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Fast photoacoustic transients from dark-adapted intact leaves: oxygen evolution and uptake pulses during photosynthetic induction – a phenomenology record

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Abstract: Using a photoacoustic technique it has been possible to observe fast oxygen evolution and uptake transients at a high time resolution (approx. 0.2 s), when a dark-adapted leaf is reilluminated. There is initially a rapid pulse of oxygen evolution, correlated with the initial fluorescence rise (total duration under the experimental conditions used about 1–2 s), corresponding presumably to the photoreduction of the plastoquinone pool. This phenomenon may be utilized to calibrate the oxygen-evolution photoacoustic signal. The first pulse is followed by a series of slower bursts of oxygen uptake and evolution, reflecting various pools which are expressed following sequential activation of various parts of the photosynthetic apparatus, until achievement of a steady state.

Key words: Oxygen (uptake, evolution) – Photoacoustic transient (fast) – Photosynthesis induction – *Spinacia* (photosynthesis).

Introduction

It is well known that during dark adaptation leaves progressively lose their photosynthetic capacity (Osterhout and Haas 1918; Rabinowitch 1956; Kelly et al. 1976; Walker 1976), an effect usually attributed to both inactivation of key enzymes in the ribulose-1,5-biphosphate (RuBP) cycle (Anderson 1979; Vu et al. 1984; Buchanan 1980; Heldt et al. 1981) and the depletion of intermediary metabolites (Walker 1976; Anderson 1979; Buchanan 1980; Heldt et al. 1981; Leegood and Walker 1981; Vu et al. 1984). Recently, another site for dark inactivation was discovered. Using photoacoustic detection, it was shown (Canaani and Malkin 1984) that at energy fluence rates as low as less than about 1 $W \cdot cm^{-2}$ there is a severe inhibition of excitation-energy transfer in photosystem I (PSI), as if the main antenna of PSI becomes functionally detached from its reaction centers. Only the small number of pigments with extended far-red absorption remain functional, as judged by the increase of oxygen evolution to normal levels by addition of far-red light.

Readaptation to light involves complex transients in measureable photosynthetic parameters following, sequentially, light activation of the RuBP enzymes (Heldt et al. 1981) and build-up of appropriate levels of intermediate substrates (Walker 1976; Leegood and Walker 1980, 1981). It was shown in wheat chloroplasts (Leegood and Walker 1980) that about 30 s were sufficient for the first stage to achieve enough activity to bring about the second stage, which then takes about 2 min until the steady state is reached. Thus, the rate of photosynthesis is slowly regained after a lag period, during which the above two processes occur (photosynthetic induction). Preliminary work on photoacoustic transients has indicated that PSI recovery is light dependent, occurring in a time scale of about 0.5–2 min (Canaani and Malkin 1984).

The aim of this work was to use photoacoustic detection to study the first steps of photosynthetic induction, at a fast time resolution (approx. 0.2 s). This method is very suitable for this purpose because of its sensitivity and fast response, and also since it monitors gross oxygen evolution at the lev-

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Abbreviations and symbols: RuBP = ribulose-1,5-bisphosphate; PSI, PSII = photosystems I, II; F_o, F_m, F(t) = initial, maximum and instantaneous chlorophyll fluorescence emission

el of the mesophyll cells (Bults et al. 1982; Poulet et al. 1983). Most other measurements of leaf photosynthesis have a time resolution which is insufficient to follow events in the range of a split second to several seconds. Fast physical observables, such as fluorescence, are useful, but their interpretation is not unequivocal. Transients in oxygen evolution from leaves, which were obtained polarographically (Blinks and Skow 1938; Prinsley et al. 1984) were probably limited by a time resolution of a few seconds. In the last case (Prinsley et al. 1984) the rate was not monitored directly but by electronic computation of the time derivative of the concentration, which could also reflect non-photosynthetic processes. For all the above reasons it was generally thought useful to add photoacoustics to the battery of existing methods for studying photosynthetic induction.

Dark-adapted photosynthetically inactive leaves are still expected, immediately upon resumption of irradiation, to evolve a brief pulse of oxygen, corresponding to the limited pool of reducible electron carriers between the two photosystems – notably plastoquinone (Kok and Cheniae 1986). Such an "oxygen gush" phenomenon was indeed previously observed, with the oxygen rate electrode, in algae and isolated chloroplasts (Joliot 1960; Joliot and Joliot 1968) but not in leaves. If both photosystems are active but the carbon-fixation cycle is not, the oxygen-evolution pulse will correspond to a larger pool, containing in addition the oxidants available to PSI (e.g. NADP and 3-phosphoglyceric acid). It was a particular aim of this work to see whether or not the photoacoustic method has the capability to record and differentiate such expected events in intact leaves. Since the plastoquinone and related electron-carrier pool is in principle measurable by other methods, it was also thought possible to use such expected phenomena for absolute calibration of both the steady-state photoacoustic oxygen evolution signal, and the various intermediary pools.

Photoacoustic transients in leaves have already been reported (Inoue et al. 1979). However, since at that time the interpretation of the photoacoustic signal in terms of gas exchange (Bults et al. 1982) was not known, the transients were discussed incorrectly in terms of changes in heat conductivity. This report was, nevertheless, valuable in discovering a phenomenological connection between the photoacoustic singal and photosynthesis, thus promoting further study. In our first studies on photoacoustic signals from leaves (Bults et al. 1982) very typical transients, attributed to gas exchange, were noticed but were not studied in any detail.

Material and methods

The photoacoustic method and equipment are described elsewhere (Bults et al. 1982; Poulet et al. 1983). In the present experimental system the modulated light was relatively strong, about 70 $W \cdot m^{-2}$, so that the resulting signal/noise ratio was quite high, and at low modulation frequencies allowed measurements with relatively very good time resolution (0.1 s setting on the lock-in amplifier – the ultimate time resolution was limited to about 0.2 s by the available recorder response). The sources for both modulated and background lights were 250-W quartz-iodine incandescent lamps properly housed and equipped with heat absorbing filters, such that the light output was limited to photosynthetic active radiation (400–750 nm). In most experiments, the modulated light passed through an interference 680 nm short-pass filter, to exclude far-red PSI light and also to allow simultaneous measurements of chlorophyll fluorescence at 715 nm. The optical path of the modulated light was equipped with a chopper and both modulated and background lights could be rapidly switched on and off by suitable fast shutters. Both lights were collected each by a corresponding arm of a triple light guide, combined at the common end and passed onto the sample. The third branch served to transmit fluorescence from the sample to a photomultiplier (EMI, Hayes, Middlesex, UK) protected by a 715-nm interference filter.

Processing of the microphone signals was made by a single-phase lock-in amplifier (model 9503; Brookdeal, Bracknell, Berks., UK). The phase angle was arbitrary, but usually manipulated to obtain good ratio of the oxygen part of the signal to the photothermal part. The fluorescence signal was fed directly into the recorder and was demodulated by a capacitor which was put across its leads.

Spinach (*Spinacia oleracea*) leaves were routinely used from plants grown hydroponically (Walker 1980). Other species were also tried, yielding very similar results. Leaf discs were cut and placed inside the O-ring of the photoacoustic cells. Alternatively, whole leaves were clamped inside the cavity with their peripheries exposed to the atmosphere. In a later stage of the work the photoacoustic cell was placed in a plastic box which could be closed tightly with a top cover. With installation of proper inlet and outlet, gas of controlled composition was passed through the box to establish an environment of known composition. In this case, when a whole leaf was placed in the photoacoustic apparatus, its main area, except for the part enclosed within the photoacoustic cavity, was in contact with the external gas environment. Streaming of CO_2 had a profound effect on the response, as if CO_2 could penetrate laterally through the inner air space to the clamped region (see *Results and discussion*).

The experimental protocol consisted of necessary adjustments of the instrumentation while illuminating the clamped sample, which was previously dark-adapted for approx. 1–3 h. During the adjustment, as was recorded, the signal reached a steady state. Another dark-adaptation period (usually 10 min, or as otherwise indicated) was then given. Experiments were often repeated with the same sample, always allowing suitable periods of dark adaptation.

Results and discussion

Figure 1a shows a typical example of a photoacoustic signal pulse obtained from a dark-adapted leaf when the modulated light was switched on rapidly (in about 10 ms). To ensure that the pulse was not an artifact caused by the electronic equip-

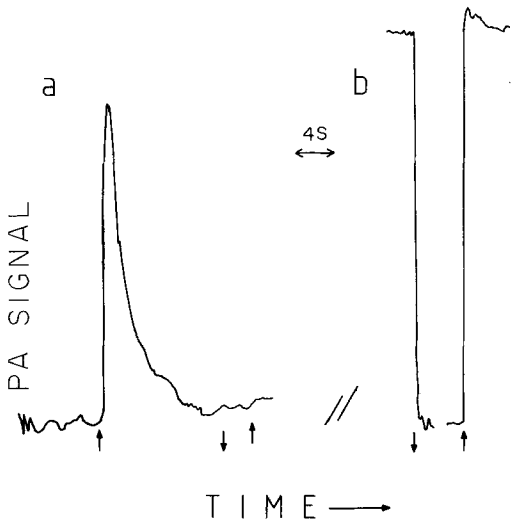


Fig. 1 a, b. Photoacoustic transient pulses of a dark-adapted (10 min) spinach leaf before (**a**) and after (**b**) achievement of the steady state in continuous modulated light. **a** and **b** are sectional traces from the same experiment. Light energy fluence rate = 70 $W \cdot m^{-2}$; modulation frequency 16 Hz; time resolution = 0.2 s. *Upward arrow* = light on; *downward arrow* = light off

ment, two checks were performed. (i) When the pulse was terminated the light was immediately switched off and on again, with no noticeable effect (Fig. 1 a, right). (ii) Similarly, following a period of illumination (approx. 2 min) and attainment of the steady state the light was again switched off and on subsequently. In this case there was a smooth immediate attainment of the same level, and no transitory behaviour was noticed within the experimental resolution time (Fig. 1 b). The latter check also defines the resolution time of the detection system to be approx. 0.2 s. The photoacoustic pulse of Fig. 1 a is therefore a meaningful effect, which is interpreted here as mainly reflecting an oxygen-evolution pulse (see later). It has a short duration (approx. 1 s half-width) and appears after dark adaptation.

Figure 2 is another typical example showing simultaneous fluorescence and photoacoustic transient events over an extended time period. The first event is again a repeat of the very rapid photoacoustic pulse. The decaying part of the initial pulse was seemingly correlated with the fast initial phase of the fluorescence induction, i.e the fluorescence rise from an initial F_o level, reaching a maximum level F_m (Fig. 2). This behaviour resembles that of the "oxygen gush" from chloroplasts or algal suspensions (Joliot 1960; Joliot and Joliot 1968). Indeed one expects (Malkin and Kok 1966; Lavorel and Etienne 1977) that the relative rate of electron transport will be linearly correlated with the parameter $f = (F_m - F(t))/F_m - F_o)$ (where F(t) is the momentary value of the fluorescence). This phenomenon probably manifests the limited-extent electron transport from water to the plastoquinone pool and should be reflected in the same way in both the fluorescence rise and in the decay

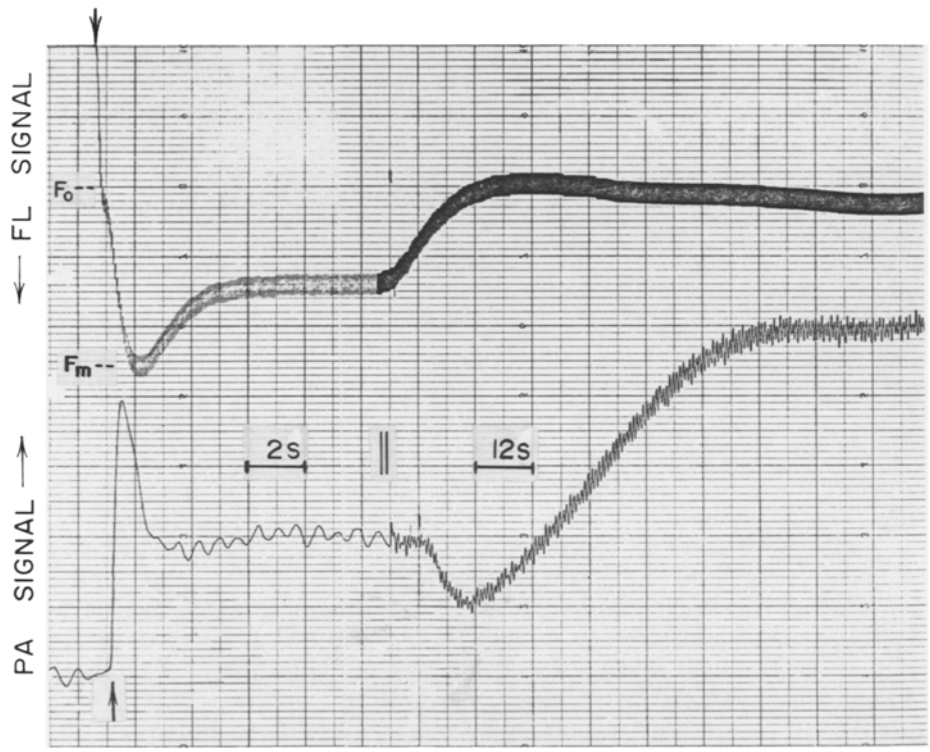


Fig. 2. Simultaneous photoacoustic (*bottom curve*) and fluorescence (*upper curve*) transients of a dark-adapted (11 min) spinach leaf. Other conditions as in Fig. 1

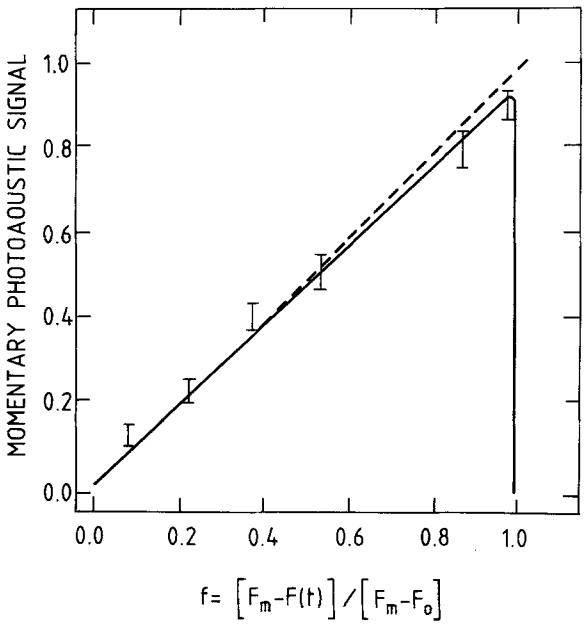


Fig. 3. Plot of the momentary photoacoustic signal versus the fluorescence function *f* (see *Results and discussion*) during the initial transient pulse phase, as in Fig. 2. Vertical bars represent the range of uncertainty in the readings from the record of raw data. Dashed line is the linear continuation of the experimental curve

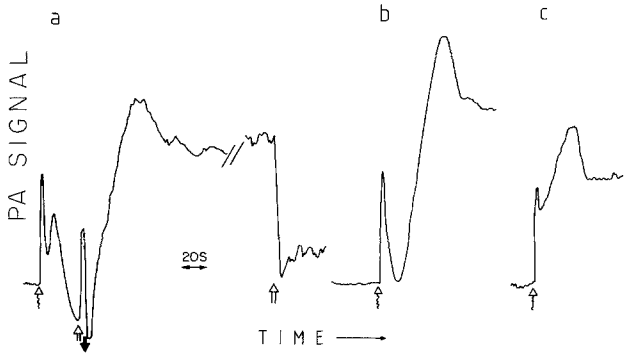


Fig. 4a–c. Demonstration of oxygen evolution and uptake pulses from a spinach leaf during the photoacoustic transients. Conditions were similar to those described in Fig. 1, except for a lower time resolution (1 s). Dark-adaptation times were: **a** 7 min; **b** 3 min; **c** 2 min (all the same sample). ⇕ = Modulated light on. Establishment of a zero baseline for the gas-exchange transients was by background-saturating non-modulated light (on ⇑, off ↓) given shortly during the transient and in the steady state

of oxygen evolution. The plot in Fig. 3 demonstrates that the two phenomena are correlated in the above manner. More accurately, this correlation does not hold for the rapidly rising side of the photoacoustic pulse (Figs. 2, 3). Otherwise, at time zero the oxygen evolution rate would be immediately at its maximum. Several factors limit the rising phase of the pulse, among which are: (i) charge accumulation processes (S-state phenomena), which precede water photolysis (Kok et al. 1970) and take a time corresponding to about an *average* of two turnovers in continous light in the rising phase (see Fig. 10 of Forbush et al. 1971), relative to the time of "filling" the plastoquinone pool, which is about 10–20 turnovers; (ii) diffusion of oxygen to the gaseous inner phase (Poulet et al. 1983) (estimated to be in a time range of about 50 ms), as well as (iii) the time limitation of the experimental system.

The "oxygen gush" can be used in principle to calibrate the photoacoustic oxygen-evolution signal in the steady state, provided that the reducible plastoquinone pool size (per chlorophyll content) can be quantitated, e.g. by using the method as developed in by Malkin and Kok (1966) for isolated chloroplasts. As an illustration, let us take a conventional pool size of 1/20 electronequivalents per chlorophyll (Kok and Cheniae 1966; Malkin and Kok (1966) and the data of Fig. 2. From the total chlorophyll, determined by extraction and spectroscopic analysis (approx. 40 nmol·cm^{-2}), the pool size per leaf unit area is estimated to be 2 nequiv.·cm^{-2}. Denoting the final steady level of the photoacoustic oxygen signal by S, the integration of the oxygen pulse gave a number around 0.36S. Hence S corresponds to 2/0.36 = 5.6 equiv. ·cm^{-2}·s^{-1}. The time average of the modulated light intensity (including the dark cycles) is about 35 nmol·cm^{-2}·s^{-1}, from which the steady-state quantum-yield is calculated to be 0.16 e^{-}/hν. The last number is smaller by a factor of about 2.5–3 from the maximum theoretical number (i.e. 0.4–0.5). This lower yield could be explained (compare Ehleringer and Bjorkman 1977) by the unfavorable gas composition in the photoacoustic cavity at the steady state, where because of the very small volume, CO_2 is expected to be rapidly depleted and arrive at a compensation point. Poulet et al. (1983), using the photoacoustic method, estimated the maximal rate of photosynthesis, from a relative saturation curve obtained by adding background light at various intensities, assuming that the quantum yield at the limiting light range is equal to the maximal theoretical value. If, however, the present estimation of the quantum yield for the given experimental conditions is more general, then the values obtained by Poulet et al. (1983) are largely overestimated.

A set of complicated, slower photoacoustic transients followed the initial oxygen pulse in the form of waves including increase and decrease phases (Fig. 2). The pattern of these waves varied, depending on preillumination/dark treatment, as shown in the several examples of Fig. 4. Frequently, following the first pulse, there is a second wave, which is much slower and usually quite shal-

low (Fig. 2), but sometimes comparable in amplitude to the first pulse (Fig. 4a). The signal height between these pulses and particularly the one following the second pulse very often reach *negative* values (Fig. 4a). This apparently surprising result forces the assignment of the decreasing waves to a transiently increasing proportion of oxygen *uptake,* which at low modulation frequencies is expected to have phase angle nearly opposite to that of oxygen evolution. Since this contribution is evidently modulated it probably reflects an uptake site very near to a photoreaction step (cf. below).

Regarding the above, it was important to check that the level of the maximal photothermal signal remained the same during the time of the transients, so as to establish a zero base line for the oxygen evolution/uptake transients. A strong background light was applied at various times during the occurrence of the transients. When the photoacoustic signal was relatively low or negative the signal then *increased* with the background light, but achieved a level very similar to that obtained with the background light at the steady state (Fig. 4a). It may be recalled that at the steady state an application of a saturating background light always caused a *decrease* of the signal, which then reflected the elimination of the modulated oxygen evolution (Bults et al. 1982). The background light effect to *increase* the signal is interpreted likewise as the elimination of the negative modulated oxygen evolution (i.e. oxygen uptake). The constant level achieved with the background light represents the non-variable maximum thermal contribution.

One anticipates that oxygen evolution coupled to plastoquinone reduction has considerable potential capacity for energy-storage. Hence the photothermal contribution to the photoacoustic signal, during the fast pulse at least, should also change transiently, increasing significantly in the same manner as the fluorescence. Therefore, hidden in the total fast-transient pulse there may be a contribution from a rising transient photothermal signal. The photothermal contribution can be avoided, however, by adjusting the phase angle of the lock-in amplifier to separate out only oxygen signals (apparently the case in Fig. 1), otherwise the oxygen-pulse part is itself obviously overestimated. To check changes in the photothermal signals caused by changes in energy storage, experiments should be conducted at a high modulation frequency where only the photothermal contribution persists (Bults et al. 1982) (e.g. approx. 400 Hz). The plastoquinone reduction could be then also reflected as a *rising* photoacoustic transient similar to the fluorescence one. Unfortunately in such high-frequency experiments the signal-to-noise ratio was

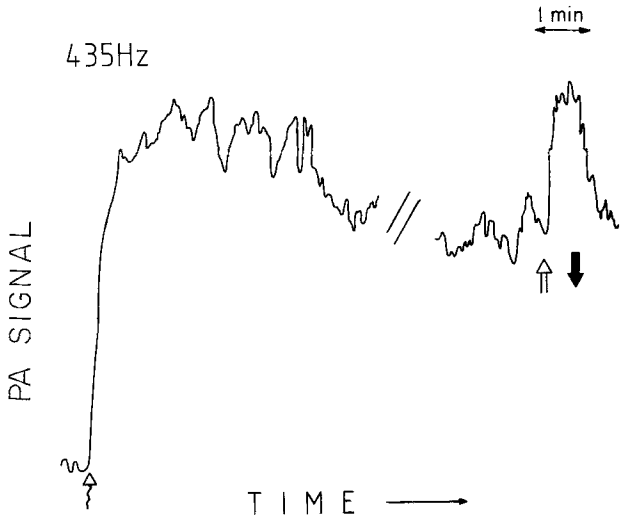


Fig. 5. Photoacoustic transients from a spinach leaf at high modulation frequency (435 Hz). Time resolution = 3 s. Dark-adaptation time = 7 min. Arrow symbols as in Fig. 4.

so unfavorable such that a very low time resolution had to be set, insufficient to distinguish any fast transients (Fig. 5). Here one can distinguish only a mild and slowly decreasing transient, which starts mainly at the time when most of the low-frequency transients have occurred and roughly parallels the main transition towards steady photosynthesis. The discovery of fast photothermal transients must await a better experimental system.

The photoacoustic measurements thus indicate complex transients in oxygen evolution and uptake, with possible contamination by photothermal transients. Oxygen uptake at steady-state conditions from leaves, isolated cells and chloroplasts has been demonstrated previously by mass-spectrometric analysis (Hoch et al. 1963; Canvin et al. 1980; Behrens et al. 1982; Furbank et al. 1982, 1983), and was shown to persist even at high CO_2 concentrations, where the RuBP-oxygenase system is less competitive, or in cell types lacking altogether the RuBP oxygenase/carboxylase (Furbank et al. 1983). This persistent oxygen uptake was taken to indicate the importance of the oxygen-uptake site near to the immediate electron-acceptor side of PSI (Mehler reaction). With the photoacoustic detection it is probably only the (transient) Mehler-type oxygen uptake which is detected, since the signal modulation amplitude for oxygen-uptake sites downstream of the electron-transport chain would tend to be extensively damped.

The total photoacoustic transients thus seem to be combinations of both evolution and uptake waves. At this stage it would be much too premature to suggest more then a plausible but tentative

working hypothesis. The transients possibly reflect the following consecutive events:

1. As light is resumed, following the dark-adaptation period, only PSII is active. This results in the pulse of fast oxygen evolution caused by the acceptor pool between the two photosystems.

2. Photosystem I becomes active in electron transport to O_2, but not to NADP, resulting in the first wave of oxygen uptake. Alternatively, oxygen uptake may occur also through the carriers of PSII alone.

3. Endogenous acceptor pools (NADP, 3-phosphoglyceric acid, etc.) become available to PSI, competing with O_2 uptake – a second slower O_2-evolution pulse results.

4. The immediate acceptor pool of PSI is depleted, O_2 uptake is resumed again and is maximal.

5. There is a slow autocatalytic build-up of the (RuBP) acceptor pool reflected by the gradual approach to the steady state.

While these propositions will be checked in more detail in future work, they are based on a previous knowledge of the system as mentioned above. Note that stages 2 and 3 depend on the fact that NADP may be not available to PSI initially, in accordance with the observation that NADP reductase requires light activation (Carillo et al. 1981; see also Horton 1983 for the interpretation of fluorescence transients regarding this proposition). It is also interesting to compare these results with those of Prinsley and Leegood (1986) on photosynthetic induction using the oxygen electrode. They were able to resolve an initial oxygen burst lasting about 30 s and suggested that this corresponds to the total acceptor pool available, including particularly 3-phosphoglyceric acid (calculating an overall pool size of about 60–100 nequiv. $\cdot mg^{-1}$ chlorophyll). Seemingly therefore, there was no resolution of the faster events. Whether this was a consequence of the experimental technique, or of the totally different conditions utilized (much lower light intensity and an atmosphere of 5% CO_2) has still to be resolved.

Although the phenomena described above generally apply to most of the samples, the degree of variability in individual experiments could be quite large. Seemingly the second oxygen-evolution pulse and the following uptake pulses depend very much on the particular history of light and dark adaptation. In some experiments, the first and second pulses intermingled, forming a usually wider pulse in which the presence of the second pulse was occasionally apparent as a secondary peak or a shoulder (Figs. 6, 7). A particular observation was that a transition occurred, in the same sample,

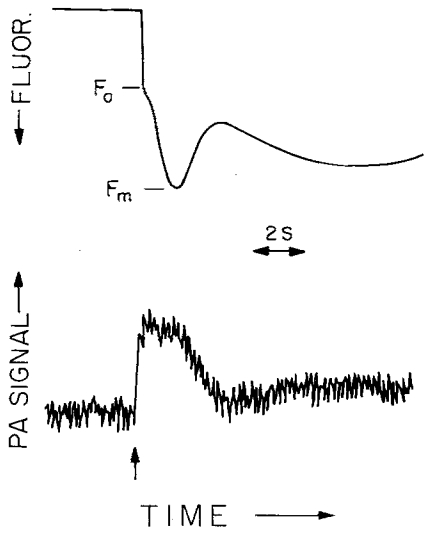
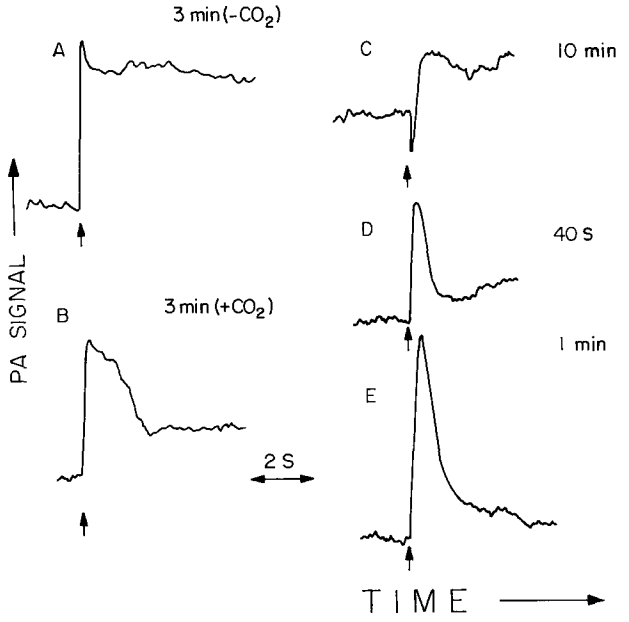


Fig. 6. Photoacoustic (*lower curve*) and fluorescence (*upper curve*) transients from a spinach leaf, in the anomalous case where the "wider" photoacoustic pulse appears (see text). *Upward arrow* = modulated light on. Other conditions similar to that of Fig. 1

from the "normal" transients to the "widened" pulse transient, as a result of passing a stream 5% CO_2/air mixture. (Fig. 7, compare A and B). For this experiment the photoacoustic cell was put in a closed container, through which a gas inlet and outlet were installed, and the leaf edges were in contact with the induced atmosphere. In terms of the above propositions, it is possible that stages 2 and 3 (PSI activation) then became so short that the first two oxygen-evolution pulses occurred almost simultaneously. The particular effect of 5% CO_2 could be partly explained by an intensive carboxylation and the formation of a large pool of 3-phosphoglyceric acid. The overall "widened" pulse area was frequently anomalous in its dependence on the previous dark time, having a relatively smaller time width at 1 min, peaking in its area at about 3 min and mostly *disappearing* at about 10 min, and finally even being replaced by a rapid oxygen uptake (i.e. negative) pulse (Fig. 7c). While expecting dark dependence of the total acceptor pool, it was strange that in this case the fast oxygen-evolution pulse was not shown after the longest dark-adaptation time, despite the normal appearance of the usual fluorescence induction transient. Strangely enough, in a small number of individual experiments this exceptional behaviour was repeated even under normal atmosphere (i.e. without streaming CO_2). This presented a real dilemma: how is it possible that the plastoquinone pool undergoes photoreduction (indicated by the fluorescence induction), while there is no equivalent oxygen evolution? One is forced



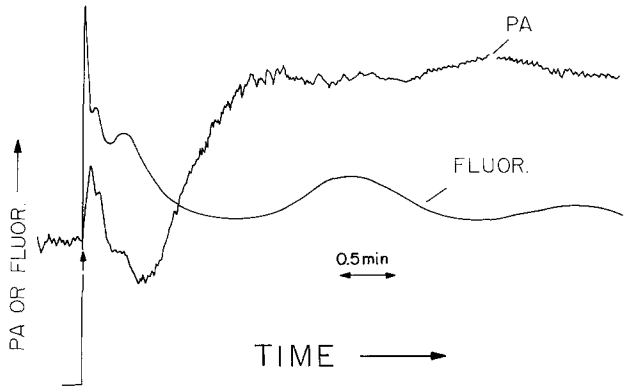


Fig. 8. An example of the correlation of photoacoustic (*PA*) and fluorescence (*FLUOR.*) transients from a spinach leaf, in a 5% CO_2 atmosphere, showing damped oscillations (see text). Other conditions similar to that of Fig. 1, except for a lower time resolution (3 s)

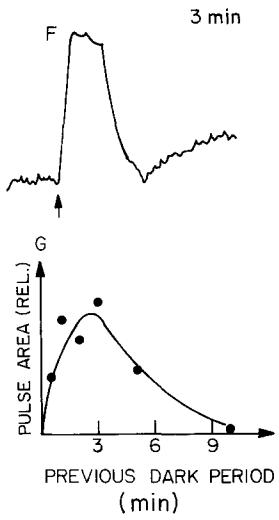


Fig. 7A–G. The effect of 5% CO_2 streaming around the photoacoustic cell. Numbers indicate dark-adaptation times. **A** Control, without CO_2; **B–F** after streaming CO_2 (**F** is a repeat of **B**). Notice that relatively narrower pulses are obtained with short dark times (e.g. < 1 min) and wider pulses appear at intermediate dark times (approx. 3 min). In **G** there is a plot of the pulse area versus the dark time. *Upward arrow* = modulated light on

to speculate that in such cases there exists another pool of reductants which replaces water.

When the steady state was nearly achieved there were frequently slow oscillations of the photoacoustic signal, accompanying fluorescence oscillations. (see e.g. Figs. 4 and 8, showing also more oscillation waves during the induction period). This effect, as with the change from "normal" to "widened" transients, described above, became much more pronounced when 5% CO_2 was streamed around the photoacoustic cell and the periphery of the leaf was in contact with the stream (Fig. 8). This observation is in accord with the results of Walker et al. (1983), who clearly demonstrated oscillations in photosynthesis and related parameters, interpreting them to be the result of an interplay of the carbon cycle, electron transport, phosphorylation and chemoismotic parameters.

From the results of this paper one may arrive at some more general statements concerning the photoacoustic signal. It is clear that not only the very fast photoacoustic and fluorescence transients are correlated, but there is also an intimate connection between the two slow types of transients in that they mark various photosynthetic events, although they do not match exactly in time (e.g. Figs. 2 and 8). In a way such results give further support for interpreting the photoacoustic signal in terms of gas exchange.

The fact that the enclosed part of a leaf responds to an external stream of 5% CO_2 in its exposed part (Figs. 7, 8), possibly indicates lateral gas diffusion inside the leaf from the exposed parts to the enclosed part. This indicates that it may be possible to achieve at least some control over the gas environment of the leaf in photoacoustic experiments (The more direct approach of streaming gas directly through the photoacoustic cavity itself led to an intolerable high noise/signal ratio).

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