Simultaneous Measurement of Oscillations in Oxygen Evolution and Chlorophyll $a$ Fluorescence in Leaf Pieces

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ABSTRACT

In spinach (Spinacia oleracea) and barley (Hordeum vulgare) leaves, chlorophyll $a$ fluorescence and O$_2$ evolution have been measured simultaneously following re-illumination after a dark interval or when steady state photosynthesis has been perturbed by changes in the gas phase. In high CO$_2$ concentrations, both O$_2$ and fluorescence can display marked dampening oscillations that are antiparallel but slightly out of phase (a rise or fall in fluorescence anticipates a corresponding fall or rise in O$_2$ by about 10 to 15 seconds). Infrared gas analysis measurements showed that CO$_2$ uptake behaved like O$_2$ evolution both in the period of oscillation (about 1 minute) and in its relation to fluorescence. In the steady state, oscillations were initiated by increases in CO$_2$ or by increases or decreases in O$_2$. Oscillations in O$_2$ or CO$_2$ did not occur without associated oscillations in fluorescence and the latter was a sensitive indicator of the former. The relationship between such oscillations in photosynthetic carbon assimilation and chlorophyll $a$ fluorescence is discussed in the context of the effect of ATP or NADPH consumption on known quenching mechanisms.

In 1949, Van der Veen (23) described what he called 'irregularities in photosynthesis' during the period when a leaf is suddenly illuminated after a period in darkness. These irregularities during the induction period, i.e. the lag before maximal photosynthesis is achieved following re-illumination (28), included 'secondary peaks' (or increases and decreases in rate which finally gave way to a more or less steady rate of CO$_2$ uptake). Similar but somewhat simpler irregularities associated with re-illumination had already been observed by McAllister and Myers (16) and by Aufdenkamper (2). They were immediately apparent when renewed attempts were made to follow O$_2$ evolution from leaf discs by polarographic methods (26). More recently (5, 10, 14, 17, 19), oscillations have been induced by changing the O$_2$ or CO$_2$ content in the atmosphere surrounding an illuminated leaf. The fact that oscillations have been detected by such disparate methods as diathermometer (23, 24), IRGA (5, 10, 14, 17), polarography (26, 28, 29), and $^{14}$C incorporation (30) suggests that they are a genuine manifestation of changes in photosynthetic rate and not artifacts of measurement. It has been suggested that they might be brought about by over-reaction of regulatory mechanisms (21, 22, 27, 28).

Chl $a$ fluorescence kinetics during induction have also been studied for many years, and many attempts have been made to relate these relatively slow changes (that normally occur after the first few s of illumination), with changes in O$_2$ evolution and/or CO$_2$ uptake (see e.g. Ref. 16). Surprisingly, Van der Veen (24) did not follow the fluorescence changes associated with the more dramatic secondary peaks in CO$_2$ fixation which he had observed (cf. Refs 23 and 24) but nevertheless concluded that 'nearly always increase of photosynthesis during adaptation (induction) is correlated with a decrease of fluorescence.' In algae, the relationship seems less clear and Chl fluorescence has been found to rise in parallel with O$_2$ evolution in some circumstances (3) but not in others (18) and Ogawa (19) has recently reported that fluorescence yield and O$_2$ evolution oscillate in phase (in parallel) in Vicia faba leaves under anaerobic conditions. In preliminary experiments with spinach, antiparallel, dampening oscillations in O$_2$ and fluorescence were observed (29). These observations have now been confirmed and further characterization of these phenomena is reported.

MATERIALS AND METHODS

Plant Material. Spinach (Spinacia oleracea L. U. S. Hybrid 424, Ferry Morse Seed Co., Mountain View, CA) was grown for 6 weeks in water culture in a glasshouse as described previously (26) and discs were cut from leaves with a sharp cork borer. Barley (Hordeum vulgare L. var. Sonjia, Nickerson RPB Ltd., Rothwell, Lincoln, U.K.) seedlings were grown for 8-9 d in vermiculite in a glasshouse under sunlight and supplementary incandescent lamps. The plant material was harvested when the leaves were between 13 and 15 cm long. Leaf pieces of 2-cm length were cut (at about 9 cm from the leaf base) using the more mature part of the first leaf but discarding the first 1.5 to 2 cm of the leaf tops.

Oxygen Evolution and Fluorescence. Methods were essentially the same as those used by Delieu and Walker (7). Temperature was kept constant at 20°C. Spinach leaf discs (10 cm$^2$) or 2-cm length barley leaf pieces (5 to 8 pieces, about 7 cm$^2$) were placed in a leaf disc O$_2$ electrode that had been modified to allow simultaneous detection of Chl $a$ fluorescence.

This apparatus (Hansatech Ltd., Norfolk, U.K.) was constructed of anodized aluminum to give improved temperature control and to minimize stray light (7). Two fiber optics were used. The first was a 13-mm-diameter bundle which brought the actinic light into the top of the chamber. A second fiber optic, inserted into the upper section of the apparatus at an angle of 40°, was used to guide light emissions to a photodiode for fluorescence measurement. The exciting light was either a 150 W tungsten/halogen source (Volpi Intralux 5000) or a light source designed by Delieu and Walker (7) powered by a Coutant regulated DC power supply and filtered through heat-absorbing glass, a heat-reflecting interference filter, and a Corning 4-96 glass filter. Emission was defined by a Corning 2-64 filter and meas-
ured using an UDT Phot-op photodiode. The fluorescence signal, O$_2$ signal, and the differential of the O$_2$ signals were fed to a chart recorder. To follow O$_2$ evolution by polarography, the leaf chamber has to be used as a closed system (the relatively low sensitivity of the O$_2$ electrode systems currently available precludes open system use). Two alternative methods were employed to permit high rates of photosynthesis in a small closed chamber for relatively long periods without undue depletion of CO$_2$: (a) capillary matting moistened with carbonate/bicarbonate buffer (7); (b) flushing the chamber with a gas mixture containing 5% CO$_2$ and 20% O$_2$ (balance N$_2$) immediately before the chamber was closed. The second method was preferred when the gas phase was to be changed during the course of the experiment.

Absolute rates of O$_2$ evolution were only occasionally determined because experience showed that they, within the limits employed, were largely irrelevant. When measured accurately, they were found to be close to 150 μmol mg$^{-1}$ Chl h$^{-1}$ which is about half the rate displayed by the same leaf material in near-saturating white light (actinic blue light was used to facilitate the measurement of weak red fluorescence). The light source and filters provided 70 W·m$^{-2}$ blue light at the leaf surface and 210 W·m$^{-2}$ of PAR when the blue filters were removed.

![Diagram](https://www.plantphysiol.org)
RESULTS

Antiparallel but Phase-Shifted Relationship between Chl a Fluorescence and Rate of O₂ Evolution in Leaves. Figure 1 shows damping oscillations in Chl a fluorescence and corresponding but antiparallel oscillations in the rate of O₂ evolution. These were initiated in a disc cut from a spinach leaf by illumination after a period of darkness. The leaf material was surrounded by an atmosphere which had been selected because it had been found to favor large oscillations and, indeed, oscillations can even be initiated in continuous light by changing the gas phase from air to one with the composition used in Figure 1 (Fig. 3). Nevertheless, these particular conditions (high CO₂, low O₂ and interrupted illumination) were only one of many combinations in which similar damping oscillations were observed (see below). Figure 1 is included because the oscillations themselves were pronounced and a number of characteristic features can be readily discerned. The antiparallel relationship between the fluorescence signal and the differential of O₂ evolution (29) are immediately apparent but it may also be noted that the fluorescence signal anticipates the changes in the rate of O₂ evolution by about 15 s (i.e. a fall in fluorescence gives warning of increase in the rate of O₂ evolution and a rise in fluorescence indicates that a fall in O₂ is imminent). How much of the delay in the O₂ signal is real and how much is attributable to any delay by the O₂ electrode in sensing a change in O₂ (see Ref. 7) is still somewhat uncertain. Present experience suggests that the extent to which fluorescence anticipates O₂ is somewhat variable (12 ± 2–3 s) but the rapidity of the electrode response (e.g. injected O₂ is sensed in less than 2 s) implies that most of the discrepancy between fluorescence and O₂ is real (i.e. that the fluorescence signals are prompted by changes in metabolism which then bring about corresponding changes in net O₂ evolution). In some experiments (not illustrated), the extent to which the fluorescence signal anticipated the O₂ response clearly varied from oscillation to oscillation and the time difference between fluorescence peak and O₂ trough was greater than that between fluorescence trough and O₂ peak.

It should be noted that we have taken the simplest view, that the signals are antiparallel but slightly out of phase. It could be argued, we believe with great difficulty, that the signals are parallel but substantially out of phase. This latter interpretation would mean that there is no obvious relationship between the size of the signals and that a large change in one could occur without a correspondingly large change in the other.

Antiparallel and Phase-Shifted Relationship between Chl a Fluorescence and Rate of CO₂ Fixation in Leaves. Figure 2 shows simultaneous measurements of Chl a fluorescence and CO₂ depletion (IRGA). As in Figure 1, the oscillations were induced by re-illumination in an atmosphere containing low O₂ (2%) and high CO₂ (960 µl/l). Again it can be seen that a rise in fluorescence anticipates a fall in photosynthetic carbon assimilation (i.e. an increase in the CO₂ content of the gas stream). Although it has not yet proved practicable to measure CO₂, O₂, and Chl a fluorescence under precisely the same conditions, Figures 1 and
2 nevertheless imply that fluorescence rises and falls slightly in advance of photosynthesis, and in an antiparallel manner and that it is largely immaterial whether photosynthesis is monitored by O₂ evolution or CO₂ fixation.

**Initiation of Oscillations by Changes in Gas Phase under Continuous Illumination. Changes in CO₂.** In Figures 1 and 2, oscillations were induced by re-illumination after a dark interval in atmospheres containing low O₂. Figure 3 illustrates an experiment in which O₂ was also low but in which the oscillations were initiated by increasing the CO₂ content from 360 to 1000 μl/l under constant illumination. Oscillations may also be initiated by either method (dark/light transition or increased CO₂ in continuous light) in 20% O₂ (Fig. 4). Data for spinach is not shown but Figure 4 (data obtained with barley) illustrates typical responses when spinach or barley leaves were subjected to these regimens. Although the external concentration of CO₂ used in Figure 4 (5%) is indeed very high, the actual concentration at the carboxylation site is not apparently deleterious for the leaf disc in the experimental conditions used, as shown by the high and constant O₂ evolution rates observed during experiments that extended over periods of hours (data not shown). It is not necessary to use such high CO₂ concentrations in order to elicit oscillations under 20% O₂ and, indeed, increasing the CO₂ content of the gas phase from 312 to 1000 μl/l produces the same sort of response (not shown).

In all of these experiments, oscillations were invariably seen when the CO₂ content was increased from about air levels of CO₂ to much higher concentrations. Oscillations could also, but with more difficulty, be initiated by increasing the CO₂ from very low concentrations to about air levels (Fig. 5) and fluctuations of a sort were even detected when the CO₂ concentration was dropped from a relatively high concentration to about air level (Fig. 6).

**Changes in O₂.** Although low O₂ favors oscillations (Figs. 1 and 2), it is not a prerequisite (Figs. 4 and 5). Indeed oscillations may be initiated, under constant illumination, either by decreasing or increasing the O₂ concentration. Thus, Figure 7 shows oscillations in fluorescence brought about by decreasing O₂ from 20 to 2% with CO₂ content held constant at 1000 μl/l. In Figure 7, the first cycle of oscillations was induced by increasing the CO₂ content from 384 to 1000 μl/l in 20% O₂, the second cycle by decreasing the O₂ to 2%, and the third cycle by increasing the...
Figures 3, 5, 6, and 7 (above), fluorescence is shown without record of the corresponding changes in O2 because (as indicated in “Materials and Methods”) the appropriate gas mixtures were passed too rapidly through the leaf chamber in these particular experiments to permit accurate measurements of O2. Nevertheless, in experiments in which the chamber was closed immediately after flushing with a new gas mixture (as in Fig. 4), O2 oscillations could be followed and these seemed to bear approximately the same qualitative and quantitative relationships to the fluorescence signals as those initiated by re-illumination when the chamber was closed. Our general experience, derived from a large number (>100) of observations, suggests that in a given leaf sample, oscillations in O2 and fluorescence can be equally as well initiated by any major perturbation (e.g. dark/light or low CO2/high CO2) and that when simultaneous O2 or CO2 measurements were precluded by practical considerations, the fluorescence signal could be regarded, with some confidence, as an indication of the changes in O2 or CO2 which would have been recorded had circumstances permitted.

The effect of lowered O2 in the presence of high CO2 was to extend the persistence of the signals and to increase the amplitude and the frequency of the oscillations. Thus, in Figure 8 the behavior of a single spinach leaf disc is followed first in 20% and then in 2% O2 with the CO2 constant at 5%. The fluorescence signals are compared in Figure 8A and the corresponding changes in the rate of O2 in Figure 8B. The fluorescence transients which are observed during the first 5 s or so after re-illumination have features which are not the subject of this report. For ease of comparison, the fluorescence signals under 2% and 20% O2 are normalized in Figure 8 so that the major secondary peaks coincide. The corresponding troughs in O2 (i.e. the first troughs after the initial O2 bursts) are similarly normalized in Figure 8B. It will be seen that the exaggeration of the oscillatory behavior in low O2 (i.e. greater amplitude, greater frequency and greater extent) is, in this instance, as apparent in the O2 signal as it is in the fluorescence signal. With the existing apparatus, however, the fluorescence signal is more readily amplified than the O2 signal without the introduction of excessive electrical 'noise' and will then continue to indicate weak oscillations when these are not discernible in the O2 trace.

Effect of Diminished Light. All of the preceding experiments were carried out at the same light intensity. When this was halved (Fig. 9), both the amplitude and the frequency of the oscillations were decreased. High light, as such, was not an essential requirement for the demonstration of oscillations. A change from air to a gas phase containing 5% CO2 and 20% O2 could still initiate oscillations in barley under light intensities as low as 10 W·m⁻² (not shown).

**DISCUSSION**

It was our objective here to make two principal points: that the oscillations in photosynthesis which have been repeatedly observed over the last 50 years (5, 10, 14, 17, 19, 23, 24, 26, 29, 30) are readily reproducible phenomena and that, at least in certain circumstances, there is a clear antiparallel but phase-shifted relationship between Chl a fluorescence and photosynthetic carbon assimilation (29).

It is now widely accepted that there are at least two quenching mechanisms (qO and qE; see Refs. 4, 8, 12, 13) which could be concerned in relating rapid changes in carbon assimilation to changes in variable fluorescence (21, 22, 27). An electron acceptor Q (8) was so called in the first instance because it quenches fluorescence (qO quenching) when it is in the oxidized state and therefore free to accept electrons. In this way, it connects with the consumption of NADPH in CO2 reduction and, if CO2 assimilation is impaired, NADPH and qE will be continuously reoxidized. Fluorescence is also quenched (qE quenching) when
the thylakoid membrane is energized and ΔpH is increased (4, 12, 13, 21, 22, 27). ATP consumption, which discharges the proton gradient, therefore tends to relax q, quenching and, in its relation to carbon assimilation, this quenching mechanism normally acts in opposition to q0 quenching (12, 21, 22, 27). Accordingly, when a leaf is transferred to CO2-free air, fluorescence first rises as NADP and Q become reduced and subsequently falls to a value lower than the initial value because q, quenching increases and predominates as shortage of ADP limits the discharge of the proton gradient (21). Inasmuch as all of the ATP and NADPH which is consumed in the reductive pentose phosphate pathway is utilized in three reactions, it is highly probable that these reactions are implicated in the oscillations and has been proposed that the interrelationship between the two kinases (phosphoribulokinase and PGA kinase) may be crucial in this regard (6, 20–22, 27, 28).

The fact that the fluorescence signal of the Chl a signal by as much as 10 s (Figs. 1, 2, and 4 and Refs. 7 and 29) is of considerable interest. When illumination is restarted after steady state photosynthesis is interrupted by a very brief dark interval, O2 evolution is detected, as in our system, within 2 s (7). This makes it most unlikely that diffusion of O2 from the chloroplast to the electrode, or the electrode response itself, could account for more than about 25% of the observed delay between a change in fluorescence and a corresponding change in O2 evolution. If, as we suspect, carbon assimilation is checked because a transient increase in ribulose-5-P opens an ATP sink and PGA phosphorylation cannot then proceed until the unfavorably low ATP/ADP ratio so created is corrected (6, 20–22, 27, 28), the two quenching mechanisms will eventually relax in concert. However, some time could elapse (depending on the size of the PGA pool) before the ATP/ADP ratio became sufficiently unfavorable to check di-PGA formation and NADPH reoxidation. Only then would the rate of O2 evolution be diminished and only then would q0 relaxation reinforce q, relaxation. On the other hand, an increase in the degree of reduction of Q which causes q0 to relax may not necessarily dictate a concomitant decrease in electron transport from water, via Q to P

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Fig. 7. Oscillations in Chl a fluorescence initiated, under constant illumination, by increasing CO2 concentration or by diminishing or augmenting the O2 concentration in the gas phase surrounding barley leaf pieces. When steady state fluorescence was achieved (see Fig. 3), cycles of dampening oscillations were induced by switching from the initial gas phase (20% O2, 384 µl/l CO2) to another with the same O2 content but augmented (1000 µl/l) CO2. Note the initial fluorescence fall, as in Figs. 3 and 5. Further cycles of dampening oscillations were induced under constant CO2 concentration by diminishing (second cycle) or by augmenting (third cycle) the O2 concentration as indicated.

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The oscillations and O₂ signals resides in the characteristics of the Clark type O₂ electrode which we have employed. We know that while the initial response of such electrodes is very rapid the speed of response can diminish significantly as the difference in partial pressure of O₂ between an initial and final value is approached (e.g., if the chamber is flushed with N₂, the O₂ signal declines very rapidly at first but much more slowly as the N₂ value is approached). Nevertheless, we are inclined to doubt that this electrode characteristic could account for all of the apparent phase shift because the oscillations in the rate of O₂ evolution can be very large initially and the frequency of the oscillations shows no tendency to decrease as the amplitude declines with time. If, however, the delays imposed by diffusion and the electrode characteristics were 4 to 5 times greater than our present estimate, the fluorescence and O₂ signals would be almost exactly reciprocal.

The initiation of oscillations by re-illumination and/or by changes in the gas phase is consistent, as is other evidence presented elsewhere (21, 22, 27, 29), with the view that many slow perturbations in fluorescence kinetics and/or O₂ evolution can derive from an imbalance introduced into the regulatory controls that govern carbon assimilation. An increase of CO₂ concentration (Figs. 3–5) would cause an increase in the availability of PGA and a consequent surge of ribulose-5-P. There is ample evidence that the reaction catalyzed by ribulose-5-P kinase can, by acting as an ATP sink, affect that catalyzed by 3-P-glycerate kinase (see e.g., Ref. 9). If ribulose-5-P increased, the ADP concentration would also increase. This, in turn, would be followed by a decrease in NADPH consumption and O₂ evolution as the increase in ADP slowed the PGA kinase reaction (6). Readjustment of ATP/ADP ratios, by cyclic or pseudocyclic electron transport during this period, would then reverse the entire sequence (6). Evidence in support of these proposals has recently been published by Horton (11) who showed, using the reconstituted chloroplasts system, that the addition of ribulose-5-P during PGA-dependent O₂ evolution caused an increase in fluorescence and a decline in ΔpH both of which anticipated the interruption of O₂ evolution brought about by the creation of an unfavorably low ATP/ADP ratio.
At limiting levels of CO₂, a decrease in O₂ to 2% (see “Results”) would presumably be as effective in increasing the availability of PGA as an increase in CO₂ concentration but at 1000 μl/l of CO₂ and greater, photorespiration is unlikely to be rapid. The initiation of oscillations by changes in O₂ concentration under high CO₂ levels and the rapidity of this initiation is also of interest and points more to a role of O₂ as an alternative acceptor to CO₂ than to an effect on photorespiration. At air levels of CO₂, a decrease in O₂ to 2% would presumably be as effective in increasing the availability of PGA as an increase in [CO₂] but at 1000 μl/l CO₂ and greater, photorespiration is unlikely to be rapid and the factors invoked by Canvin (5) to explain the inhibition of photosynthesis by decreased O₂ (see also Refs. 10, 17, and 25) may well be important. These include ATP production via pseudocyclic phosphorylation and promotion of cyclic electron transport by the maintenance of electron carriers at a suitable redox level. In the present experiments, oscillations were initiated not only by the procedures used by other workers (5, 10, 14, 17, 19, 23, 30) but also by making many other changes. In particular, we found that, although high light intensity, low O₂, and high CO₂ concentrations favored oscillations, they were not a prerequisite. Clearly, any explanation of the oscillatory behavior of photosynthesis and/or fluorescence has to accommodate this broad range of experimental conditions.

While some of our results are in accord with those of Ogawa (19), others do not support his inference that oscillations can only be observed in low O₂ nor are we in agreement with his views about the relationship between O₂ evolution and Chl a fluorescence which we believe, as stated above, to be antiparallel but slightly out of phase.

Moreover, Ogawa (19) states, “The absence of a complementary relationship between the rate of O₂ evolution and fluorescence yield indicates that the observed fluorescence cannot be regarded as an inefficiency index of photosynthesis . . .” This, we believe, is too sweeping. It is true that there may be circumstances in which such an inverse relationship does not hold (3, 18) and, particularly in the first seconds of illumination (which did not concern us here), changes in fluorescence are determined more by the photochemistry than the reactions of carbon assimilation. Nevertheless, we have been impressed by the fact that there is often a very close, essentially reciprocal or antiparallel relationship between the slow fluorescence signals and photosynthetic carbon assimilation whether the latter is measured by O₂ evolution or CO₂ fixation. This, together with the fact that this reciprocal relationship appears to hold even when no oscillations are observed (or after oscillations have subsided) implies that, at least in well defined circumstances, it may well become possible to use fluorescence as a measure of photosynthetic performance.

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