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Technical note

Demonstration of a highly-sensitive portable double-flash kinetic spectrophotometer for measurement of electron transfer reactions in intact plants

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Abstract

A highly sensitive, portable spectrophotometer for use in measuring flash-induced absorbance changes in intact leaves is demonstrated. The design of the instrument is modified for whole plant use from that suggested by Joliot and Joliot (Biochim. Biophys. Acta 765, 210-218). The spectrophotometer uses trifurcated light guides to deliver measuring and actinic beams to two comparable areas of the leaf. The measuring beam is provided by a series of short, relatively intense light pulses from a xenon flashlamp in place of the constant weak measuring beam used in conventional machines. The use of a flash measuring beam and differential detection allows for a high signal-to-noise ratio (noise levels of 10^{-5}A) without significant actinic effects. The time resolution of the instrument is 2 μsec and the noise level is independent of the experimental time range. The instrument is battery or mains powered, computer operated, and has a liquid crystal display for computer-user interface and dialogue, and to show the kinetic traces graphically. Wavelength selection is provided by interchangeable interference filters. The instrument can communicate with a laboratory-based computer, receiving programming information and sending experimental data to be processed and plotted. The instrument is demonstrated by following the kinetics of the electrochromic shift, the change in redox states of cytochrome *f* and the *b* cytochromes in an intact cucumber leaf, and in the same leaf after infiltration with DCMU.

Introduction

Functional aspects of photosynthesis in plants in their natural state and the effects of different environmental changes and stresses can best be approached by studying the processes *in situ*. The present level of understanding of higher plant photosynthesis has come about mainly through work on *in vitro* systems. Extending this understanding to plants in their natural state has been limited by the instrumentation available to make biophysical measurements of photosynthesis *in situ* and the ability to interpret the measurements made. Although direct methods for measuring oxygen evolution and CO_2 uptake are available for field use, there have been only a few indirect methods for measuring a limited number of electron transport reactions under field conditions. The methods that are available are based on measurements of fluorescence (Schreiber, 1986; Robinson, 1986), and interpretations of these data are complicated.

The most direct method of measuring light-induced electron transport in plants is to follow the kinetics of the absorbance changes due to the redox components of the electron transport chains. Through appropriate deconvolution of the visible and ultraviolet spectra, the kinetics of cytochromes *b*, cytochrome *f*, plastocyanin and other components can be determined (e.g. Joliot and Joliot, 1984, Rich, et al., 1987). Absorption spectroscopy

can also be used for measurement of the kinetics of charge transfer across the thylakoid membrane through the absorbance changes resulting from the electrochromic shift of carotenoid and chlorophyll molecules of the bulk pigments (reviewed in Witt, 1975). Such measurements require instrumentation of high sensitivity and fast response time. The difficulties in meeting these requirements are compounded by the fact that the probe beam used in conventional absorbance measurements must be kept at low incident intensity to avoid actinic effects; as a consequence, the signal-to-noise ratio is usually limited by the number of photons detected. Since the number of photons measured per time point decreases with increasing response times, the signal-to-noise ratio of measurements made on conventional instruments decreases as the resolution time of the measurement becomes faster.

In most cases, higher signal-to-noise ratios are achieved by signal averaging. For many measurements, however, signal averaging is impractical due to the long time required, and difficulties in preparing and maintaining the samples needed. In some cases, the signal-to-noise ratios are so small that hundreds of traces must be averaged to achieve acceptable data. The difficulties arising from the need to sample many experiments are especially acute for experiments where the condition of the sample is modified by the actinic flash used in the experiment. This is true for many *in situ* experiments, where the physiological condition of the leaves can be altered by extended dark periods, or where measurements are needed of processes like the two-electron gate, or the S-states, where a prolonged dark adaptation is needed between actinic flashes to allow the system to reset to a known state.

Joliot and Joliot (1984) have developed a laboratory-based kinetic spectrophotometer that achieves a very high signal-to-noise ratio (noise levels of less than 10^{-5} A) by utilizing differential optics (by dividing the measuring beam through fiber optics), and a series of relatively intense light pulses from a xenon flashlamp instead of a continuous beam to probe the sample. This allows the measuring beam to have a higher intensity, and thus to allow a higher signal-to-noise ratio, at the discrete time points where the measurements are made, while the integrated intensity over the course of the experiment remains low, and non-actinic. With this instrument, no signal averaging is necessary even for very small signals. In addition, since the amount of light per discrete time point is constant, the noise level is not affected by the trace time.

In this paper, we describe a completely portable instrument for the measurement of the kinetics of flash-induced absorbance changes in intact leaves, based on the flash measuring beam/differential optics technique of Joliot and Joliot (1984). The instrument has a sensitivity (noise levels of about 10^{-5}A) approaching that of the laboratory-based instrument described by Joliot and Joliot (1984), but is designed to hold intact leaves, and is battery-powered and computer controlled to provide portability and ease of use for studies on whole plants in the field. Our aim in developing this instrumentation is to be able to make parallel measurements with laboratory-based instruments on *in vitro* systems, and with portable instruments on intact plants in the field. We anticipate that use of this instrument will extend our options for measurement of photosynthesis *in situ*, and further our knowledge of photosynthesis in the intact plant, and its modification in response to changes in the environment. We demonstrate the versatility of this instrument by showing the effects of DCMU on the kinetics of the flash-induced electrochromic shift, and on electron transport through cytochrome *f* and the *b* cytochromes in an intact cucumber leaf. This instrument represents a significant improvement over an earlier prototype which has been briefly reported elsewhere (Kramer, et al., 1987).

Materials and methods

Plant Growth Conditions. Cucumber plants (*Cucumis sativus* L. cv. Ashley) were raised from seed in a soil/peat/vermiculite mixture, watered daily and fertilized weekly. The plants were grown in a controlled environment chamber (600–800 μE m^{-2} s^{-1}, 14 hour photoperiod, day/night temperatures of 23°C/20°C) as described in Martin and Ort, 1985. Attached leaves which had reached almost full expansion were used for the experiments.

Double-flash kinetic spectrophotometer.

General overview. The instrument uses differential

optics to measure the absorbance of two similar areas of the leaf. One of these areas is illuminated by an actinic flash. Measurement of the absorbance change induced by the actinic flash is achieved by measuring the difference in absorbance between the sample (illuminated) and reference (dark) areas of the leaf. The measuring beam is provided by a series of relatively intense measuring flashes at a particular wavelength selected by an interference filter. The light from these flashes is divided evenly into reference and sample beams by a specially constructed fiber optics bundle. The sample and reference beams are passed through two different but closely-spaced spots on the leaf. The intensities of the transmitted light are detected by photodiodes, integrated and measured.

In a typical experiment, the leaf is first probed with a few measuring flashes; after each flash, the gains of the two channels are adjusted so that the response is the same on sample and reference channels. Any differences in the optical densities of the leaves are accounted for by analog circuitry, which allows the computer to automatically set and balance the gains for both the reference and sample circuits. After the gains of the two channels are correctly balanced, a baseline is taken, using two or more additional measuring flashes to determine a baseline differential signal. These are followed by actinic illumination of the sample area, and by a train of measuring flashes which detect any change in the differential signal. The fiber optics bundle allows either an actinic flash or constant actinic illumination to reach the spot on the leaf probed by the sample beam, although in the present apparatus, only the flash activation was implemented. The actinic flash(es) are followed by a series of measuring flashes at user-selected intervals. The time resolution of each point is limited by the duration of the measuring and actinic flashlamps, about 2 μsec, and this also determines the shortest interval between the actinic flash and the first detecting flash, to establish a limit to the time resolution of the spectrophotometer. The spacing between successive flashes used to measure a series of time points in a kinetic trace is limited by the time required to recharge the flash-lamp discharge capacitor. In the present machine, the recharge time for the measuring flashlamp is approximately 10 msec. The instrument is shown in use in the field in Fig. 1.

Fig. 1. Photographs of the portable double-flash spectrophotometer. Left: The instrument consists of three portable boxes. The first of these contains the two flash lamps, one for the actinic flash, another for the measuring flash, high voltage power supplies to run them, and optics leading to a specially constructed fiber optics network. The fiber optics bundle carries the light to the leaf chamber which is mounted on the second box. This box contains the photodiode detectors and blocking filters, and the analog electronics. The third box contains a microcomputer to run the experiments and to collect, store, manipulate and display data. Right: Detail showing leaf clamp/detector holding a leaf. The total weight of the instrument, including batteries, is about 17 Kg.

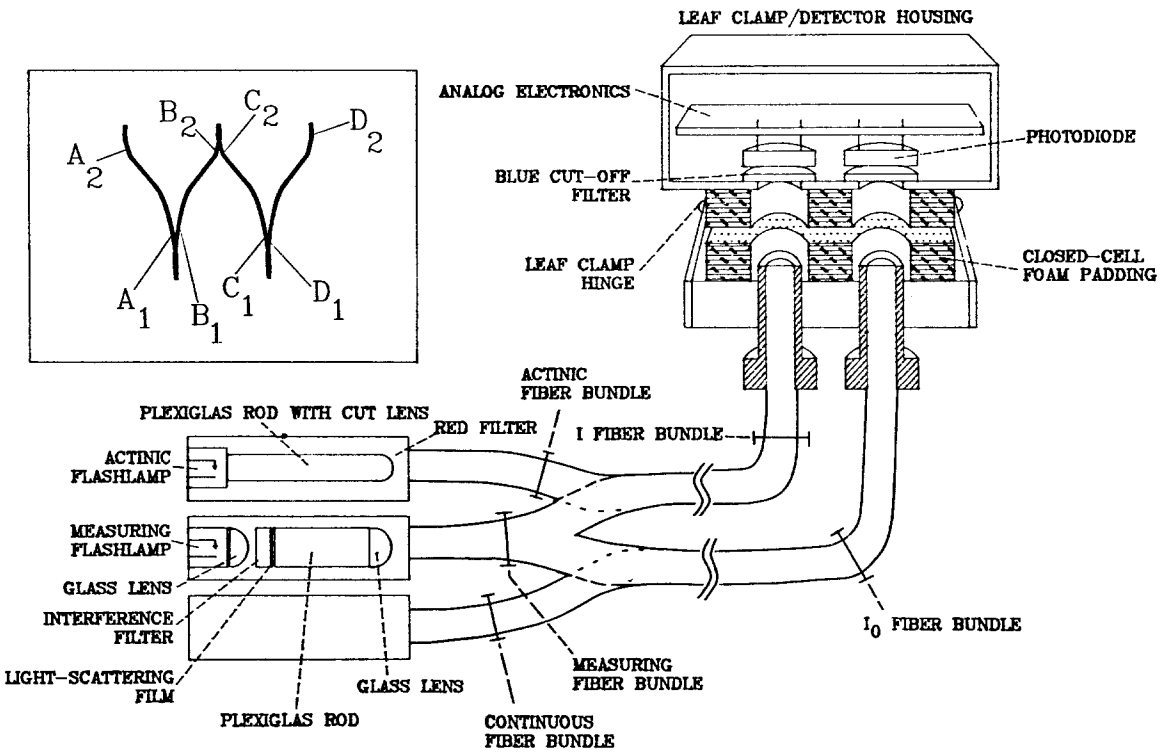

Fig. 2. Diagram of the optics of the spectrophotometer. Detailsare described in the Materials and Methods section. Inset: Representation of the construction technique used in constructing the fiber bundles. The lines represent individual optical fibers glued as described in the Materials and Methods section.

Optics. A schematic of the optics is illustrated in Fig. 2. Both the actinic and measuring flashes are provided by xenon flash lamps (FX802, EGG, Boston. Ma.). The flash lamp discharge is powered by two rechargeable 12 V lead acid batteries (EP 1218-26, Technacell Elpower, Santa Ana, Ca.), which drive a high voltage DC-DC converter/trigger circuit (PS350, EGG, Boston, Mass.) to provide from 300 to 1500 VDC. The actinic and measuring flashes are powered respectively by the discharge of a 1 μF and a 0.25 μF high voltage capacitor (Plastic Capacitors, Inc.). The duration of the actinic flash is 2 μsec, and that of the measuring flash is slightly shorter.

Wavelength selection for the measuring pulses is provided by inter-changeable interference filters (Omega Optical, Brattleboro, Vt.). For the work in this paper, we used the following wavelengths: 515, 545, 554, 563, and 572 nm. Filters were blocked to 1000 nm and had a 2 nm bandwidth, except the 515 nm filter which had a 5 nm bandwidth. Due to absorption by a leaf, the transmission spectrum of the 515 nm filter was distorted somewhat. A spectrum of the light passing through the 515 nm filter and a cucumber leaf showed a shift of the maximum transmission of about 0.5 nm to the red and a slight broadening. However, no significant transmission beyond 7.5 nm on either side of the peak could be observed. The transmission spectra of the remaining filters did not show significant distortions when the transmitted light was passed through a leaf. Light from the measuring flashlamp was collected by a 1 inch diameter, 1 inch focal length glass lens, and passed as a parallel beam through the interference filter. To homogenize the light, it was then passed through a light-scattering film followed by a 1 inch diameter light pipe constructed from a Plexiglas rod. The light was then focused by a lens onto the fiber optics bundle network.

The ability to evenly divide the measuring flashes into reference and sample beams is perhaps the most crucial design feature of the instrument. This is made difficult because of the inherent instability of the xenon flash arc. A workable solution was

adapted from that described by Joliot and Joliot (1984) who divided the beams using a bifurcated fiber optics bundle. Light from the measuring flash-lamp was introduced into a bundle of thin optical fibers. Half of these fibers were used for the reference beam and the remaining are used for the sample beam.

Light from the actinic flash lamp is collected in a light pipe constructed out of a 1/2 inch diameter plexiglas rod with a lens cut into the exit end. The light is passed through a red color filter (Kodak Wrattan 70) and then into the fiber optic bundle network.

Because of the geometry of the intact leaf, actinic illumination must be given perpendicular to the surface, ie. parallel to the direction of the measuring beam. This is accomplished by trifurcating the entrance end of the fiber optics bundles as shown in Fig. 2. Fibers that collect the actinic flash light are intermixed with the sample beam fibers. Thus, when the actinic flash is fired, only the leaf spot probed by the sample beam is illuminated. Fibers are similarly intermixed with the reference fibers in order to keep the density of fibers on both channels equal. These fibers form a separate bundle that can be used for continuous actinic illumination.

Several schemes for constructing the fiber bundle networks were attempted. The following scheme was found to be both effective and relatively easy to implement. Please refer to the inset of Fig.2. First, about 600 '*V*-shaped' fiber networks were constructed by attaching one end each of two fibers (0.02 inches diameter, 3 feet long plastic optical fiber, Fiber Optics Development, Santa, Barbara, Ca.) with a small amount of cyano-acrylate glue. This is illustrated in Fig. 2 inset where fiber ends A_1 are attached to B_1, and C_1 to D_1 to make vertices A_1-B_1 and C_1-D_1, respectively. The networks were separated into two equal parts (each with about 300 pairs of networks). From these pairs, '*W*-shaped' networks were constructed by similarly gluing together one of the free ends of each '*V*-shaped' network. This is illustrated in Fig. 2, where fiber ends B_2 and C_2 are attached to make vertex B_2-C_2. The networks were gathered at random at the two vertices, A_1-B_1 and C_1-D_1 to make the two exit ends. The three remaining bundles A_2, B_2-C_2 and D_2 were gathered at random to make the actinic flash entrance end, the measuring flash entrance end and the continuous light entrance end, respectively. All the gathered ends were placed in brass housings, glued with epoxy resin (Epo-tek 310 epoxy resin, Epoxy Technology, Inc., Billerica, Ma.), and polished to a glass finish using a series of fine sandpapers and emery cloth. The effect of this method of construction was to equalize the distribution of light between pairs of pathways, with a haphazard (rather than statistically random) geometry. Experiments done with statistically randomized bundles did not show lower noise levels (data not shown).

The two exit ends of the fiber network are inserted into holes in a hinged leaf housing. Intact leaves are placed in this housing and shielded from stray light by soft closed pore foam padding. The atmosphere around the leaf can be kept constant throughout the experiments by passing a stream of moistened air or other gas mixture into the housing. Light from the two exit bundles is passed through the leaf at two closely-spaced (2 cm center-to-center) spots and the transmitted light is detected by two photodiodes with active areas of 100 mm^2 each (UDT-UV100L, United Detector Technology, Hawthorne, Ca.) protected from the actinic light by blue-green color filters (CS 4-96, Corning Glass).

Analog Electronics. A simplified schematic for one channel of the analog electronic circuit is shown in Fig. 3. The circuit is repeated for the other channel. The power supplies (CP-3611 and CP-3601, TDK, Industries, Skokie, Ill.) were energized using two 12 V rechargeable lead acid batteries (Technacell Elpower, Sant Ana, Ca.). The first analog stage, centered around an operational amplifier (op amp, OPA111, Burr Brown, U2 in Fig 3), serves to convert the current output of the photodiodes to a voltage (I-V converter), and to integrate the signal. Integration serves to capture the transient voltage pulse as a stable voltage level so that the signal can be measured accurately using more slowly responding circuitry. The second analog stage is a computer-controlled gain stage. It consists of an operational amplifier (OPA111, Burr Brown, U3 in Fig 3) with one half of a dual multiplying digital to analog converter (DAC, AD7528, Analog Devices, U4 in Fig 3) in the feedback loop. This circuit provides a variable gain, selected by the computer, of between 1 and 255. The output from the variable gain stage is passed through a low pass RC filter and input to a precision track-and-hold

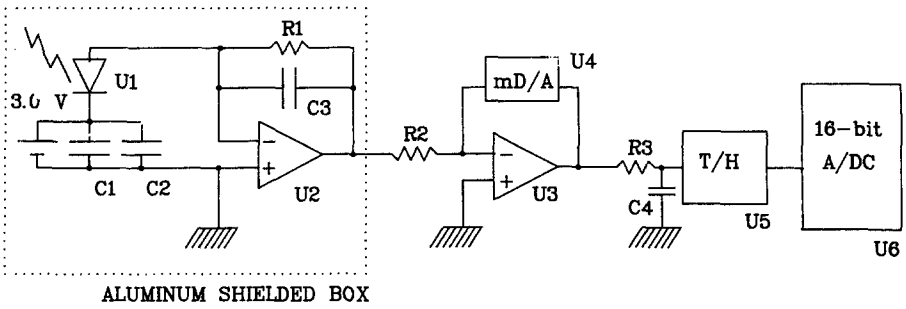

Fig. 3. Simplified schematics of the analog electronics of the spectrophotometer. The values of the resistors and capacitors are as follows: R1 = 1 MΩ, R2 = 5 KΩ, R3 = 100Ω, C1 = 10 μF (tantalum), C2 = 1000 pF (polystyrene), C3 = 220 pF (polystyrene), C4 = 1000 pF (polystyrene). Further details are described in the Materials and Methods section. A complete set of schematics can be obtained from the authors.

(T/H) amplifier (MN373, Micro Networks, Worchester, MA, U5 in Fig 3). This device holds the integrated voltage for conversion by a 16-bit analog to digital converter (ADC, 2D569, Burr Brown, U6 in Fig 3).

Digital Electronics and Computer Control. All digital electronic components are complementary-metaloxide-semiconductor (CMOS) to reduce the current drain on the batteries. The timing is performed in hardware using nine 16-bit digital timers (on three Intel 82HC54 timer chips). Programming of the timers and gain stages as well as acquisition, processing, display and storage of data are performed by a microcomputer (MSI-CZ81, MSI, Inc., Batton-Rouge, LA.). The processor chip is a Zilog CMOS Z80. Data is stored in 64 Kbytes of battery-backed random access memory (RAM, MSI-C764, MSI, Inc., Batton-Rouge, LA.). This memory can hold data from several hundred experiments, and retain it even when the power to the microcomputer is off.

Complete schematics of the apparatus, including an outline of the mechanical and optical design, electronic circuits, schematics of the portable computer, and computer source code can be obtained from the authors. The total cost of the parts for the instrument were approximately $7500 (US).

The program for control of the apparatus has been written in FORTH and compiled for transfer to three 16 Kbyte erasable programmable read only memory (EPROM) chips using a FORTH target compiler (Robinson, et al, 1985). The program is menu-driven and consists of subroutines to run the experiments, an editor that allows experimental routines to be set up, display routines, and a terminal program that allows the microcomputer to communicate with a laboratory-based computer over a standard RS-232C port. Through this port, the microcomputer can receive programming data from and send experimental data to a laboratory--based computer. The menus and graphics are displayed on a liquid crystal display (LCD) panel (LM-213, Hitachi).

Infusion of reagents into intact leaves. Under dim room light, the upper and lower surfaces of the intact leaves were lightly abraded with 400 grit carborundum, and tissue paper soaked with the reagent solutions was applied to both sides of the leaves. The covered leaves were supported by plastic weighing dishes and an additional small amount of the reagent solution was added to the dishes. To aid the infiltration, all solutions included 0.1% Tween 80 as a surfactant. This low concentration had minimal effects on the kinetics of the electrochromic shift whereas a 10-fold higher concentration increased the decay rate slightly. The reagents were allowed to soak into the leaves in the dark for approximately 30 minutes.

Results and discussion

The kinetics of the electrochromic shift in an intact

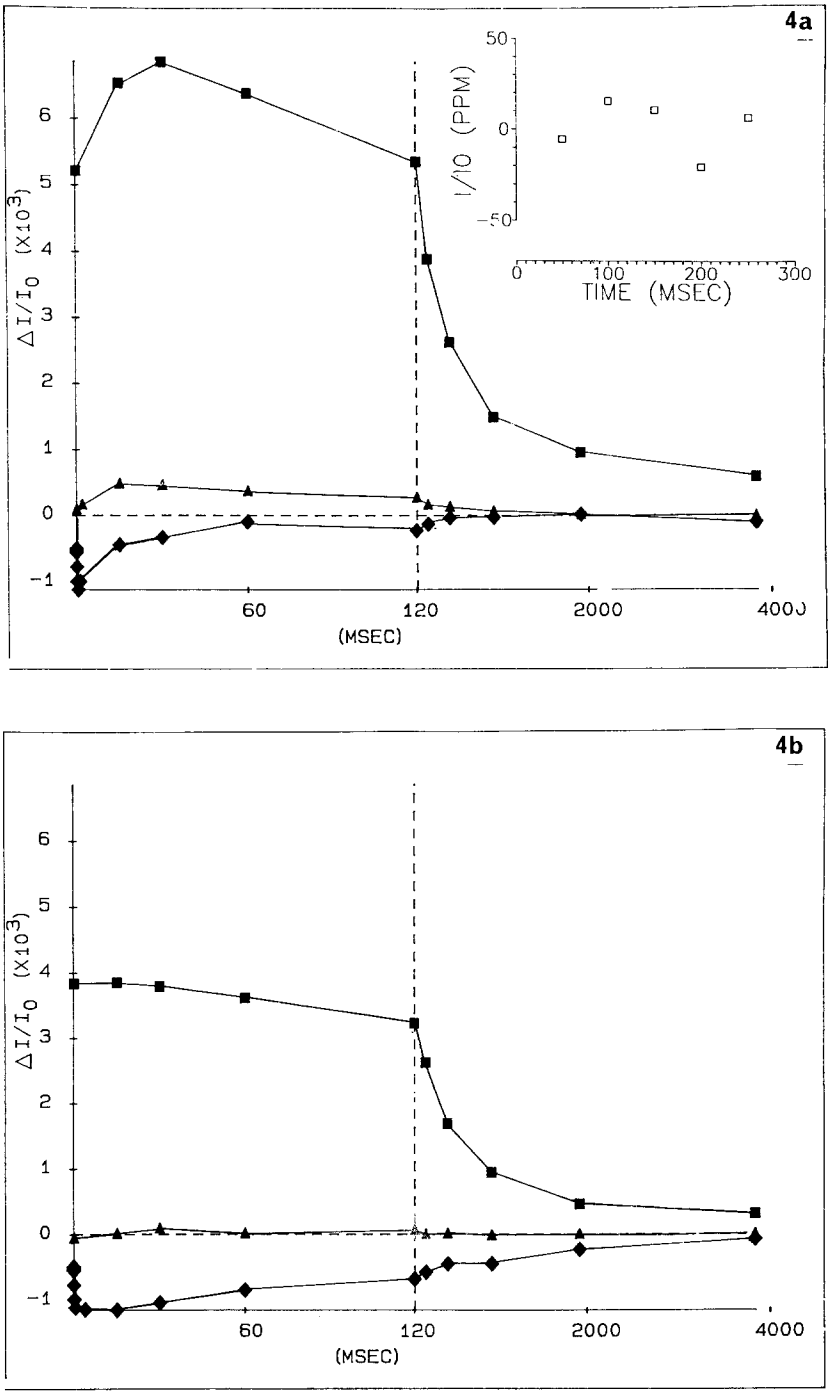

Fig. 4. Kinetics of the electrochromic shift ($\Delta I/I_{515\,nm}$, squares), cytochrome *f* ($\Delta I/I_{545}$-$\Delta I/I_{554}$+0.1($\Delta I/I_{515}$), diamonds) and the *b* cytochromes ($\Delta I/I_{563}$-$\Delta I/I_{572}$, triangles) in an intact cucumber leaf. a) control leaf with no additions, b) same leaf as in a) but infused with 10 μM DCMU in 0.1% tween 80. Actinic flashes were given at time zero. Please note the split time base starting at the vertical dashed line. Inset to 4a) Typical baseline of five points taken before the actinic flash to show the noise level of the instrument. This baseline was taken at 515 nm and has a standard deviation of 14.9 parts per million (ppm) of $\Delta I/I_0$; most baselines had a standard deviation between 10 and 20 ppm, though longer baselines (up to several seconds) showed larger deviations of up to 25 ppm, probably due to drifts induced by small movements of the sample leaf.

cucumber leaf induced by a single actinic flash of about 50% saturation and measured using the present instrument are shown in Fig. 4a. The noise levels of the baselines of single measurements ranged between 10 and 25 ppm (standard deviation of $\Delta I/I_0$ of four consecutive baseline points, 50 msec apart, a typical baseline for the absorbance change at 515 nm as shown in the inset to Fig. 4a). The average of the noise levels for ten baselines was 14.9 ppm. When the baselines were extended over longer time spans, the noise levels were slightly higher. The average noise level for ten traces 3 seconds long was 21.6 ppm.

The plant was dark-adapted for two hours and given one actinic flash followed by a train of 10 measuring flashes at exponentially increasing time intervals. The kinetics of the electrochromic shift in the control leaf (Fig. 4a) are typical for those seen in intact chloroplasts (see references in Joliot and Joliot, 1986), or in intact leaves when measured with conventional instrumentation (Morita, et al., 1981). These kinetics consist of several phases. The fastest phases are due to the initial charge separations in the reaction centers of both PSI and PSII, with a $t_{1/2}$ less than 100 nsec (Wolf et al., 1969), and are therefore completed by the time the first point in the above traces is taken at 100 μsec after the actinic flash. It is generally accepted that the slow phase of electrochromic shift is due to the electrogenic turnover of the cytochrome b_6f complex (reviewed in Joliot and Joliot, 1986), although the nature of the actual electrogenic step is disputed.

When the intact leaf was treated with 10 μM DCMU, followed by a two hour dark adaptation, and then excited by a single actinic flash (Fig. 4b, squares), the slow phase of the electrochromic shift had almost completely disappeared while the fast phase was unaffected. DCMU specifically blocks the flow of electrons from Q_A to Q_B in PSII. The failure to detect a slow phase can therefore be interpreted as showing that no electrons were available for turnover of the cytochrome b_6f complex, and suggested that, in the dark-adapted leaf, the plastoquinone pool was almost completely oxidized before the actinic flash. The residual slow phase presumably reflects the fraction of chains in which plastoquinol was available for oxidation by the cytochrome b_6f complex.

We also observed the kinetics of cytochrome *f* and the *b* cytochromes of the cytochrome b_6f complex. Since the $t_{1/2}$ of oxidation of cytochrome *f* is on the order of 200–300 μsec (Bouges-Bocquet, 1977), a composite of ten experiments was performed to resolve the kinetics from 10 μsec to 10 msec (Fig. 5). Bouges-Bocquet (1977) previously noted a 50–70 μsec delay between the actinic flash to the onset of the cytochrome *f* oxidation (see Fig. 5, inset). The spectrum of the electrochromic shift overlaps that for the cytochrome *f* changes, so we took advantage of the lag in cytochrome *f* changes to estimate the percentage of the absorbance change at 515 nm needed to correct the cytochrome *f* changes. At 10 μsec after the actinic flash, the fast phase of the electrochromic change is complete, whereas, because of the lag in cytochrome *f* oxidation, minimal changes in the cytochrome *f* kinetics should be seen. We found that about 10–13% of the 515 nm change should be added to correct the traces. Upon flash excitation of a normal dark-adapted leaf, cytochrome *f* became transiently oxidized, with an apparent $t_{1/2}$ of about 100-150 μsec, after a lag of 20–40 μsec (Fig. 5), and then rereduced with an apparent $t_{1/2}$ of 35 msec by turnover of the cytochrome b_6f complex (Fig. 4). These values are similar to those seen for isolated chloroplasts (Bouges-Bocquet, 1977), though the rise kinetics are somewhat faster, and may reflect the more intact system in the present work. Treatment with DCMU slowed the rereduction of cytochrome *f* to a $t_{1/2}$ of 70 msec, since it blocks the turnover of the cytochrome b_6f complex by inhibiting the reduction of plastoquinone at the Q_B site as seen previously for isolated chloroplasts (Crowther and Hind, 1980), and in the electrochromic changes above. The inhibition of turn-over of the b_6f complex after addition of DCMU is also reflected in the kinetics of turnover of the *b* cytochromes. In the control leaf, they are transiently reduced upon flash excitation (Fig. 4a), but in the DCMU-treated leaf, no significant changes were observed (Fig. 4b).

The instrument has been successfully used for both laboratory (Kramer and Crofts, 1989) and extensive field work (Kramer et al., 1989; Wise et al., 1989). The instrument performed well in the laboratory, even when placed inside a growth chamber. Field conditions introduce several additional problems. When experiments lasting longer than the 2–3 hour battery life were to be performed, mains power was used. External batteries of higher capacity would have been an alternative. The in-

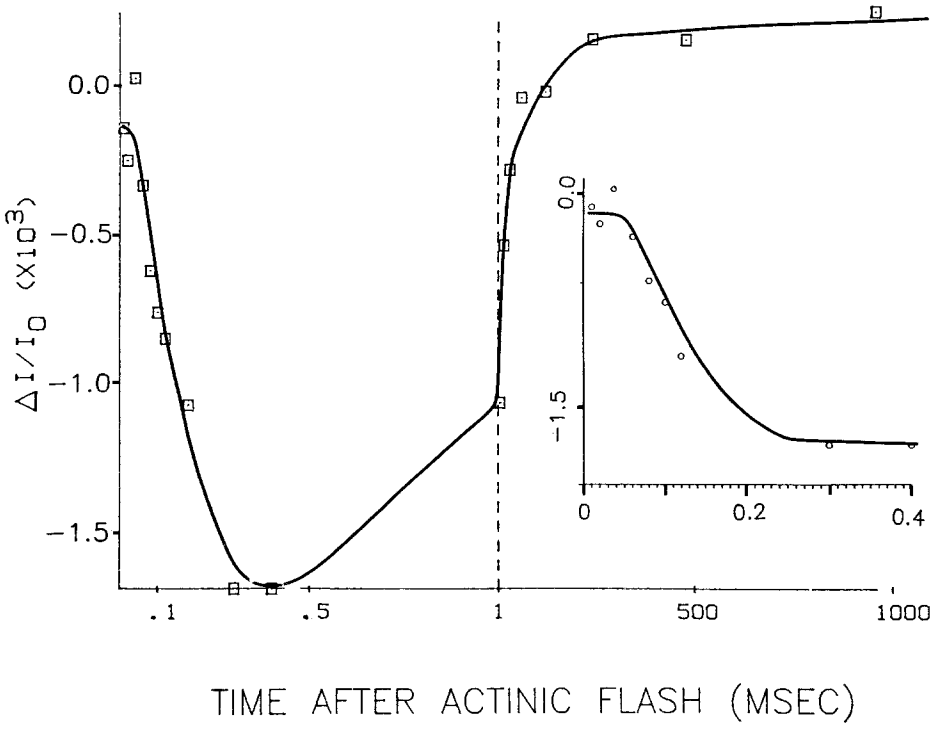

Fig. 5. Fast kinetics of the oxidation of cytochrome *f* of an intact cucumber leaf after one single-turnover actinic flash. Inset: a faster time scale allows the resolution of the lag before oxidation of cytochrome *f*. The kinetics were obtained by subtracting the absorbance change at 545 nm from that at 554 nm and adding 0.13 of that at 515 nm. This plot is a composite of ten experiments on the same leaf. Since the time between the first and second measuring flash must be greater than 10 msec, the first time point of each experiment was varied to obtain data in the microsecond range. The fastest time point was taken at 10 μsec after the actinic flash. The time base is split at 1 msec as indicated by the vertical dashed line.

strument performed well under Illinois summer conditions (high light (full sunlight) and high temperature (above 42°C)). A simple plastic cover was used to protect the instrument from water (e.g.: under wet leaf canopies) and dirt. The most difficult problem encountered in the field was leaf movement caused by wind. On a particularly windy day, leaf movement introduced significant drift in the measurements. This problem could mostly be alleviated by securing the leaf petiole with a clamp attached to the leaf housing.

By observing the kinetics of electron transport in an intact cucumber leaf, and the effects on these of an inhibitor of known action, we have demonstrated the usefulness of the instrument. This instrument, in conjunction with previously available techniques, should aid in giving useful information not only about the rate of photosynthesis in intact plants, but also on the mechanisms by which the rates vary. It should allow researchers to verify data gained in the laboratory on plants in the field.

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