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Pulsed photoacoustic detection of flash-induced oxygen evolution from intact leaves and its oscillations

(photosynthesis/S states/*Nicotiana tabacum*/*Triticum kotschyi*)

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ABSTRACT **Photoacoustic signals from intact leaves, produced upon excitation with single-turnover flashes, were shown to be dependent on their position in the flash sequence. Compared to the signal obtained from the first flash, all the others were time-shifted and had increased amplitudes. The signal from the third flash had the largest deviation, whereas that from the second flash deviated only minimally. The amplitude difference of the signals relative to that from the first flash was measured at a convenient time point (5 ms) and showed oscillations of period 4, similar to the O_2-evolution pattern from algae. These oscillations were strongly damped, tending to a steady state from about the seventh flash on. The extra photoacoustic signal (relative to the first flash) was shown to be inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea, heat treatment, or water infiltration. Its change with flash number, its saturation with increasing flash energy, and the above inhibition criteria indicate that it originates in pulsed O_2 evolution. The sound wave produced by the first flash, however, arose by a photothermal mechanism only, as shown by its linear dependence on the flash intensity and insensitivity to the above treatments. The above flash pattern demonstrates that the photocycle of the S states (i.e., positive charge accumulation before two water molecules can be oxidized in a concerted way to produce molecular oxygen) occurs in intact leaves. It proves the applicability of the photoacoustic method for mechanistic studies of O_2 evolution in leaves under physiological conditions. Water content of leaves is readily measured by this method.**

The discovery of the photocycle of the S-states' charge accumulation required to form O_2 (1, 2) advanced our understanding of the mechanism of O_2 production in photosynthesis. Good reviews are available (3, 4). This work has resulted in the hypothesis of a manganese valence cycle (5). The characterization of the S states is most conveniently carried out by polarographic measurements of the O_2 evolved with a large-area electrode (6). However this method restricts the biological material to chloroplasts and single cells or near-unilamellar algae. It would be of great interest to have a method for determining the S-state pattern in other tissues, such as intact leaves. We have now found that this can be done by photoacoustic detection with pulsed light excitation.

Conventional photoacoustic measurements are based on formation of sound waves produced by periodically modulated light. The most common mechanism of sound production involves the conversion of the absorbed modulated light energy to modulated heat, which ultimately produces periodic contraction and expansion of a thin layer of the surrounding gaseous phase adherent to the sample, forming a pressure wave in the bulk gas (7). This pressure wave, sensed by a suitable detector (most commonly a microphone), depends on the optical and thermal properties of the sample (7) but also contains information on photochemical processes and the related energy storage (8). Photosynthetic systems are particularly suited to study of this last aspect, because the photochemical processes can be saturated with continuous light of sufficiently high intensity, allowing a convenient reference to determine the efficiency of energy conversion (9).

O_2-evolving photosynthetic systems give rise to another mechanism of photoacoustic signal generation. In leaves, one can distinguish a separate strong photoacoustic signal resulting from the direct production of a gas (O_2). This signal follows the light intensity modulation and thus results also in a pressure wave (10–13). The specific morphology of leaves particularly favors this kind of signal generation in that the diffusion path of the O_2 in the aqueous phase from the chloroplasts to the periphery of the mesophyll cell is quite short (about 1 μm). For low modulation frequencies (up to about 100 Hz) there is relatively small damping in the modulated flux of O_2 to the gaseous phase (the inner air phase), and a photoacoustic signal can be formed.

Conventional photoacoustic measurements are usually made by tuning the detection system to a single frequency of the exciting light through the use of a lock-in amplifier. In this way most of the unrelated ambient noise is filtered out. In the present work, however, we were able to demonstrate by direct observation photoacoustic pulses due to O_2 production by single-turnover flashes of light in leaves. This required a special experimental system, constructed to minimize noise and vibrations. As shown in this report these signals behaved according to S-state phenomena. This capability of the photoacoustic method makes a wide variety of plant tissue available for direct study of the photochemical state of the O_2-producing system.

MATERIALS AND METHODS

The flash-photoacoustic detection system consisted of a 10-μs-flash light source (E.G. & G. FX-132 lamp, 2.4 μF capacitor charged to 1–2 kV from a Voltronix power supply, and a triggering unit). The light flash was filtered through a Corning 4-96 glass (400- to 600-nm-wide band) and focused onto a branch of a bifurcated light guide coupled to a photoacoustic cell that has been described (10). The second branch of the light guide served to lead nonmodulated background light (from a 250-W tungsten iodine lamp) to the sample. This light was filtered through either a Corning 4-96 blue filter or a 680-nm interference filter (Ditric optics; 10-nm bandwidth). The photoacoustic signal was detected by a microphone (Knowles) whose current was preamplified 10-fold (Princeton Applied Research model 113) with low- and high-pass filters set between 1 Hz and 1 kHz. The output was connected directly to a Tektronix storage oscilloscope.

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Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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Special efforts were made to filter low-frequency vibrational noise by using rubber damping (tire) on a heavy table.

The pulsed photoacoustic signal is the time derivative of the pressure in the cell, as confirmed by separate experiments. For example, it was found that an input of a triangular sound wave became a square-wave output (data not shown). The positive part of the signal (see Fig. 1*a*) reflects the pulsed heat, generated by conversion of the light energy, which is transduced into a gas pressure pulse. The signal shape and amplitude thus depend on the optical and thermal properties of the sample. The amplitude is also inversely dependent on the cell volume. The cell dimensions in these experiments were 1-cm diameter × 1-mm thickness. The negative part of the signal reflects the dissipation of the pressure pulse in the cell. The pressure pulse produced by gas formation follows a similar pattern. The thermal signal from the intact leaf disc had a similar shape as that from a black tape (larger amplitude, 30% faster rise), white filter paper, or a suspension of algae on filter paper.

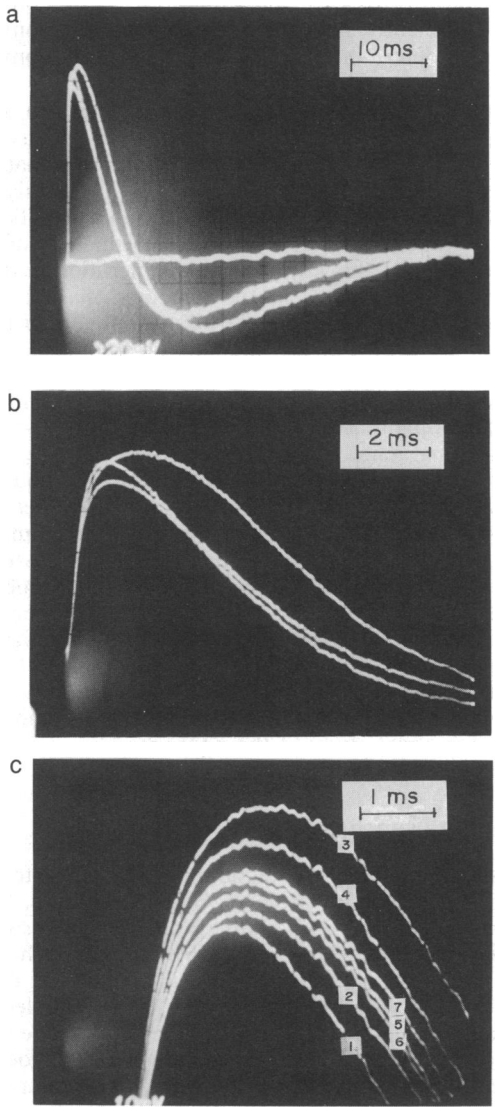

FIG. 1. (*a*) Oscilloscope traces of pulsed photoacoustic signals from a tobacco leaf after three successive flashes. The time between the flashes was 0.3 s. Time scale is as indicated (5 ms per division on the oscilloscope). (*b*) As in *a* but in 5 times faster time scale, as indicated. The signal maximum after the second flash is slightly higher than the first one, and after the third flash, the signal is yet larger and time-shifted (≈2 ms). (*c*) Pulsed photoacoustic signals after each of seven successive flashes. Flash interval was 1 s. Time scale is as indicated (0.5 ms per division on the oscilloscope). Signals were monitored on 2-times-expanded scale compared to *a* and *b*. Signals are labeled according to flash number.

The flash energy delivered onto the sample area (≈0.8 cm^2) was measured by an Ophir laser power model 38A/C lightmeter and was about 0.15 mJ.

Tobacco (*Nicotiana tabacum*) and wheat (*Triticum kotschyi*) plants were grown in daylight, as described (13). Discs from intact leaves were cut and placed in the photoacoustic cell, which has been described (10). In some cases the leaf discs were infiltrated with water under vacuum for several minutes. DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] was administered either by spraying a leaf in a whole plant and allowing it to stand for 48 hr or by incubating a leaf disc in 10 μM DCMU for ≈1 hr.

RESULTS

Fig. 1 *a* and *b* show the results of an experiment in which a dark-adapted tobacco leaf was exposed successively to three single-turnover flashes and the acoustic signal resulting from each flash was recorded. The general shape of each acoustic signal was that of a damped wave beginning with a positive sharp rise that is instrument-limited (≈100 μs), reaching a crest, and then decreasing to negative values, reaching a trough and finally decaying to the baseline. The total signal time was about 40 ms with a time width of about 5 ms for the positive part and of about 15 ms for the negative part. There was, however, a striking difference between the signals caused by the first two flashes and the signal caused by the third. The third clearly had a time-shifted (≈2 ms) component. Evidence that such difference was caused by oxygen evolution will be presented below.

However, the pattern of oscillations with the flash number is already a sufficient indication for this characterization. (Fig. 1*c* reveals the pattern obtained with seven successive flashes measured on an expanded scale for a better visual

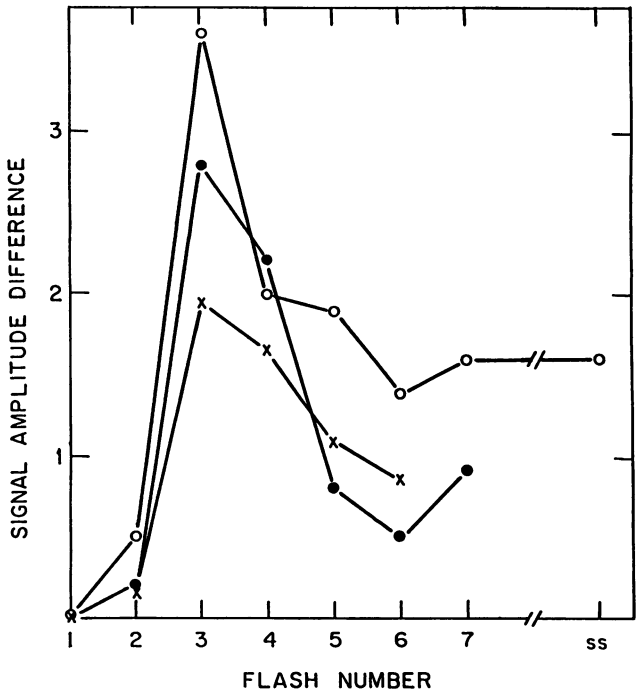

FIG. 2. Signal difference between the *n*th and first flashes as a function of flash sequence. Values were calculated from experiments similar to that of Fig. 1*c*. Three separate experiments with different tobacco leaves are shown. Signal units are arbitrary, measured in mV directly from the oscilloscope. ss, Steady state.

discrimination of the different experiments. Fig. 2 shows the difference between signals following the *n*th and the first flash at a time point chosen as most suitable (5 ms), as a function of the flash number. The difference is low for the second flash, maximal on the third, decreases gradually to a minimum on the sixth, and increases on the seventh. Further oscillations were within the noise of the measurement, and thus the steady-state signal was about equal to that of the seventh flash. The ratio of the signal of the third flash to that in the steady state was about 2.2.

These properties are characteristic of the S-state cycle obtained with algae and chloroplasts by measurement with the O_2 polarographic method (6). However, the damping of the oscillations is stronger in the intact leaf than in isolated chloroplasts. The additional signal on the third flash and the flash oscillation pattern are very reproducible and can be repeated over and over again, provided that the leaf is dark-adapted prior to the measurements.

The relaxation of the higher S states in the dark was measured by the recovery of the acoustic signal of the third flash obtained after various dark periods following previous illumination. Fig. 3 shows an example of such a measurement. The recovery kinetics had a half-time of about 30 s and was monoexponential over at least 80% of its extent. Increasing the dark period to 90 min did not reveal any further recovery. In spinach chloroplasts these kinetics were reported to be biphasic with a first-phase half-time of about 45 s (6).

The effect of an additional continuous background light on the flash response depended on the light intensity. Red light of very low intensity (0.18 W/m^2, 680 ± 10 nm) resulted in a greatly reduced difference between the third and first flashes (Fig. 4*b*). This reflected the mixing of the S states by the continuous light. High light intensity (75 W/m^2, 400–600 nm) removed any oscillation (Fig. 4*c*). This intensity was sufficient to saturate photosynthesis and thus prevent flash-induced O_2 formation. The half-width of the photoacoustic signal in the presence of continuous strong background light (Fig. 4*c*) increased from 3.5 ms in the control (Fig. 4*a*) to 4.8 ms. The half-width in the presence of dim red light was 4.3 (Fig. 4*b*). These differences are not presently understood and may be caused by photothermal effects occurring in a time range of about 5 ms.

Photothermal and O_2-evolution signals can also be distinguished on the basis that the latter must saturate with increasing flash energy, whereas the former is a linear function of energy (Fig. 5*a*). The amplitude of the signal observed on the first flash indeed increases linearly with flash energy, whereas the difference between the signal from the third and first flashes shows saturation (Fig. 5*a*). For a single-turnover flash, one usually expects that the normalized saturation curve of the resulting electron transfer will have

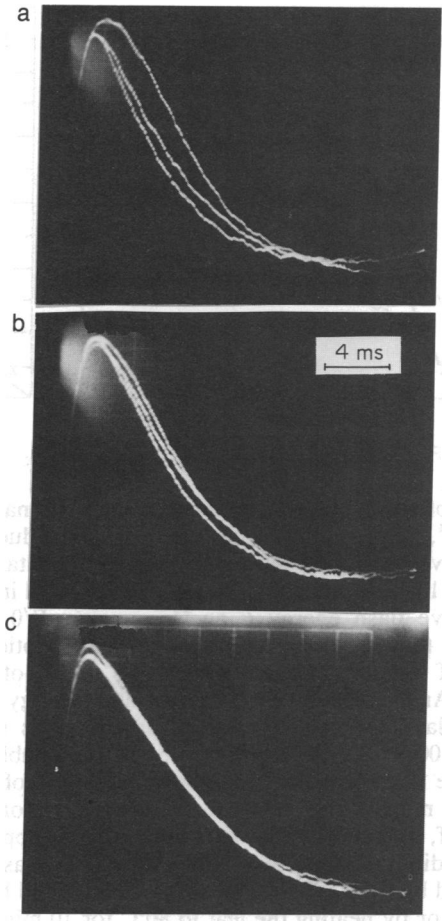

FIG. 4. Mixing and saturation of the S states in a tobacco leaf. (*a*) Photoacoustic signals in a dark-adapted leaf after three successive flashes, 0.3 s apart. (*b*) Leaf preilluminated for 5 sec with 680-nm d.c. background light (0.18 W/m^2) and then exposed to three successive flashes as in *a*. (*c*) Three successive flashes as in *a*, in the presence of 400- to 600-nm d.c. background light (75 W/m^2). Signals appear in the same order of their height as in Fig. 1 *a* and *b*. Time scale is indicated (2 ms per division on the oscilloscope).

the form $1 - \exp(-x)$, where x is the average number of hits per reaction center (14–17). The higher-energy points of the data roughly fit this algebraic form, but the low-energy points clearly fall well below the theoretical curve (Fig. 5*a*). This can be rationalized by noticing that, in order to produce the expected effect from the third flash, three successive hits should have occurred in any particular reaction center. Thus, flash energies below several hits per center will not advance the S states of all centers. To a good approximation, the

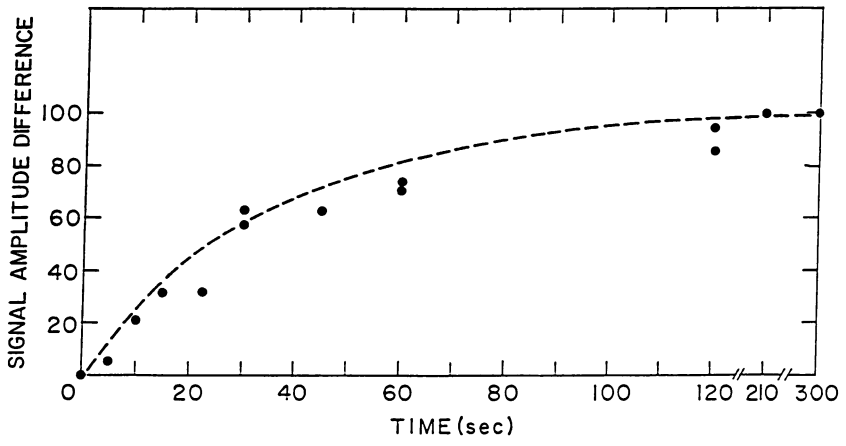

FIG. 3. Relaxation kinetics of the S states (S_3) in a tobacco leaf. The signal difference between third and first flashes (at 5 ms) was plotted as function of dark-adaptation time (●). The curve (– – –) is a theoretical plot of $y = 1 - \exp(-t/\tau)$ to fit the experimental points, where t is time and the half-time τ = 35 s.

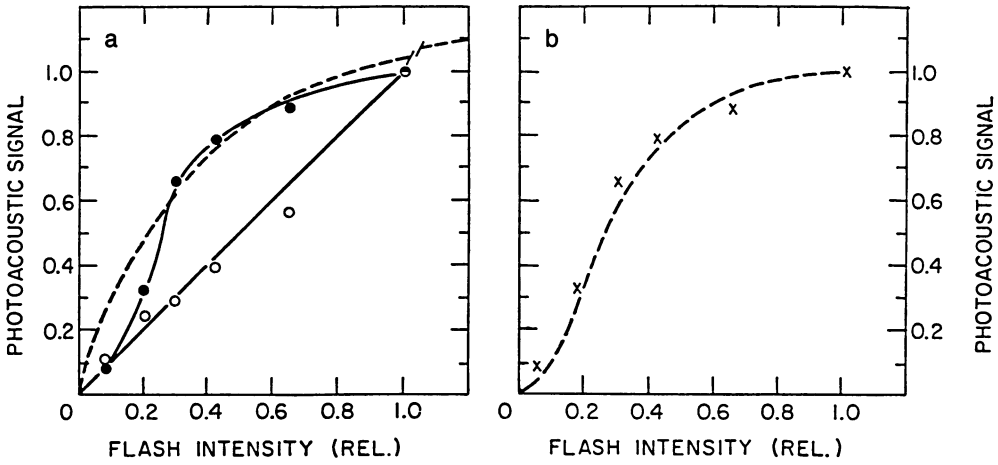

FIG. 5. (*a*) Saturation curves of the photoacoustic signals of the first flash (○) and of the difference between third and first flashes (●), with an attempted theoretical fitting by an exponential function passing through points of higher flash energy (*E*), $y = 1 - \exp(-E/0.35)$ (– – –). (*b*) Theoretical curve according to $y = [1 - \exp(-E/0.17)]^3$ and experimental points reproduced from *a*. Rel., relative.

advance of the S state three times is proportional to $[1 - \exp(-x)]^3$, since the hits from the flashes are due to independent events. Fig. 5*b* shows that the experimental points fit a relation like the one above, which is expressed in terms of the relative flash energy E as $[1 - \exp(-E/0.17)]^3$. By following this reasoning we can obtain the optical cross-section of the units, since we know the energy of the flash (14–17). An average of one hit occurs at an energy of 0.17 of the full flash energy. An average optical cross section of roughly 200 $Å^2$ is calculated, which is a reasonable number when one considers the broad-band blue light of the flash (16). One must bear in mind also the optical inhomogeneity of the leaf, so that the above equations are only approximate.

The additional signal seen on the third flash was shown to be caused by O_2 evolution, since it was inhibited by DCMU (Fig. 6*a*) or by heating the leaf to 80°C for 10 min (data not shown). It is interesting that the half-width of the signal was 4.8 ms, the same as in strong continuous light (Fig. 4*c*). Use of a water-infiltrated leaf furnished another proof for the assignment of the above-mentioned signal to gas evolution. In this case (Fig. 6*b*) excess signal was practically abolished, since the diffusion path for O_2 was increased about 30-fold [from ≈1 to ≈30 μm (10)].

The above samples were also measured by the conventional, modulated-light photoacoustic method. The DCMU- and heat-treated leaves showed an O_2-evolution signal of about 5% and the water-infiltrated leaf showed an O_2 signal about 10% that of the control leaf. The residual signals were probably caused by incomplete treatment. All three samples showed an expected thermal signal as large as in the control. The difference between the behavior of the thermal signal and that of the additional signal is strong evidence that the latter is indeed caused by O_2 diffusing out from the chloroplasts to the inner air phase of the leaf—hence its disappearance in the water-infiltrated leaf. This conclusion is further strengthened by an experiment showing an increase in time needed to reach the maximum of the O_2 signal, from ≈3 ms in a tobacco leaf (Fig. 1*b*) to ≈5 ms in a wheat leaf (Fig. 6*c*). The anatomical structure of the wheat leaf is consistent with this increased diffusion path (18).

The slower diffusion of O_2 compared to the thermal diffusion accounts for the lag in the signal seen on the third flash (Fig. 1 *a* and *b*). Hence the measure of the O_2 signal is chosen at the inflection point of the photothermal signal of the first flash. An attempt to measure an O_2 signal from the unicellular algae *Chlamydomonas reinhardtii* led to negative results. However, it is expected that such a signal would be 10–20 times lower in an algal suspension than in intact leaves (19, 20), owing to the longer diffusion path, so that the signal-to-noise ratio in the pulsed photoacoustic method is still insufficient to detect it.

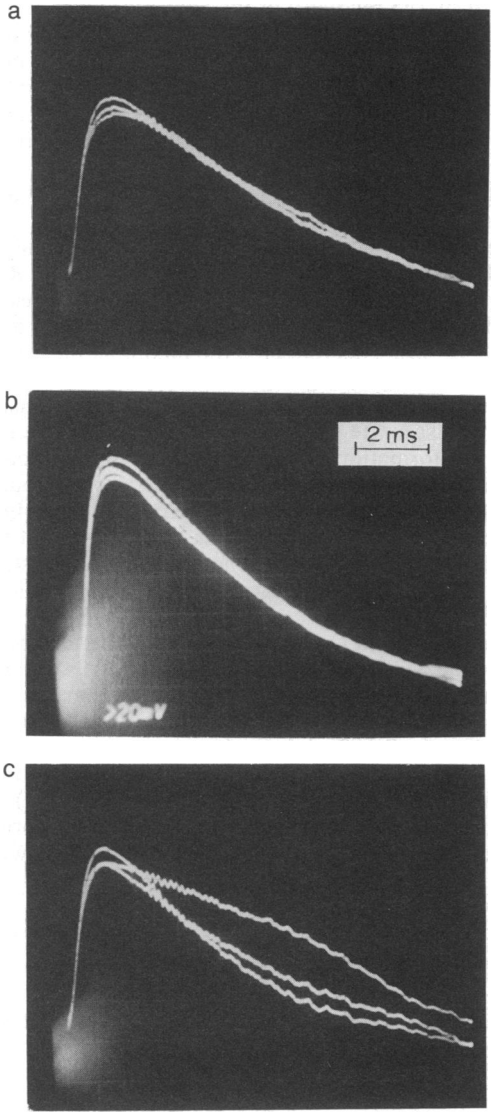

FIG. 6. Effect of treatments and leaf variation on the pulsed photoacoustic signals. (*a*) Tobacco leaf treated with 10 μM DCMU. Three flashes were 0.3 s apart. (*b*) Water-infiltrated tobacco leaf, dark-adapted 2 min. Three flashes were as in *a*. (*c*) Wheat leaf (*Triticum kotschyi*), untreated. Three flashes were as in *a*. The signals' heights are ordered as function of the flash number as in Fig. 1 *a* and *b*. Time scale is indicated (1 ms per division on the oscilloscope).

DISCUSSION

This work presents direct experimental demonstration of the S-state phenomenon in an intact leaf. Photoacoustic signals due to pure photothermal effects, excited by single flashes in leaves immersed in water, were detected previously by a fast piezoelectric transducer placed in the water. However, no O_2 signal could be observed under such conditions (21). Oscillations in both thermoluminescence and delayed light emission in intact leaves were interpreted to monitor the charge accumulation process in photosynthesis (22), but no method was available to measure directly O_2 produced by flashes in leaves.

The photoacoustic signal resulting from single-turnover flashes was shown to be dependent on the flash sequence. It was concluded that the sound wave produced by the first flash is purely photothermal, because of the linear dependence on the flash intensity and the insensitivity to treatments that inhibit photosynthesis or abolish the inner air phase. The second flash produced a slightly increased signal, but there is a large increase in the sound signal occurring on the third flash, which is also time-shifted by about 2 ms. This additional signal was shown to result from O_2 evolution by five criteria: oscillations resembling the S-state oscillations in algae and chloroplasts, saturation with increasing flash energy, and inhibition by DCMU, by heat treatment, and by water infiltration.

The delay of the O_2 signal reflects the diffusion of the gas from the leaf matrix to the inner air space, which varies significantly between leaves with different morphologies. By using the diffusion equation and known diffusion coefficients, estimates of *in vivo* diffusion paths in different leaves could be derived. Photoacoustic monitoring of the S states gives a direct measure of the photochemical activity of photosystem II. This observation provides a basis for monitoring the photochemical activities in leaves under different physiological conditions.

The photothermal signal was found to increase monotonically with a decrease in leaf water content, as expected because of decreased thermal capacity of the sample. Desiccation of the leaf was followed by a total loss of O_2 signal and by a further increase of the photothermal signal (data not shown). This observation, with a suitable calibration, can be readily used for a fast and easy measurement of leaf water content.

This type of flash photoacoustic method for the detection of O_2 evolution actually monitors a time domain on a time scale of micro- to milliseconds and is a complementary technique to the conventional modulated-light photoacoustic technique used in the frequency domain, which is usable in time scales of about 10–100 ms. To our knowledge, the flash photoacoustic method is so far the only one that can detect O_2 evolution from intact leaves in flashing light, and as such it is complementary to the O_2-electrode method, which is ideal for chloroplasts and unicellular algae. Pulsed photoacoustics will be suitable for quantitative analysis of absorption cross-sections of photosynthetic units in leaves through the use of monochromatic flashes of varying intensities (16).

This paper is dedicated to Prof. Ernst Fischer (Structural Chemistry Department, The Weizmann Institute) on the occasion of his 65th birthday. This work was partly supported by the Erna and Victor Hasselblad Foundation, Gothenburg, Sweden. D.M. wishes to thank the Rockefeller University–Weizmann Institute joint program for a personal fellowship.

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