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THE RELATION OF OXYGEN EVOLUTION TO CARBON ASSIMILATION WITH ISOLATED CHLOROPLASTS

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SUMMARY

1. In order to determine the relationship to carbon assimilation, the progress of oxygen evolution by isolated chloroplasts was followed using the oxygen electrode.

2. An oxygen uptake was observed in the dark. This was attributable both to the chloroplast preparation and to autoxidation of the medium.

3. In the absence of added Calvin-cycle intermediates the rate of oxygen evolution reached a maximum only after several minutes illumination.

4. In the presence of ribose 5-phosphate the induction period was shortened. In the absence of bicarbonate, evolution came to a stop and could be restarted by the addition of bicarbonate. The oxygen produced corresponded to the CO_2 fixed when the oxygen uptake was allowed for.

5. When illumination of the chloroplasts was interrupted by a short period of darkness, oxygen evolution ceased. When illumination was resumed the induction period was very nearly eliminated.

6. In the presence of substrate amounts of 3-phosphoglycerate no induction period could be observed suggesting that the progress of oxygen evolution is related to the formation of the natural hydrogen acceptor, presumably 1,3-diphosphoglycerate.

7. The results provide direct evidence that there can be a carbon dioxide-dependent oxygen evolution with isolated chloroplasts under aerobic conditions.

INTRODUCTION

The green plant in light can produce an intracellular tension of oxygen considerably above that obtaining in the atmosphere. The production of oxygen corresponds with the carbon dioxide which is assimilated. In the original experiments with chloroplasts[1–4] in the presence of their associated hydrogen acceptors the production of oxygen could only be detected at very low pressures of this gas. DAVENPORT[5], by using the haemoglobin of Ascaris, was able to show that oxygen could be produced from the chloroplast preparations in considerable amounts but at an exceedingly low pressure. As the pressure was increased the oxygen became absorbed resulting in no net production. In the presence of suitable hydrogen acceptors, however, such as $K_3Fe(CN)_6$ the reduction to $K_4Fe(CN)_6$ can produce, in light, a pressure

of oxygen above that in the atmosphere. ARNON and his co-workers[6] were first to show that isolated chloroplasts could carry out the complete assimilatory process with carbon dioxide. They were able to measure the oxygen in a manometer and to characterise the gas by luminescent bacteria. Even in these experiments the initial pressure of oxygen was at a very low level, being below that required for the bacterial luminescence. Here again it seemed that unless the pressure of oxygen was low there could be no net production of this gas.

Subsequent work on the fixation of carbon dioxide by chloroplast preparations[7,8] has shown that significant rates can be obtained even in presence of atmospheric oxygen. Rates of up to 67 μmoles CO_2 per mg chlorophyll have been obtained[9]. This compares favourably with rates of oxygen production with certain hydrogen acceptors as given in a table by RABINOWITCH[10] (p. 1596).

The present work is an attempt to find out how far the assimilation of carbon dioxide by preparations of significantly intact chloroplasts[11] depends upon hydrogen transfer from water as compared with that derivable from some organic source. We have concluded that the measurements of changes in oxygen concentration as measured by a polarographic method show a correspondence with the carbon dioxide assimilated and that the equivalents of hydrogen required for its reduction are derived from water.

EXPERIMENTAL

Plant materials

Peas (*Pisum sativum* L. var. Laxton Superb) were grown in moist vermiculite for 14 days (11 h light, 13 h dark) at 18° under low light (approx. 200 foot candles) provided by five 40-W fluorescent lamps. Spinach (*Spinacia oleracea* L.) was grown in the Chelsea Physic Garden or purchased from local markets. Leaves of Good King Henry (*Chenopodium bonus henricus* L.) and Fat Hen (*Chenopodium album* L.) were collected from the field in June and July.

Preparation of chloroplasts

50 g of leaf were macerated for 5 sec in a Waring blendor in 200 ml ice-cold grinding medium and the juice squeezed from the homogenate through two layers of muslin and filtered through a further eight layers. The filtrate was poured into four 50-ml tubes, placed in a high-speed angle head centrifuge and the chloroplasts separated by allowing the centrifuge to accelerate to 6000 rev./min and then bringing it to rest as rapidly as possible by manual braking (the total spinning time was approx. 90 sec). The pellets were washed in resuspending medium and finally resuspended in a total of 5 ml using a glass rod and cotton wool. In the experiment illustrated in Fig. 1, chloroplasts were prepared from market-bought spinach which had been stored overnight in the dark at 5°. Prior to separation of the chloroplasts leaves were stripped of their mid-ribs and two 50-g samples were immersed in water. One sample was returned to the dark at 20° and the other illuminated (1000 foot candles) for 4 h at the same temperature.

Grinding and resuspending medium

The grinding medium contained 22.8 g sucrose, 200 mg NaCl, 200 mg $MgCl_2$

and 216 mg sodium isoascorbate in 200 ml of 0.1 M phosphate buffer at pH 6.8. In the resuspending medium the sucrose was increased to 30.8 g and the phosphate buffer replaced by 200 ml of water.

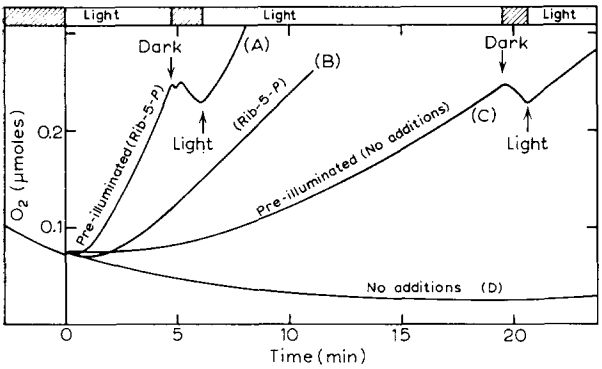


Fig. 1. Induction periods in oxygen evolution; effects of added ribose 5-phosphate and pre-illumination. The complete reaction mixtures (2 ml) contained ribose 5-phosphate, 12 μmoles; reduced glutathione, 6 μmoles; $NaHCO_3$, 17.7 μmoles; incubating medium, 0.3 ml; spinach chloroplasts, 0.6 ml. In A and C the chloroplasts were from pre-illuminated leaves and contained 318 μg chlorophyll. In B and D (from dark leaves) the chloroplasts contained 282 μg chlorophyll. In C and D the ribose 5-phosphate was omitted; (OG) 18°.

Incubating medium

The incubating medium (pH 7.5) added to the reaction mixtures contained the following additions in μmoles/ml. Sucrose, 600; disodium hydrogen phosphate, 10; $MgCl_2$, 5; $MnCl_2$, 10; EDTA, 10; Tricine–NaOH, 150. The Tricine (trishydroxymethylglycine) was prepared according to GOOD[12] and twice recrystallized from aq. ethanol.

Oxygen electrode

Two types of electrode were used. The "Rank" electrode indicated by (R) in the legends to figures, has a stationary platinum electrode separated from the reaction mixture by a polythene membrane. A closely fitting lid with a small hole limits gaseous exchange between the reaction mixture and the atmosphere. The reaction vessel is of perspex and is surrounded by a water jacket. The reaction mixtures (5 ml) are stirred by a magnetic stirrer.

The G.M.E. Oxygraph (Model KM), indicated by (OG), uses a rapidly oscillating naked platinum cathode, without auxilliary stirring, in a glass water-jacketed cell. In both instruments the reaction vessels were illuminated by a 500-W slide-projector fitted with a red perspex filter which transmits 90 % of the incident light at wavelengths longer than 610 mμ.

The electrodes were calibrated by taking 0.25 μmole/ml as the difference between the reading for air-saturated dilute KCl solution and the same solution following prolonged flushing with oxygen-free nitrogen.

Measurements of radioactivity

Samples (10 μl) were withdrawn from reaction mixtures with a microsyringe and injected into 40 μl of HCl (2.5 %, v/v). Aliquots (20 μl) were dried on lens tissue discs on aluminium planchettes and their radioactivity measured with a Nuclear-

Chicago gas-flow counter (Model D47). A standard radioactive sucrose solution sampled and counted in the same way was used as a basis for converting counts/min to μmoles CO_2 fixed[8]. The validity of this method had previously been confirmed by comparison of samples with known standards in a scintillation counter.

RESULTS

Induction periods in oxygen evolution

When chloroplasts were illuminated in the absence of added Calvin-cycle[13] intermediates, oxygen evolution reached its maximum only after a lag, or induction period, lasting several minutes (see *e.g.* Fig. 1C). The length of this induction period depended on the pretreatment of the leaves. Freshly gathered leaves which had been pre-illuminated yielded chloroplast preparations in which the induction period was relatively short. Conversely, after dark storage of leaves, the lag might be lengthened to such an extent that no net O_2 evolution was observed (Fig. 1D) but the ability to evolve oxygen could be restored by pre-illumination of the leaves (Fig. 1, *cf.* C and D). The induction period could also be shortened considerably by the addition of ribose 5-phosphate, though again the length of the lag was affected by the pretreatment of the leaves (Fig. 1, *cf.* A and B). In reaction mixtures containing chloroplasts from freshly gathered pre-illuminated leaves the induction periods in the presence of ribose 5-phosphate were as short as 1 min.

Requirement for carbon dioxide

If bicarbonate was not added to the reaction mixture the course of oxygen evolution was at first similar to that in the presence of bicarbonate (Fig. 2 *cf.* A and B). However, it soon declined and stopped. It could then be restarted by the addition of bicarbonate (Fig. 2B). The initial oxygen evolution in the absence of added bicarbonate may be attributed to the utilisation of carbon dioxide already present in the reaction mixtures.

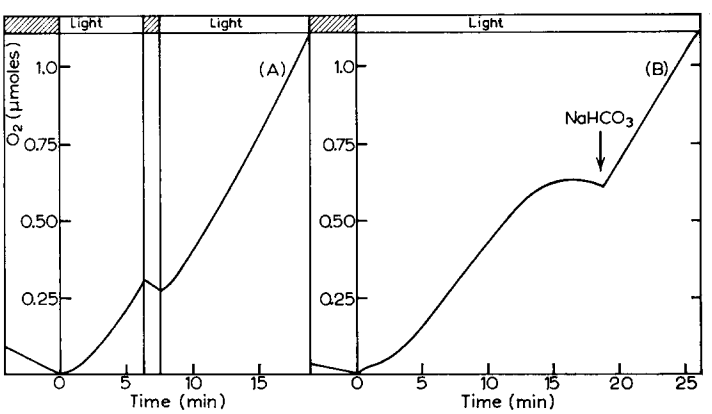


Fig. 2. Dependence of oxygen evolution on presence of carbon dioxide. The reaction mixtures (5 ml) contained ribose 5-phosphate, 20 μmoles; reduced glutathione, 10 μmoles; resuspending medium, 2.0 ml; incubating medium, 0.5 ml; and spinach chloroplasts, 1.0 ml (approx. 500 μg chlorophyll). In addition, in A, 30 μmoles of $NaHCO_3$ were added prior to illumination. In B no bicarbonate was added initially, 15 μmoles at the time indicated; (R) 16°.

Interrupted illumination

If the light was switched off once the chloroplasts had started to produce oxygen at, or near, maximum rates the evolution of O_2 ceased; when the illumination was resumed after a short dark interval the induction period was either greatly diminished or entirely eliminated (Figs. 1–5).

Relation of oxygen evolution to carbon dioxide fixation

The induction periods in oxygen evolution illustrated in Figs. 1 and 2 closely resembled those observed in carbon dioxide fixation in similar reaction mixtures[11]. Fig. 3 shows the results of an experiment in which CO_2 fixation and oxygen evolution were followed simultaneously in the same reaction vessel. Part of the added bicarbonate was replaced by $Na_2{}^{14}CO_3$ and samples were withdrawn and acidified at the times indicated. The residual radioactivity following acidification and drying is a measure of the $^{14}CO_2$ incorporated into photosynthetic products (see EXPERIMENTAL). It will be seen that an initial induction period was observed in both CO_2 fixation (Fig. 3 curve A) and oxygen evolution (Fig. 3 curve B) and that the progress curves followed a similar course. When the light was switched off, fixation ceased and oxygen evolution was replaced by an oxygen uptake. Following the dark interval, both evolution and fixation were resumed without an appreciable lag (*cf.* ref. 11).

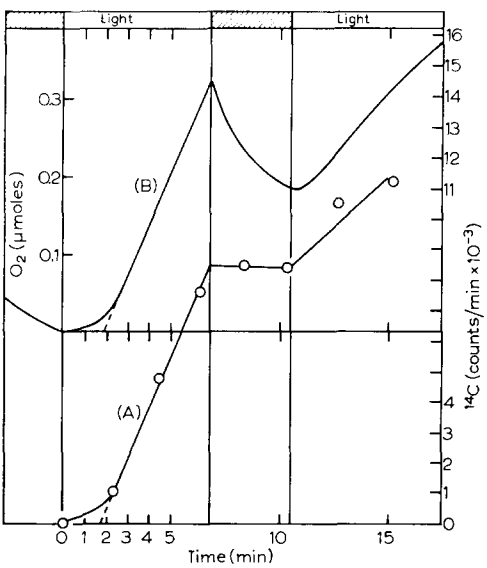


Fig. 3. Progress curves for oxygen evolution (B) and carbon dioxide fixation (A) in the same vessel. Reaction mixture (1.8 ml) as for Fig. 1 (containing 12 μmoles ribose 5-phosphate) with pea chloroplasts (230 μg chlorophyll) and a proportion of the added bicarbonate as ^{14}C (120 μC); (OG) 20°. Samples for counting (A) were withdrawn with a microsyringe at the times indicated. A count of 1.2×10^4 is equivalent to 1.0 μmole CO_2 fixed.

Factors contributing to oxygen uptake

A mixture of the incubating medium and resuspending medium (see EXPERIMENTAL) showed an appreciable oxygen uptake in the absence of chloroplasts, presumably because of a manganese catalysed oxidation of ascorbate. In the dark this

was increased in the presence of chloroplasts. Addition of heat-inactivated chloroplasts (see below) produced a more rapid oxygen uptake in the dark which was further increased in the light.

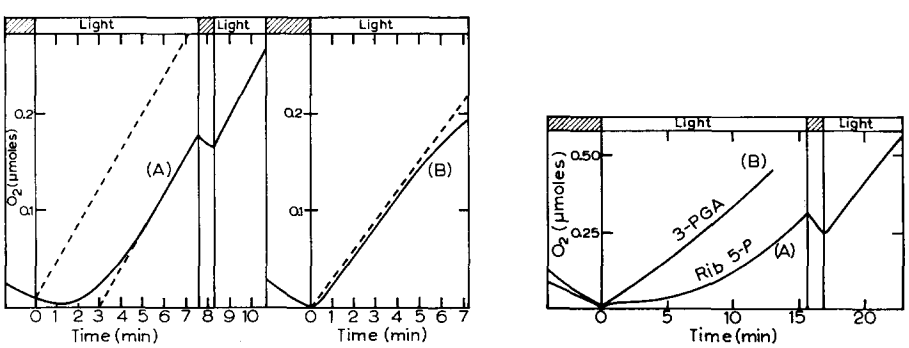
In complete reaction mixtures oxygen was normally taken up at a fast rate immediately after cessation of illumination. This uptake then slowly declined towards the rate observed prior to illumination.

Ratios between oxygen produced and carbon dioxide fixed

In Fig. 3, the maximum rate of carbon dioxide fixation was 38 μmoles per mg chlorophyll per h and the maximum net rate of oxygen evolution 16 μmoles per mg chlorophyll per h. In order to calculate the actual rate of oxygen production it is necessary to make a correction for oxygen uptake. It will be seen in Fig. 3 that immediately following the cessation of illumination the dark uptake was approximately equal in rate to the preceding evolution. If it is assumed that the rate of O_2 consumption in the light is the same as the uptake recorded on darkening, the value for oxygen production would therefore be 32 μmoles per mg chloropyll per h. We regard this as a reasonable agreement between oxygen evolution and carbon dioxide fixation in view of the nature of the data obtained.

Oxygen evolution in the presence of 3-phosphoglycerate

In carbon dioxide fixation by isolated chloroplasts in reaction mixtures similar to those described here ribose 5-phosphate diminished the lag more effectively than any other cycle intermediate tested[11,14]. In the presence of 3-phosphoglycerate the induction period in fixation is diminished but the maximum rate was still only reached some minutes after the start of illumination[14]. In contrast, for oxygen evolution this order of effectiveness was reversed and in the presence of 3-phosphoglycerate the lag was entirely eliminated. Fig. 4 shows this effect recorded on the G.M.E. Oxygraph for pea chloroplasts and Fig. 5 shows it measured on the Rank electrode (with the polythene membrane; see EXPERIMENTAL) using spinach chloroplasts. In both Figs. 4 and 5 the course of O_2 evolution in the presence of 3-phosphoglycerate (B) is compared with that in the presence of ribose 5-phosphate (A) in



Figs. 4 and 5. Progress curves for oxygen evolution; effect of 3-phosphoglycerate (3-PGA) compared with that of ribose 5-phosphate (Rib-5-*P*). Fig. 4, reaction mixtures as for Fig. 1, with pea chloroplasts (408 μg chlorophyll); 17° (OG). Fig. 5, reaction mixtures as for Fig. 2, with 30 μmoles $NaHCO_3$ and 6 μmoles ribose 5-phosphate; (R) 16°. In Fig. 4B and Fig. 5B, equimolar 3-phosphoglycerate substituted for ribose 5-phosphate.

otherwise identical reaction mixtures. The rapidity with which the maximum rate was reached in the presence of 3-phosphoglycerate may be compared with the resumption of evolution following a dark interval (Figs. 1–5).

Chloroplast preparations from different plant material

Essentially similar results to those reported in this paper were also obtained using chloroplasts prepared from spinach purchased from a number of different sources in London and Cambridge and from leaves of *Chenopodium bonus henricus* L. and *Chenopodium album* L.

Thermolability and sensitivity to DCMU

Oxygen production was no longer observed when chloroplasts in resuspending medium were exposed to 45° for 2 min prior to illumination in a full reaction mixture. Evolution was also rapidly stopped by the addition of DCMU to a final concentration of $3 \cdot 10^{-7}$ M in reaction mixtures containing approx. 250 μg chlorophyll.

DISCUSSION

In the experiments designed to show the assimilation of CO_2 by isolated chloroplasts in the light there is an uptake of oxygen in the dark when the experimental mixtures are in contact with air. In light, there can be a net production of oxygen if the preparation is sufficiently active. It may be assumed that the uptake of oxygen can mainly be accounted for in terms of the suspending medium for the chloroplasts, together with the presence of a certain fraction of broken chloroplasts in the preparation. GIBBS AND BAMBERGER[7] found that in the presence of atmospheric oxygen the rate of fixation of CO_2 is increased if ascorbate and glutathione are added. It was found in the present investigation that the addition of ascorbate and glutathione gives an increased rate of CO_2 fixation and also an increased rate of oxygen uptake. In the absence of these additions there can still be a net production of oxygen, though at a lower rate. In view of this dark uptake there must be a critical limit for the activity of the system for fixation below which there will be no net production of oxygen (*e.g.* Fig. 1, D) though CO_2 is being assimilated[11]. The oxygen uptake seems to depend upon the pressure of oxygen so that, if there is a low activity for the fixation of CO_2 there will be no net production of oxygen unless the pressure of oxygen is at a sufficiently low level.

In general the experiments have shown that in air a high rate of CO_2 fixation in light is accompanied by a high rate of oxygen absorption in the dark (*e.g.* Fig. 3), though there is no obvious quantitative relationship between the light and dark processes. The whole problem of the oxygen uptake deserves further study. For the present discussion we have assumed that the absorption of oxygen is independent from the fixing of the CO_2.

It was found by GIBBS AND BAMBERGER[7] and by WALKER[11] that the chloroplasts when illuminated in the appropriate medium always show a lag period before the final and sensibly uniform rate of CO_2 fixation is attained. The lag period could be shortened by supplementing the chloroplasts with components of the Benson–Calvin cycle[13] such as fructose 1,6-diphosphate, 3-phosphoglycerate and ribose 5-phosphate[7,11,14]: the last mentioned appearing to act most rapidly. When a steady state

was attained, if the light was interrupted by a short dark interval there was found to be no further lag when illumination was resumed[11]. The changes in the rate of CO_2 fixation were found to correspond to changes in the oxygen concentration when the system was followed with the polarographic methods. The correspondence, as can be seen from Fig. 3, would be nearly exact if the rates of dark uptake are added to the rate of change in oxygen concentration in the light. This would indicate that the hydrogen for the reduction of the CO_2 is derived from the water and not from organic sources such as the ascorbic acid RABINOWITCH[10] (p. 1537).

When bicarbonate was not added to the medium the production of oxygen was found to diminish after an interval in light and to give place to an oxygen uptake. At this point, addition of bicarbonate restored the rate of O_2 production. The production of O_2 in the complete system can thus be seen to depend directly upon the presence of CO_2.

While addition of small amounts of ribose 5-phosphate or 3-phosphoglycerate diminished the lag phase in CO_2 fixation when present before the initial light period they did not abolish it[11,14]. In contrast to this, addition of 3-phosphoglycerate in substrate amounts before illumination immediately gave a steady rate of O_2 production from the start of the light period. This is evidence that, as regards the whole chloroplasts 3-phosphoglycerate can act apparently as a direct hydrogen acceptor in the light, before the full capacity of the system to fix CO_2 has been attained. If the reduction of 3-phosphoglycerate is assumed to take place *via* a photophosphorylation, a phosphokinase reaction and reduced NADP, this process would be independent of the other carbohydrate components of the cycle which act in a catalytic manner for the CO_2 fixation[11,14]. Regarded in this way there could hardly be a more striking demonstration that the Calvin–Benson cycle is operating as the major process in the fixation of CO_2 with these chloroplast preparations.

RABINOWITCH[10] (p. 1418) has offered an explanation of the induction phenomena of photosynthesis in terms of an autocatalytic adjustment of the concentrations of the intermediates of the Calvin cycle. The experiments described here with the intact chloroplasts would appear to support this analysis of the induction process.

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