responsible for the storing of modulated light energy. ES after 25 minutes in atrazine has been shown to be reduced to less than 10 % of that in the uninhibited sample (figure 6.1). Inhibition of ES is still not complete after 45 minutes of incubation in atrazine. The remaining energy storage may be the result of either incomplete inhibition of PS II or the presence of cyclic electron transport around PS I as there are no other energy-storing processes leading to stable photochemical products known to occur (Herbert *et al.*, 1990).

Inhibition of ES with atrazine in *V. americana* is similar to the inhibition of ES in pea leaves by DCMU (Havaux *et al.*, 1990). DCMU blocks the flow of electrons at a site very close to that of atrazine (Arntzen *et al.*, 1979). Havaux *et al.* (1990) found that inhibition of ES was dependent on the time the leaf was exposed to DCMU as well as its concentration. Their results show complete abolition of ES after 20 minutes with 10^{-4} M DCMU but only partially after 55 minutes with 6×10^{-5} M DCMU.

When measuring ES in the presence of atrazine, Q_s was found to rise close to the level of Q_m . Q_m unexpectedly fell rapidly initially and then at a decreased rate (figure 6.3). The combined effect of this is a sharp decline in ES. A decline in Q_m

under these conditions indicates either a reduction in the absorption of the modulated light or an increase in the attenuation of the photothermal gradient.

Photographic evidence has shown that the epidermal chloroplasts from the periclinal walls to the anticlinal walls after repeated ES measurements. This results in the creation of large transparent areas in the epidermal cells. The creation of these transparent areas could result in a decreased absorption of modulated light which would lead to the reduction observed in Q_m .

The decrease in Q_m and movement of the chloroplasts were also observed in the absence of atrazine but there was no movement of chloroplasts if the sample was kept in the dark. It appears that the movement of the chloroplasts (and its related decrease in Q_m) is a response to repeated bright light exposure and not related to the addition of atrazine. This phenomenon will be more closely examined in the next chapter.

CHAPTER SEVEN
THE EFFECT OF CHLOROPLAST MOVEMENT
ON ENERGY STORAGE IN *V. americana*.

Introduction:

Phototrophic organisms have developed many mechanisms which allow them to optimize light harvesting for photosynthesis. One of these mechanisms is the movement of chloroplasts within photosynthetic cells in response to changing light environment experienced by plants and algae (Haupt and Scheuerlein, 1990). Izutani *et al.* (1990) have found that chloroplasts in the epidermal cells of *Vallisneria gigantea* showed movement patterns which exhibited a fluence response. Under low light intensity, chloroplasts move to become evenly distributed against the outer periclinal wall (figure 7.1). Izutani *et al.* (1990) also found that upon exposure to high intensity light the chloroplasts moved toward the anticlinal walls. The changes in the position of the chloroplasts lead to substantial changes in the absorption cross-sectional area of the chloroplasts (Izutani *et al.*, 1990).

The ecological significance of this response is unclear. Is this response a protective measure which acts to prevent photoinhibition at high light intensities or is it a mechanism which acts to optimize photosynthetic activity?

In the following experiments, the phenomenon of chloroplast movement will be studied with respect to its effect on the PD signal and ES in epidermal cells of *V. americana*.

Materials and Methods:

Several experiments were performed to determine the effect of chloroplast

Figure 7.1 Schematic diagram showing the position of the chloroplasts during the high light and low light responses (from Haupt and Scheuerlein, 1990)

LOW LIGHT INTENSITY
ARRANGEMENT

HIGH LIGHT INTENSITY
ARRANGEMENT

movement on ES and the PD signal. Cytocholasin B (CB) was used to inhibit chloroplast movement and to verify its effects on Qm and ES. Leaf sections were vacuum infiltrated with the appropriate medium and incubated in the dark in this medium for 30 min. The sample was then placed in the sample cell and covered with the same filtered medium. The phase angle was adjusted and the sample was incubated in the dark for 5 min further.

ES was measured before and after exposure to a bright light using the following modified light regime. The modulated light ($110 \mu\text{E m}^{-2} \text{s}^{-1}$) was added for a period of 40 minutes. The background light ($425 \mu\text{E m}^{-2} \text{s}^{-1}$) was added after five minutes after the application of the modulated light for a period of 30 minutes. This allowed measurement of ES at the start and end of the background light exposure.

Leaf sections were examined with the modified light regime after being infiltrated with APW, APW + DMSO (0.5% v/v), or APW + 20 $\mu\text{g/ml}$ cytocholasin B. Vacuum infiltrated leaves were prepared by cutting a 1 cm X 2 cm section of leaf and placing it in a 10 cc syringe. Experimental medium was drawn into the syringe then all air was expelled from it. The entry to the syringe was blocked and then the plunger pulled back which caused gas to escape from the leaf. When the plunger was released experimental medium moved into the leaf where the gas was initially.

Cytocholasin B (CB) (Sigma, St. Louis) was dissolved in dimethyl sulfoxide (DMSO) and used at a concentration of 20 $\mu\text{g/ml}$ while the final concentration of

DMSO was 0.5% (v/v).

The position of chloroplasts in the epidermal cells of *V. americana* were observed in leaf sections which were infiltrated with APW plus the substances as indicated. Photographs of the epidermal cells were taken before and after illumination with the modified light regime used to test ES.

Results:

Microscopic examination showed that the epidermal chloroplasts were evenly distributed over the periclinal walls of the epidermis before being treated with the modified ES light regime. This occurred regardless of the medium that the leaf section was infiltrated with (figures 7.2 a,c, and e).

After the application of the modulated light there was a transient decrease in the PD amplitude. This occurred regardless of the medium that the leaf section was infiltrated with (figure 7.3). The subsequent application of the non-modulated background light allowed an initial measurement of ES. The initial value of ES was not found to be statistically different in the presence and absence of CB (Student t-test, $n=6$ for each treatment).

When the leaf sections were tested with APW there was a decrease in Q_m and Q_s after the application of the non-modulated light (figure 7.3, Trace A). ES increased after the application of the background light by $60 \pm 21\%$ (mean $\pm$ sd, $n=6$) when compared to the initial value of ES. The phase lag was found to increase

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PRECISIONSM RESOLUTION TARGETS

Figure 7.2 Photographs of the adaxial epidermal cells before (a, c, and e) and after exposure (b, d, and f) to the light regime described in figure 7.3. Figures a and b show the distribution of the epidermal chloroplasts when the leaf was tested with APW. Figures c and d were taken of samples tested with APW + 0.5% DMSO. Figures e and f are photographs of samples tested with APW + 20g/ml CB (Bar =100 μ m.)

Figure 7.3 The effect of Cytocholasin B on the normalized PD signal from infiltrated leaf sections of *V. americana*. Traces A and B show the photothermal signal obtained when the leaf was infiltrated and tested in APW and APW + 0.5% DMSO respectively. Trace C shows the signal when the leaf was infiltrated and tested with APW + 20 μ g/ml Cytocholasin B. The background light was added and removed where the up and down arrows indicate. All traces were normalized such that the value of each photothermal signal when the background light is turned on is taken to be unity.

after being treated with this light regime by $10 \pm 3.5^\circ$ (mean $\pm$ sd, $n=6$).

Photographic observations of the leaf sections after this treatment showed transparent areas where chloroplasts were once observed (figure 7.2 b)

Statistically similar results concerning Q_m and ES were obtained when the sample was tested with DMSO included in the test medium. Chloroplasts were also observed to reorient themselves along the anticlinal walls of the epidermal cells (figure 7.2d).

In the presence of CB, Q_m showed no decrease, on average, compared to its initial level (figure 7.3, Trace C). ES increased by $20 \pm 16\%$ and the phase lag increased by $6 \pm 2^\circ$. Both of these values were statistically smaller at the 95% confidence level (Student t-test) than those of either of the controls. Microscopic examination of the epidermal cells following the treatment with the modified light regime showed that the chloroplasts remained evenly distributed (figure 7.2 f).

Discussion

Chloroplast movement in the epidermal cells of *V. gigantea* has been shown to exhibit different responses when illuminated at low and high fluence rates (Izutani *et al.*, 1990). The mechanism responsible for movement of chloroplasts has been shown to occur by their movement along microfilament bundles which are present in the vicinity of the cytoplasmic membrane (Yamaguchi and Nagai, 1981). These microfilaments are composed of F-actin (filamentous actin) and could be destabilized

by the addition of CB (Yamaguchi and Nagai, 1981). The movement of chloroplasts within the cells of *V. gigantea* has been inhibited by the addition of 100 $\mu\text{g/ml}$ CB (Izutani *et al.*, 1990).

The results shown here indicate that chloroplasts are initially spread evenly along the outer periclinal wall of the epidermal cells (figures 7.2, a,c.&e). After exposure to bright light for a period of 40 min the chloroplasts have moved to form clumps along the anticlinal walls (figures 7.2 b & d). This movement in response to bright light was inhibited by 20 $\mu\text{g/ml}$ CB (fig 7.2 f)

Chloroplast movement should have a twofold effect on the PD signal. Movement of the chloroplasts to the anticlinal walls in response to high light intensity results in the clumping of chloroplasts along the anticlinal walls (figures 7.2 b&d). This would result in an increased average distance between the chloroplasts and the probe laser beam. This would produce an increased attenuation of the PD signal (lower Q_m) and an increase in the phase lag, both of which have been observed in the controls. In addition, the formation of transparent areas would lead to a decrease in the modulated light absorbed by the adaxial epidermis of the leaf and hence a decrease in Q_m .

The clumping of chloroplasts would also lead to a decrease in the light intensity received by some chloroplasts due to shading by others. Chloroplasts receiving a reduced modulated light fluence would show higher values of ES as compared to those chloroplasts which were not shaded. This is based on the hyperbolic relationship between ES and the modulated light intensity seen in figure

5.2. These results show that chloroplast movement in response to high light has led to increased values of ES when compared to leaf sections where chloroplast movement has been inhibited by CB.

These results indicate that chloroplast movement increases the efficiency at which the modulated light was used. It appears that this was not in an effort to evade photoinhibition because when it was inhibited by CB there was no apparent inhibition observed. These results are similar to those of Rojas and Garcia (1993) who found an increase in the rate of $^{14}\text{CO}_2$ uptake when the light intensity is increased up to 200 W m^{-2} (approximately equivalent to $800 \mu\text{E m}^{-2} \text{ s}^{-1}$) in *V. spiralis*. This light intensity was also found to cause the movement of chloroplasts to the anticlinal walls. However, at higher light intensities (up to 800 W m^{-2}) $^{14}\text{CO}_2$ uptake decreased even though chloroplast movement occurred (Rojas and Garcia, 1993). At these high light intensities it appears that chloroplast movement was unable to prevent photoinhibition.

CHAPTER EIGHT
GENERAL CONCLUSIONS

The PDS technique has been successfully applied here to measure ES in the leaves of four aquatic plants. The factors influencing ES were investigated in *V. americana*. ES has been characterized with respect to the modulated light intensity. It has also been shown to reflect the differential ability of *V. americana* to use CO_2 and HCO_3^- from the bathing medium. These experiments have laid the groundwork for future studies of *V. americana* with this technique.

Experiments are currently being performed to measure ES as a function of the DIC concentration at different pHs. This will help to characterize the uptake of inorganic carbon by this plant. These experiments in conjunction with those presented here should result in the characterization of the factors which affect ES in the leaves of *V. americana*. These studies will show how ES varies as a function of the two substrates of photosynthesis (light and CO_2).

The PDS technique is one of several methods presently available which measure the thermal energy released by photosynthetic tissues. A closely related technique, photoacoustics (PA) measures the acoustic wave produced as a result of the thermal emission.

PDS has several advantages over PA in the measurement of heat release. First it is much more sensitive to thermal emission (Havaux *et al.*, 1989). Secondly, PA measurements are sensitive to a gas exchange component which complicates the interpretation of the results. Finally, PA measurements are made in a closed system with a typically small volume (0.08-0.7 cm^3) (Havaux, 1990).

The small volume of the PA cell dictates that only low intensity illumination

be used or that the use of high intensity light be used for short periods of time. This is due to the exhaustion of available of CO_2 in the cell.

PDS is however technically more difficult to use as it requires more precise alignment of the optical components to obtain its maximal sensitivity. Also samples must not be exceedingly rough as this will limit the offset between the laser and the sample (Jackson *et al.*, 1981).

The results of this work have shown that the use of PDS in the study of aquatic plants is ideal. This type of study allows the measurement of thermal gradients in water where both the PDS technique and the aquatic plants work best.

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