

# Photosynthesis by Isolated Chloroplasts, Simultaneous Measurement of Carbon Assimilation and Oxygen Evolution<sup>1</sup>

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**Abstract.** Photosynthetic carbon assimilation by isolated chloroplasts and its associated oxygen evolution were measured simultaneously in a single simplified reaction mixture. Each showed an initial lag prior to the attainment of the maximal rate and the photosynthetic quotient was unity during the period of illumination. Following illumination an oxygen uptake was observed in the dark which was not accompanied by any net release of newly fixed carbon dioxide.

Oxygen evolution during carbon dioxide fixation by isolated chloroplasts was first observed in the classic experiments of Allen *et al.* (1). Oxygen was measured manometrically under anaerobic conditions and characterized by using luminescent bacteria. Subsequently improvements in technique (see *e.g.* ref. 9) led to the separation of chloroplasts which could sustain a higher rate of CO<sub>2</sub> fixation and to the first measurements of the associated oxygen evolution in a fully aerobic system (Walker and Hill, 10). This paper reports similar measurements, made aerobically in the absence of exogenous ascorbate, with carbon dioxide as the sole added substrate.

## Materials and Methods

**Isolation of Chloroplasts.** Chloroplasts were isolated as described previously (6) from spinach leaves in a medium containing sorbitol, MgCl<sub>2</sub>, and sodium pyrophosphate-HCl as buffer. The chloroplasts were resuspended and stored at 0° if necessary in a solution containing sorbitol, MgCl<sub>2</sub>, MnCl<sub>2</sub>, EDTA and HEPES-NaOH as buffer (6). Sodium isoascorbate was not used.

**Assay Medium.** Oxygen evolution and carbon dioxide fixation were assayed simultaneously in 1.0 ml of a solution containing sorbitol, 0.33 M; EDTA, 2.0 mM; MgCl<sub>2</sub>, 1 mM; MnCl<sub>2</sub>, 1 mM, NaH<sup>14</sup>CO<sub>3</sub>, 7.5 mM (400 μC); HEPES, 50 mM adjusted to pH 7.6° with NaOH and chloroplast suspension containing 100 μg chlorophyll. The chloroplast suspension was added after the mixture had reached the assay temperature of 20°, 30 seconds before

illumination. The radioactive bicarbonate was replaced by NaHCO<sub>3</sub> (10 mM) for measurements which only involved oxygen.

**Measurement of Oxygen Evolution.** Oxygen evolution by the chloroplasts was measured polarographically as described previously (5) using a perspex electrode cell illuminated by heat filtered light from Quartz Iodine slide projectors and cooled by circulation of temperature controlled water through the integral water jacket. The output of the electrode system was recorded electrically.

**Calibration.** The oxygen electrode was calibrated by taking the difference between the output of the system when the cell contained sodium dithionite in water and when it contained air saturated water at 20° to represent 0.28 μmole O<sub>2</sub>/ml. This method of calibration and also the linearity of response of the system was checked by injecting small quantities of standardized H<sub>2</sub>O<sub>2</sub> into suspending medium containing catalase.

**Measurement of CO<sub>2</sub> Fixation.** The progress of carbon dioxide fixation in the same reaction mixture was followed by withdrawing 10 μl samples through a capillary in the top of the electrode cell. The samples were then mixed with 40 μl of approximately 0.3 N HCl. Samples (20 μl) were transferred to uniform lens tissue discs on aluminum planchettes, dried under a heat lamp, then their radioactivity was estimated using a gas-flow counter.

**Calibration.** The values of the specific activity and radioactivity of the NaH<sup>14</sup>CO<sub>3</sub> provided by The Radiochemical Centre (Amersham, Bucks) were accepted without further verification. The radioactivity of a solution containing (<sup>14</sup>C) sucrose (The Radiochemical Centre) was checked against standards in a scintillation counter. Small quantities of this solution were added to reaction mixtures containing chloroplasts that were then sampled and measured as described above. This procedure, which eliminates errors resulting from variable geometry

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and self-absorption, was used as a basis for equating counts/min recorded with carbon dioxide converted into an acid-stable form.

## Results

Figure 1 illustrates simultaneous measurements of  $\text{CO}_2$  fixation and oxygen evolution. The chloroplasts were prepared and assayed in the absence of any added reductant (such as cysteine or ascorbate) and without added substrate other than  $\text{CO}_2$ . It will be seen that there was a close correspondence between the progress curve (continuous line) for oxygen and the  $\text{CO}_2$  fixation (circles). Both evolution and fixation started slowly and gradually accelerated until they reached a maximum rate after some minutes. When illumination was stopped, oxygen evolution was replaced (after a delay) by oxygen uptake. The immediate post-illumination uptake was rapid but the rate gradually decreased until after some minutes it had returned to the value observed prior to illumination (see also fig 2). There was no corresponding release of  $\text{CO}_2$ . We also measured post-illumination values of acid-stable radioactivity, under other, different, experimental conditions (see *e.g.* ref. 5). Our measurements were not sufficiently accurate to detect minor fluctuations ( $\pm 5\%$  of the  $\text{CO}_2$  fixed) but they would allow us to make the general statement that we have been unable to find evidence of any appreciable post-illumination release or uptake of  $^{14}\text{CO}_2$ .

*The Effect of Ascorbate.* Exogenous ascorbate increased oxygen uptake in the dark prior to illumination. Net evolution in the light was often unchanged or slightly higher than that observed in the absence of ascorbate, implying that the gross production of oxygen was greater. The effect varied with the season (possibly as the endogenous ascorbate varied). The rate of evolution in the presence of added ascorbate also tended to fall off less rapidly (see terminal rates of evolution in fig 2). Immediate post-illumination oxygen uptake was largely unchanged by added ascorbate but its rate declined more slowly (compare curves in fig 2).

as indicated and assayed for radioactivity (5). Oxygen (continuous line) was measured simultaneously in the same vessel (see Methods). Temp.  $20^\circ$ .

FIG. 2. (middle) Oxygen evolution and uptake in the presence and absence of exogenous ascorbate. Both reaction mixtures contained spinach chloroplasts ( $100 \mu\text{g}$  chlorophyll) in suspending medium (1 ml) containing  $\text{NaHCO}_3$  ( $10 \mu\text{moles}$ ). In addition one (continuous line) contained added isoascorbate ( $4 \mu\text{moles}$ ). Oxygen was recorded simultaneously in both vessels.

FIG. 3. (bottom) Post-illumination oxygen uptake. The effect of the duration of the preceding light period and the oxygen tension. Reaction mixtures *etc.* as for figure 2 but without added ascorbate.

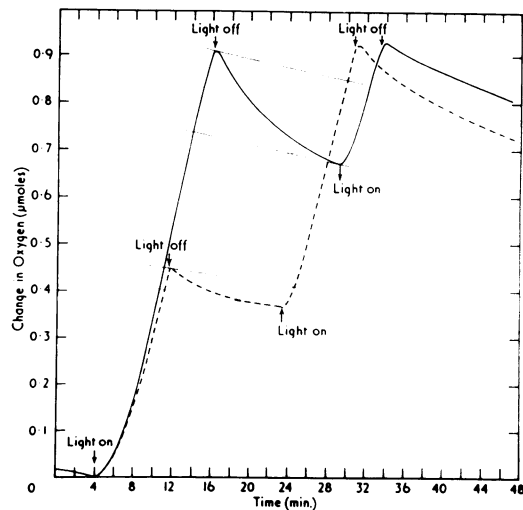
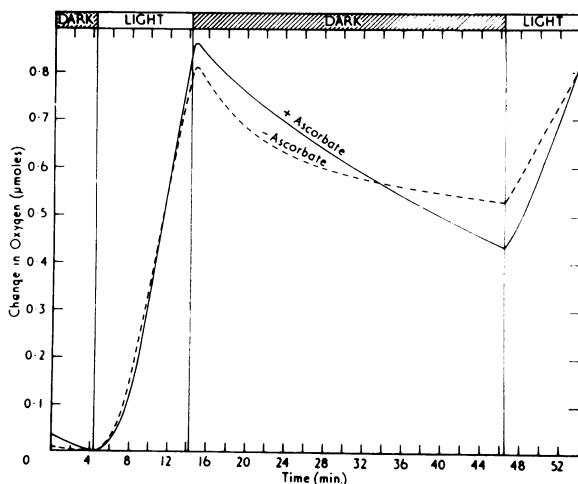
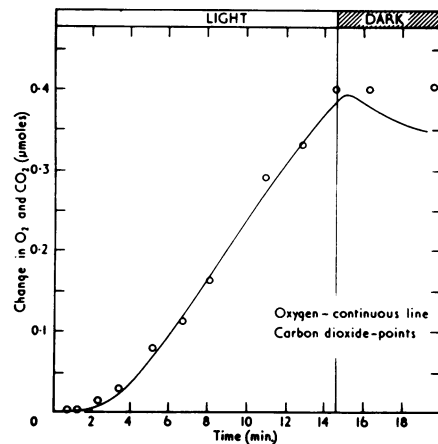


FIG. 1. (top) Simultaneous measurement of the oxygen evolved and the carbon dioxide fixed by isolated chloroplasts. The reaction mixture contained spinach chloroplasts ( $100 \mu\text{g}$  chlorophyll) in 1 ml of suspending medium (see Methods) to which was added  $\text{NaH}^{14}\text{CO}_3$  ( $400 \mu\text{c}$ ,  $7.5 \mu\text{moles}$ ). Samples (points) were removed

*The Effect, on Post-illumination Oxygen Uptake, of Oxygen Tension and the Duration of the Preceding Light Period.* In a number of experiments we found that there was an apparent degree of correlation between the rate of post-illumination oxygen uptake and the duration of the preceding light period. In figure 3 two identical reaction mixtures were illuminated simultaneously. After 8 minutes, illumination was stopped in one (broken line) and continued in the other (continuous line) for a further 4 minutes. It will be seen that the post-illumination oxygen uptake was greatest in the vessel (continuous line) which had been illuminated for the longer period. However, the question which then arises is whether or not this is attributable to the duration of illumination rather than to the increased oxygen tension. It is possible that both factors may be involved. Thus it will be seen in figure 3 that when a second shorter period of illumination was given to this vessel (continuous line) the post-illumination oxygen uptake was diminished although the oxygen tension at the onset of darkness was unchanged. Conversely, in the other vessel (broken line) a second, equal, period of illumination led to an increased oxygen uptake at the higher oxygen tension then prevailing.

*Extent of the Post-illumination Uptake.* Since the oxygen uptake after illumination was variable, it is clearly impossible, at present, to define its magnitude in anything but general terms. However, the progress curves given here are typical of our present experience and it will be seen that initially the rate of post-illumination uptake was about 3 times the dark uptake and about 25% of the preceding evolution. Its rate declined in 3 to 5 minutes until it had fallen to that of the dark uptake observed prior to illumination. The oxygen evolved in this period was of the same order (approx. 0.1  $\mu$ mole) as the chlorophyll content of the reaction mixtures (100  $\mu$ g).

## Discussion

In the experiments carried out by Walker and Hill (10) the chloroplasts were prepared and assayed in mixtures which at that time had given the highest published values for carbon dioxide fixation. These mixtures contained reduced glutathione and ascorbate (which were added to promote fixation and to slow chloroplast deterioration). The fact that these compounds also promoted a considerable oxygen uptake (even in the absence of chloroplasts) was of little consequence in fixation studies but when  $O_2$  evolution was measured it was necessary to correct for the oxygen uptake. Accordingly the rate of post-illumination oxygen uptake was added to the rate of net evolution in order to derive a value for actual oxygen production. This gave a stoichiometry (between  $O_2$  evolved and  $CO_2$  fixed) of approximately one. In the present work the chloroplasts

were prepared and assayed in new mixtures containing no added ascorbate or reduced glutathione and in which the initial oxygen uptake in the dark was negligible. Also in the previous experiments (10) ribose-5-P was added to shorten the initial induction period and to increase the maximum rate. It has since been shown (3,4) that the induction period is increased by the presence of orthophosphate in the separating medium (or in the reaction mixture) and that if orthophosphate is replaced by inorganic pyrophosphate (*cf.* 3,4,5,7,8) it is possible to obtain high rates of photosynthesis without adding cycle intermediates. The results illustrated in figure 1 therefore represent simultaneous measurement of fixation and evolution in a much simplified system, containing no exogenous reductant, and  $CO_2$ -bicarbonate as the sole added substrate. The agreement between  $O_2$  and  $CO_2$  is excellent and the stoichiometry between net evolution and fixation is close to unity throughout the period of illumination. It is probable, therefore, that most of the post-illumination oxygen uptake seen under these conditions actually starts when illumination ceases and is not simply made apparent by the cessation of evolution. This would in turn imply a reoxidation of some compound (or compounds) which had become reduced in the light.

Because of the magnitude of the observed changes which, on a molecular basis, are of the same order as the chlorophyll in the reaction mixtures it is unlikely that an explanation could be entirely based on some sort of post-illumination peroxidation (though peroxidation of ascorbate might be a contributory factor). Other proposals would need to take into account the apparently excellent correspondence between  $O_2$  evolved and  $CO_2$  fixed in the preceding light period and the fact that there is no appreciable net release of newly fixed  $^{14}CO_2$ , in the dark, from acid-stable products. The possibility that the oxygen uptake may be associated with post-illumination changes in the concentration of metabolites such as dihydroxyacetone phosphate cannot be discounted. A post-illumination change in dihydroxyacetone phosphate has been reported by Bassham and Jensen (2) which bears a remarkable resemblance to the post-illumination oxygen uptake which we have observed. This resemblance may be entirely fortuitous but it does provide an example of a known change which could be an oxidation and which would not necessarily involve a release of  $CO_2$ .

The results confirm our previously expressed view (3) that under adequately defined conditions the oxygen electrode provides a convenient and accurate means of measuring photosynthesis by isolated chloroplasts.

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