

# Simultaneous Measurement of Oxygen Evolution and Chlorophyll Fluorescence from Leaf Pieces<sup>1</sup>

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## ABSTRACT

An apparatus is described which permits the simultaneous measurement of O<sub>2</sub> evolution and chlorophyll *a* fluorescence from illuminated discs or pieces of green leaves. O<sub>2</sub> is measured in the gas phase in a temperature-controlled chamber of approximately 5-milliliter capacity. Calibration is effected by injection of air through vents. Response time is approximately 1.5 seconds for O<sub>2</sub>, and full scale deflection, in normal operating mode, is approximately 10 micromoles O<sub>2</sub>. The apparatus may also be used to monitor fluorescence alone, in an open mode, in which gas is passed continuously through the chamber.

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Although photosynthetic carbon assimilation in leaves is usually measured by IRGA<sup>2</sup> of CO<sub>2</sub> uptake, it is also possible to follow O<sub>2</sub> evolution by polarography in the gas phase (2). No O<sub>2</sub> electrode system currently available can match the sensitivity of IRGA but if a leaf segment is illuminated in a small chamber, the change in O<sub>2</sub> content can be readily detected. Accordingly, a simple apparatus was constructed for this purpose (2) and it has now been improved and modified to permit simultaneous measurement of Chl *a* fluorescence and O<sub>2</sub> exchange.

## MATERIALS AND METHODS

**The O<sub>2</sub> Electrode.** The O<sub>2</sub> detector is a conventional Clark-type Pt/Ag/AgCl<sub>2</sub> electrode (1) manufactured by Hansatech (Hansatech Ltd., Paxman Road, Hardwick Industrial Estate, King's Lynn, Norfolk). The Pt cathode is mounted in an araldite dome across which a Teflon membrane is stretched. The Ag anode lies in a well beneath the dome and a KC1/borate/bicarbonate solution is used as the electrolyte (2).

**The Leaf Chamber.** The leaf chamber (Figs. 1 and 2) is made of fairly massive anodized aluminum which affords good temperature control by circulated water. The electrode rests on a base which presses it against an O-ring so that a seal is effected, yet the cathode is exposed to the atmosphere in the leaf chamber through a small hole in its floor. The chamber itself is cylindrical (163 mm high × 370 mm radius) and accommodates a leaf disc of 10-cm<sup>2</sup> area or smaller pieces of leaf carried on a perforated stainless steel plate, with an entire center, which prevents light falling directly on the cathode. The roof of the chamber is the Perspex (Plexiglas) floor of the upper water jacket through which the leaf is illuminated. The top window in this water jacket is

either plain Perspex or an aspheric lens held in place by an opaque nylon (Nylatron 901, black) or aluminum collar supporting an electronically operated shutter (Uniblitz model SD 122B shutter drive unit). A nylon probe, fitted with its own Perspex window, is inserted into the side of the upper water jacket at an angle of 40° and allows fluorescence to reach a photodiode (UDT 500 Phot-op photodiode) protected by filters (*e.g.* Corning 2-64 filter) which exclude the blue (Corning 4-96) actinic light which is delivered to the distal side of the electronic shutter by a fiber optic. When the chamber is to be closed, the roof is drawn down into position by two clips. O-rings are used to give an air-tight seal. The fluorescence signal is amplified within the probe, from which it is passed to a pen recorder.

**Actinic Light.** Although any commercial light source, combined with adequate heat filtration, may be used, we have preferred an actinic light source of our own design based on a 12 v, 150 w, or 250 w GEC quartz halogen lamp with built-in parabolic reflectors which favor visible light (a proportion of the IR output of the lamp passes directly through the reflectors). A 'cold-mirror,' OCLI (Optical Coating Laboratory Inc.), MR16 Specification 6021014, at 45° and a wide band 'hot-mirror,' OCLI 6022001 at right angles to the light path, together with several cm of water, minimize increases in leaf temperature consequent upon bright illumination. Electrical noise is avoided by using a stabilized power supply (Coutant ASC R10).

**Calibration.** The leaf chamber is fitted with two taps which communicate with the external atmosphere. These allow the chamber to be flushed with N<sub>2</sub>, or air, or other gas mixtures. The difference between the electrical output of the electrode in air and N<sub>2</sub> is a measure of the partial pressure of O<sub>2</sub> in the atmosphere (2) although, for the greatest accuracy, attention must be paid to barometric pressure and water vapor (2). The quantity of O<sub>2</sub> in the chamber is obviously governed by volume as well as concentration and since the effective volume is diminished by the volume of the leaf (and anything else present in the chamber) and since this is not as readily determined as it is in conventional polarography (in which a precise volume of solution can be added to a measuring vessel), it is necessary to use an indirect procedure. This involves closing one tap and inserting a known volume of air into the leaf chamber with a gas-tight syringe through the other (the taps have female luers for this purpose). This causes an excursion of the electrode trace (the electrical output of the O<sub>2</sub> sensor is applied to a pen recorder as a voltage in the mv range) which is proportional to the number of μmol of O<sub>2</sub> present in the added air (2). If carried out before and after a leaf disc is placed in the chamber, this procedure also gives a measure of leaf volume (2).

**Carbon Dioxide Source.** The CO<sub>2</sub> content of the leaf chamber is rapidly exhausted by an actively photosynthesizing leaf disc unless it is replenished. For this reason, a carbonate/bicarbonate buffer is usually employed (1, 9) as a source of CO<sub>2</sub>. This is best

<sup>1</sup> This work was supported by a grant from the Royal Society.

<sup>2</sup> Abbreviation: IRGA, infrared-gas analysis.

carried on capillary matting to facilitate rapid equilibration and, if necessary, equilibration can be speeded by adding carbonic anhydrase to the buffer. Leaf tissue is best protected from direct contact with very alkaline buffers and for this reason a second perforated stainless steel disc and a foam rubber disc are also inserted into the chamber. Accordingly, the matting carrying the bicarbonate lies on the floor of the chamber and is separated from the leaf tissue above it by the foam rubber disc sandwiched between the two perforated plates. The second perforated plate is not shown in Figure 1 but would lie immediately above the matting and below the sponge. As illustrated, this sponge has a central hole but this hole is not essential and, provided that the sponge is not allowed to become completely water-saturated, it offers no discernible barrier to diffusion within the chamber. The use of the sponge, particularly in conjunction with two perforated steel discs has the added advantage that the foam rubber, when compressed, acts like a spring, pressing the leaf tissue lightly against the temperature-controlled roof of the chamber.

**Operation.** After the chamber is loaded, closed, and calibrated, the recorder pen is returned to a position at one side of the chart by an electrical 'back-off' device (1, 2) in order that most of the chart can be used to record O<sub>2</sub> evolution. Additional sensitivity can be derived by using the highest electrical output that the potentiating circuit (1, 2) will give and then switching the pen recorder to a lower range and backing-off most of the signal (*e.g.* if the electrical output in air is 3.5 mv and 3.4 mv is backed-off then the remaining signal will, if recorded on the 1-mv rather than the 5-mv range, be effectively amplified by a factor of 5). Obviously a pre-amplifier can also be used for this purpose and then, as in many other circumstances, the extent of amplification will be dictated by whatever degree of electrical noise is acceptable.

Some period of temperature equilibration is necessary (usually not more than 2 to 3 min). During the first part of this period an apparent O<sub>2</sub> uptake will normally be recorded. This will be caused partly by temperature equilibration and partly by respi-

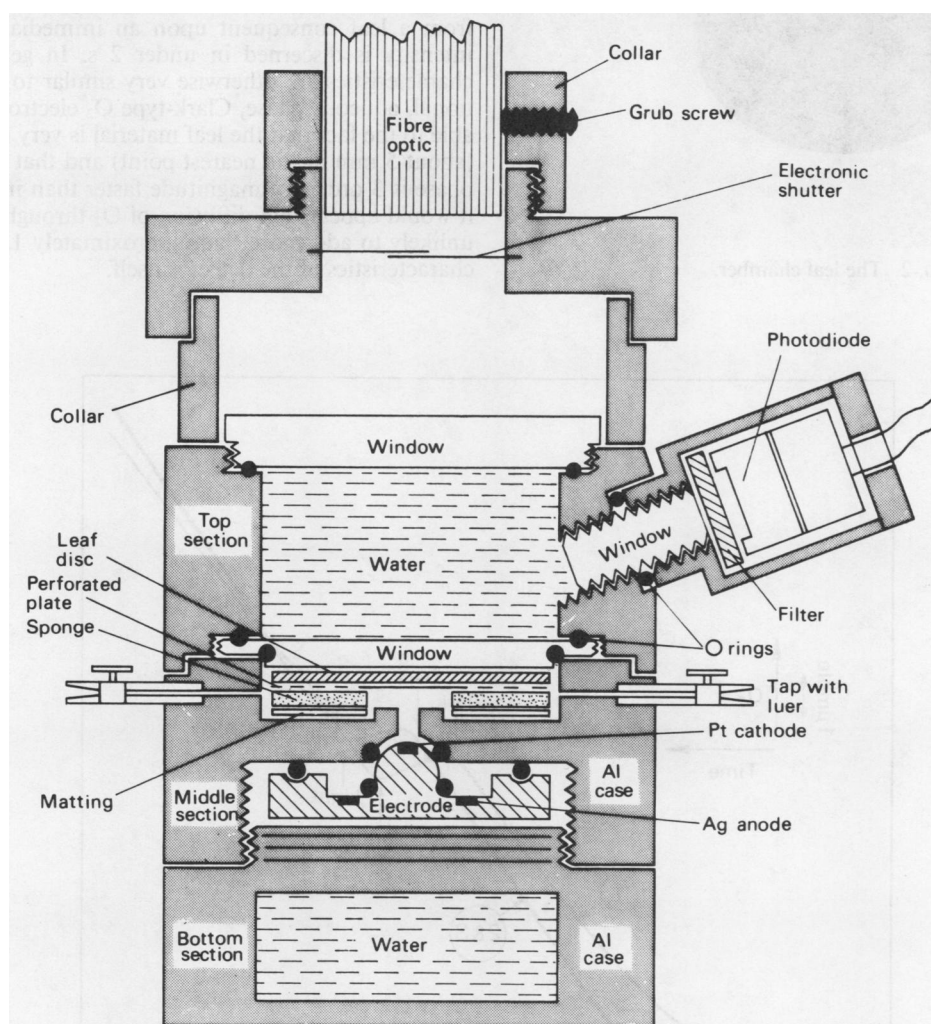


FIG. 1. Schematic diagram of gas phase O<sub>2</sub> electrode and fluorescence probe. The leaf disc or leaf pieces are supported on a stainless steel mesh in the chamber which is located in the middle section of the apparatus (see "Materials and Methods" for a more detailed description). The O<sub>2</sub> sensor (Clark-type electrode) lies beneath the leaf chamber with its Pt cathode exposed to the atmosphere within it. The leaf tissue is pressed lightly against the temperature-controlled roof of the chamber by a foam rubber disc which also separates it from the carbonate/bicarbonate buffer carried on capillary matting. The tissue is illuminated through this window which also allows fluorescence to reach a probe (inserted at an angle of 40°) where it is monitored by a photodiode. Blue actinic light is delivered either directly, or via a fiber optic (as shown), to the top of the apparatus, through an electronic shutter. The photodiode is protected from the actinic light by a suitable filter or filters. The clips which draw the top section onto the middle section (so that the roof of the leaf chamber is sealed against an O-ring) and the tubes which carry temperature-controlled water to the top and bottom sections are not shown. The taps (with luers) are for calibration and adjustment of the gas phase.

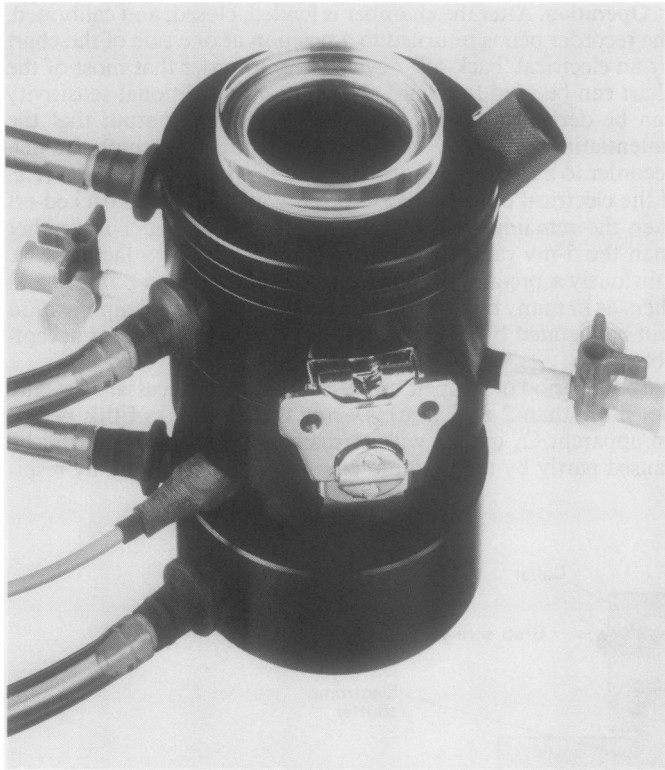


FIG. 2. The leaf chamber.

ration. It should be noted that if the leaf material has been brightly illuminated immediately prior to measurement the uptake of  $O_2$  in the dark will be more rapid than it would be if the leaf had been previously kept in darkness or low light. In the experiments illustrated, the temperature was kept at  $20^\circ C$  except where indicated.

When the shutter is opened,  $O_2$  evolution will commence after an initial lag or induction period (11). Fluorescence will rise swiftly to an early maximum and then decline to a quasi-steady state or terminal value (for reviews, see Refs. 3 and 5). Both  $O_2$  and fluorescence may display secondary kinetics. The precise relationship between the two remains to be determined but in many circumstances (13, 14) the fluorescence change anticipates the  $O_2$  change by several s and behaves in an antiparallel or reciprocal fashion (*i.e.* a fall in fluorescence signals an increase in the rate of  $O_2$  evolution).

**Response Time of  $O_2$  Detection and Diffusion with the Leaf Chamber.** If a small quantity of air ( $10 \mu l$ ) is injected into the leaf chamber, the initial response of the  $O_2$  electrode is recorded in approximately 1.5 s and the change in rate of  $O_2$  evolution from a leaf consequent upon an immediate increase in light intensity is discerned in under 2 s. In general, the response characteristics are otherwise very similar to those of a conventional, aqueous phase, Clark-type  $O_2$  electrode. This is attributable to the fact that the leaf material is very close to the cathode (about 5 mm at the nearest point) and that diffusion in the gas phase is 3 orders of magnitude faster than in solution. In short, it would appear that diffusion of  $O_2$  throughout the chamber is unlikely to add more than approximately 1.5 s to the response characteristics of the detector itself.

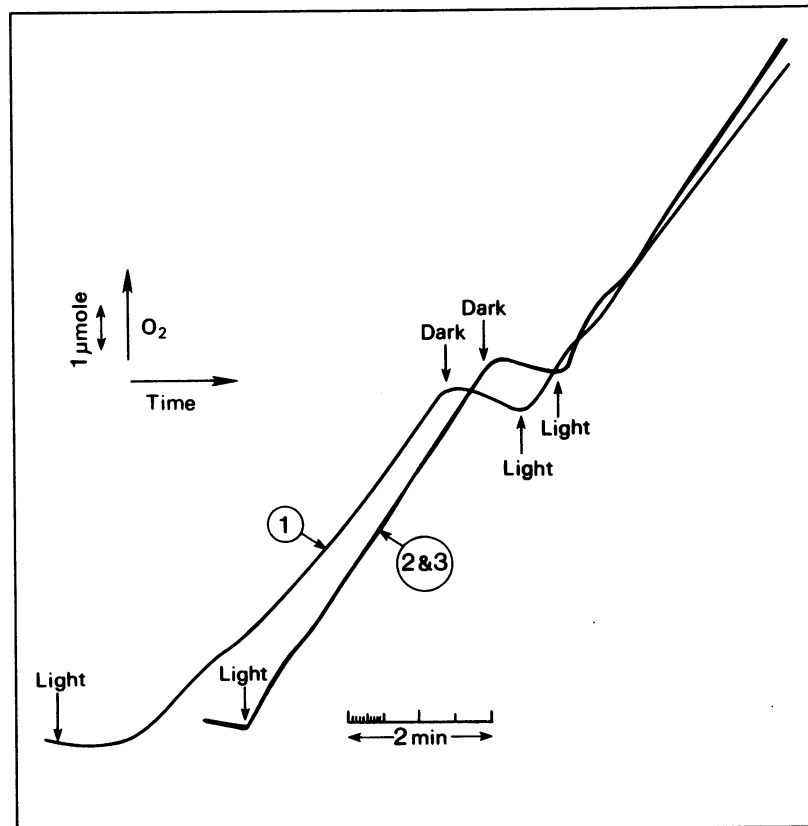


FIG. 3. Reproducibility. All three traces in this figure were obtained using the same leaf disc. After the first (interrupted) period of illumination, the  $O_2$  content was returned to the starting value and the disc then illuminated twice again, each time in precisely the same manner. Once a particular regimen was established, the leaf disc behaved in a highly reproducible fashion (*cf.* traces 2 and 3), even displaying virtually identical secondary kinetics.

The fact that the leaf disc is pressed lightly against the roof of the chamber does not appear to constitute a significant barrier to rapid diffusion. Thus, in saturating light, it is immaterial whether a leaf, like spinach, which has most of its stomata on the lower surface is illuminated from above or below. Moreover, the O<sub>2</sub> evolution from small discs (in which the cut edge might be presumed to make a larger contribution to gaseous exchange) is the same, on a *pro rata* basis, even though gases would then appear to have more unimpeded space through which to circulate. Similarly, the central hole in the foam rubber sponge (Fig. 1) is not essential and the sponge offers no sensible barrier to diffusion unless completely water-saturated.

In 5% CO<sub>2</sub>, inspection confirmed that the stomata were closed but, in saturating light, rates of O<sub>2</sub> evolution as high as 300 to 400 μmol mg<sup>-1</sup> Chl h<sup>-1</sup> have been recorded. These rates, on average, were about 25% higher than those observed when the same material was subjected to conventional IRGA in augmented (1000 μl/l) CO<sub>2</sub>. This we attribute to the fact that CO<sub>2</sub> reaches the chloroplast in saturating concentrations and that O<sub>2</sub> detection is similarly unimpeded, despite the closed stomata. What proportion of the gaseous exchange occurs via the closed stomata, because of steepened diffusion gradients, and what part through the cut edge of the disc, or discs, is not known. But the fact that such high rates can be measured suggests that the apparatus may prove to be a useful means of determining the maximum rate of photosynthetic carbon assimilation in leaf tissue. Preliminary experiments indicate that the rates of O<sub>2</sub> evolution which we have observed are consistent with maximal

rates of coupled electron transport, through both photosystems, in chloroplasts isolated from the same tissue.

As already noted (see "Carbon Dioxide Source"), a limitation can be imposed by the rate of equilibration of carbonate/bicarbonate buffers with the gas phase when CO<sub>2</sub> consumption by the leaf is particularly fast. This can be detected by a fall in the rate of O<sub>2</sub> evolution with time which can be corrected by addition of carbonic anhydrase to the carbonate/bicarbonate buffer.

**Differentiator.** For some purposes, it is convenient to differentiate the O<sub>2</sub> signal in order to obtain an immediate record of change in rate. This was achieved by following the O<sub>2</sub> electrode signal on a Rikadenki R-14 recorder in which a monitor gave a linear output of 0 to 1 in proportional relationship to the position of the pen recording the O<sub>2</sub> signal. This output was powered through a normal passive RC differentiator, comprising a 2.3-μf polyester capacitor (in series with the positive output from the monitor) and a 30 kΩ resistor (across the positive and negative output from the monitor).

## RESULTS

It is frequently held that leaf pieces sustain damage during cutting and handling which makes them unreliable experimental material, but present experience indicates that they will behave in a stable and reproducible fashion. For example, Figure 3 shows three traces which were obtained with one leaf disc. Immediately after cutting (the leaf had previously been 30 min in low light, at about 10 w·m<sup>-2</sup>), the induction period was long

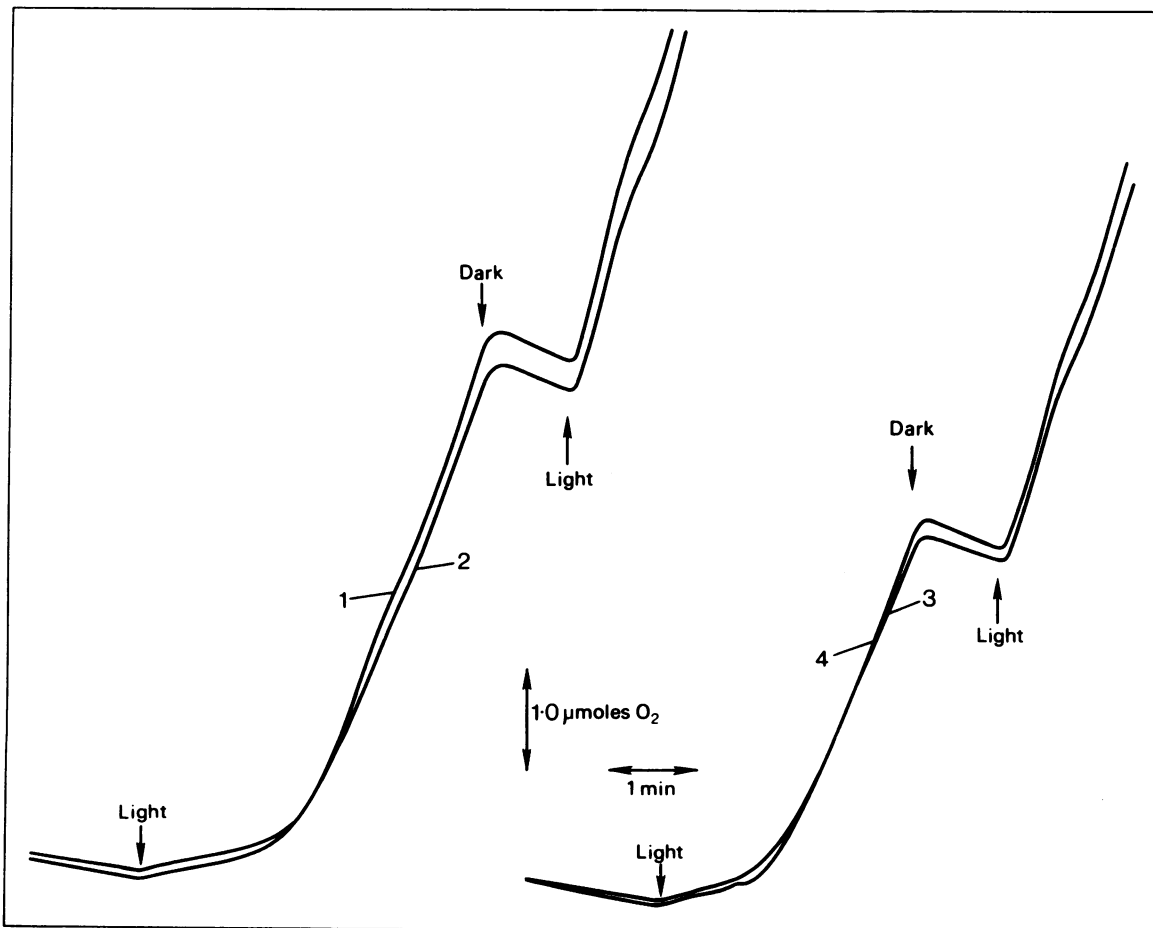


FIG. 4. Reproducibility. Spinach leaf discs cut from either side of the midrib of a single leaf behaved in a very similar fashion when treated in the same manner. The first two traces were obtained with two discs taken from a leaf in the glasshouse. The second two were obtained with two more discs from the same leaf which had been kept in the relatively subdued light of the laboratory during the first measurements, thereby extending the initial induction period.

and the rate relatively slow. Following the period of illumination shown (10 min), illumination was stopped, the  $O_2$  content of the chamber was returned to the starting level (by flushing with air), and the leaf then was re-illuminated. When this latter procedure was repeated, a virtually identical trace was recorded (*cf.* traces 2 and 3 in Fig. 3). Such behavior was typical of discs from spinach and a number of other species including sunflower, barley, and maize. Thus, provided a disc was treated in precisely the same manner each time, it would respond in almost exactly the same way, including the secondary kinetics (which can be clearly discerned in Fig. 3). Eventually, prolonged continuous illumination might lead to some falling-off in rate but such deterioration was not normally more pronounced than we have seen in IRGA experiments with attached leaves. Similarly, when discs were cut from corresponding positions in either side of the midrib, in broad-leaved species such as spinach, they also behaved in much the same manner (Fig. 4). Like chloroplasts and protoplasts, the discs responded to illumination after long and short periods in the dark, to high and low light intensities (Fig. 5) and to high and low temperatures (Fig. 6) in a way which has been recognized as typical since the experiments of McAlister

(4). It may be noted that the  $O_2$  traces in Figure 5 permit interpolation which is fully consistent with McAlister's concept of 'induction loss,' *i.e.* that the lag can be quantified in terms of the extent to which photosynthesis would have occurred had photosynthesis immediately reached its maximal value.

Although, in the above experiments, carbonate/bicarbonate was added to the capillary matting in molar concentrations that would be in equilibrium with  $CO_2$  in the gas phase as high as 5% (9), experience (M. Hills, personal communication) suggests that, unless the chamber is subsequently flushed with an appropriate gas mixture prior to illumination, this partial pressure is rarely realized, because of the rapid loss of  $CO_2$  from the capillary matting during assembly. We have not yet had an opportunity to make a detailed examination of either  $CO_2$  limitation or  $CO_2$  inhibition in these circumstances but when near-saturating white light has been employed with summer-grown spinach we have observed rates of photosynthesis in excess of  $300 \mu\text{mol of } O_2 \text{ mg}^{-1} \text{ Chl} \cdot \text{h}^{-1}$  and a decline in rate with time which can be corrected by the addition of carbonic anhydrase to the carbonate/bicarbonate solution. These and other results suggest that in the experiments illustrated here, we have operated the apparatus at

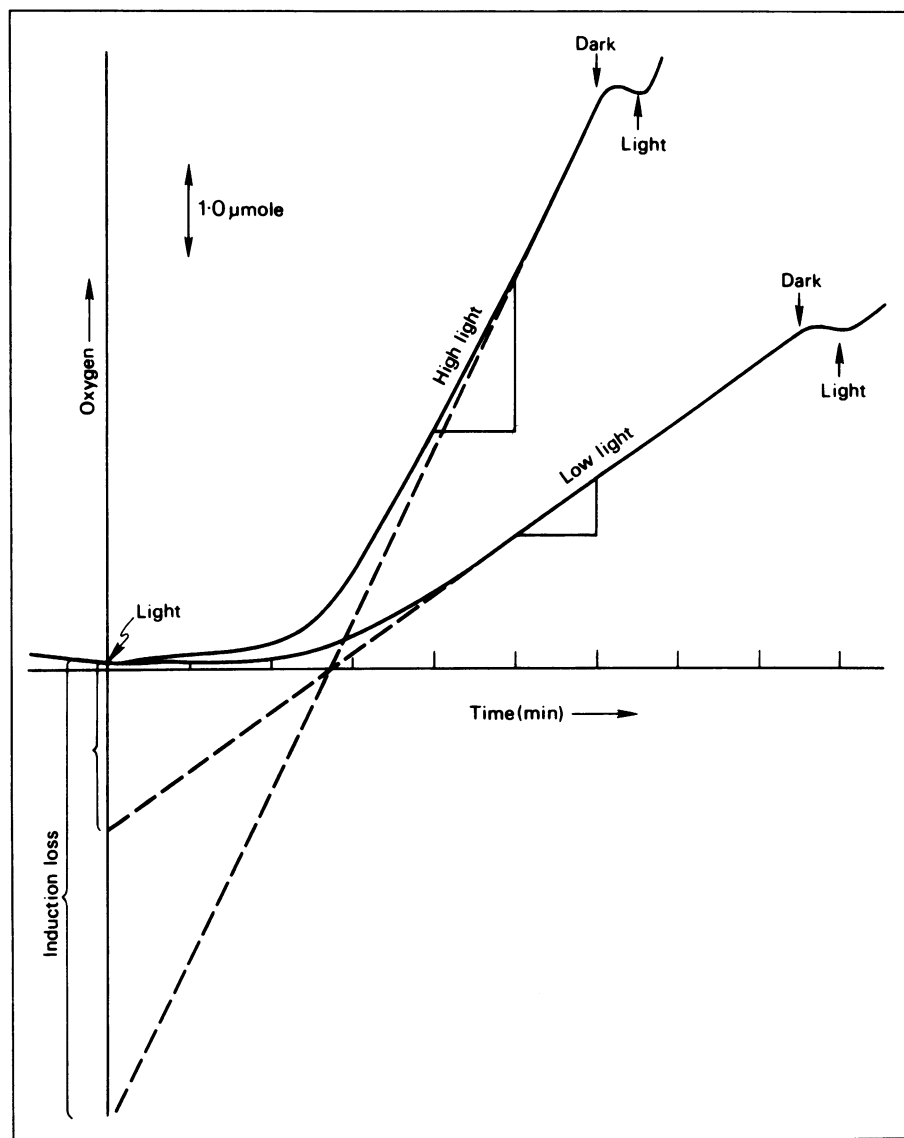


FIG. 5. Illumination in high ( $180 \text{ w} \cdot \text{m}^{-2}$ ) and low ( $60 \text{ w} \cdot \text{m}^{-2}$ ) light. Following illumination in relatively low white light, the rate of  $O_2$  evolution by a spinach disc was increased in high light but the lag (11) was unchanged and the induction loss increased.

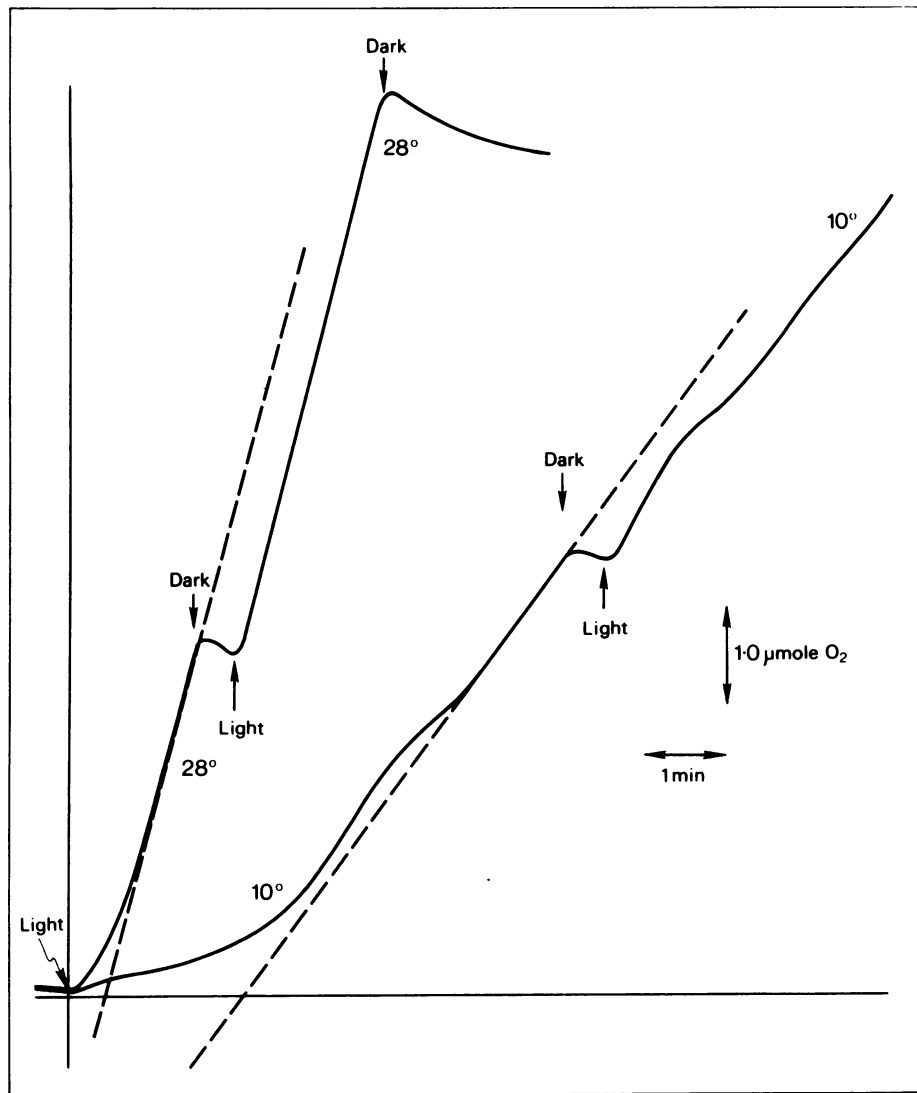


FIG. 6. Illumination at high and low temperatures. Following illumination at 10°C an increase in temperature to 20°C caused an increase in rate and a decrease in the induction lag (11).

near-optimal CO<sub>2</sub> concentration. However, in any work in which it was necessary to establish the [CO<sub>2</sub>] in the gas phase with accuracy, a combination of carbonate/bicarbonate buffers and gas-flushing prior to commencement of measurement could be used here in the same way as it has been employed for many years in Warburg manometry (9).

**Secondary Kinetics in O<sub>2</sub> Evolution.** These are described in more detail in a second paper (14). They have been noted on many previous occasions (see *e.g.* Ref. 10) and earlier attempts at electronic differentiations of the signal have been made in order to allow changes in rate to be directly plotted as a function of time (12). A very simple electronic device (see "Materials and Methods") was employed here and changes in rate, which can sometimes be observed only with some difficulty when amount of O<sub>2</sub> is plotted against time, were now immediately apparent. In Figure 7, the dampening oscillations in both O<sub>2</sub> and Chl *a* fluorescence are very striking and emphasize the intrinsic potential of simultaneous measurements.

**Temperature Artifacts.** Some increase in temperature during strong illumination in white light is often unavoidable even when most of the infrared is removed from the actinic light by filters. When a piece of black matting was used in place of a leaf, an artifactual 'O<sub>2</sub> evolution' was observed which would normally

have added less than 5% to that evolved by an actively photosynthesizing leaf during the first 5 min of illumination. Thereafter, the electrode would stabilize at the new temperature and would display a corresponding decrease on darkening. Experiments with killed leaf tissue confirmed that this temperature artifact will, in most circumstances, be smaller for leaves than for a black body. Similarly, the size of the artifact can be diminished by improved heat 'filtration' of the actinic light source.

## DISCUSSION

Our present understanding of whole leaf photosynthesis owes much to the development of IRGA and it is not our purpose to detract, in any way, from the usefulness of this technique or to ignore the advantages which derive from its application by skilled operators, to whole plants. IRGA is not, however, a procedure which is undertaken lightly and, for the plant biochemist/physiologist who wishes to learn more about the photosynthetic performance of leaf tissue without making it his life's work, the prospect of introducing IRGA into the laboratory can be daunting. Similarly, the measurement of Chl *a* fluorescence has, until recently, been more for specialists than an everyday technique

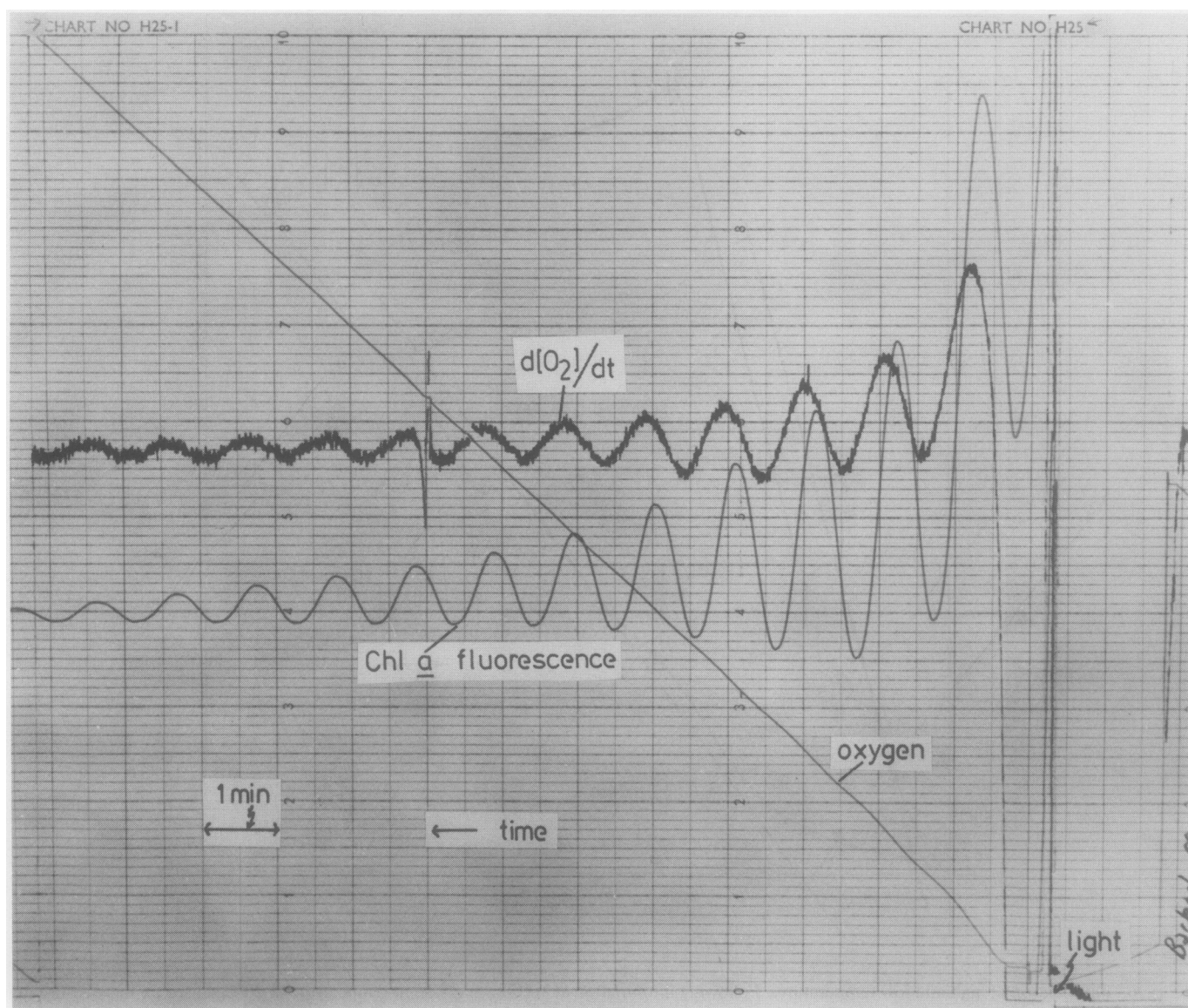


FIG. 7. Actual traces of  $O_2$ ,  $dO_2/dt$ , and Chl *a* fluorescence showing antiparallel (reciprocal) relationship between dampening oscillations in Chl *a* fluorescence and rate of  $O_2$  evolution. It should be noted that the displacement of the recorder pens is such that the fluorescence signal should be shifted approximately one vertical division to the right in order to permit direct comparison of the traces. (Spinach leaf disc at  $20^\circ\text{C}$  in 2%  $O_2$  and 5%  $CO_2$  following re-illumination after a dark interval of 2 min. For other details, see Ref. 14.)

like pH measurement. This latter situation has been largely rectified by the commercial availability of an apparatus originally designed by Schreiber (the Brancker Plant Productivity Meter) which, by an ingenious combination of light-emitting diodes and photodiodes, has brought many essential requirements together into one probe and its associated circuitry (6). There is, however, an obvious need for an equally simple and inexpensive apparatus which combines both the simultaneous measurement of photosynthetic gaseous exchange and Chl *a* fluorescence and, ideally, one which will allow the employment of high light intensities and give reasonable control of temperature and gas phase. This we believe we have achieved. An earlier version of the leaf disc electrode (2) is useful but lacks the refinements (better temperature control, better control of the gas phase, easier calibration) afforded by the new apparatus and does not offer the advantages which can derive from combined  $O_2$  and fluorescence measurements. Further examples of these are described in the following paper (14). The tight, near-reciprocal relationship between fluorescence and  $O_2$  opens up a great many possibilities because of

the sensitivity and immediacy of fluorescence measurements. Until recently, the impact of carbon assimilation on fluorescence has attracted little attention because of the complexity of the signals and the fact that interpretation seemed remote. Ultimate interpretation (7, 8, 14) of slow fluorescence signals is now at least conceivable and the value of the nonintrusive nature of fluorescence measurements will be enhanced once their relationship with other photosynthetic events is more clearly understood. The combination of gas phase polarography and photodiode-based fluorescence measurements described here should promote such understanding.

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