Effects of pH and Oxygen on Photosynthetic Reactions of Intact Chloroplasts

Received for publication April 29, 1975 and in revised form September 25, 1975

Ulrich Heber, T. John Andrews, and N. Keith Boardman
Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, and Research School of Biological Sciences, Australian National University, Canberra, Australia

ABSTRACT

Oxygen inhibition of photosynthesis was studied with intact spinach (Spinacia oleracea L.) chloroplasts which exhibited very high rates of photosynthetic CO₂ reduction and were insensitive to additions of photosynthetic intermediates when CO₂ was available at saturating concentrations. Photosynthetic rates were measured polarographically as O₂ evolution, and the extent of the reduction of substrate was estimated from the amount of O₂ evolved. With CO₂ as substrate, inhibition of photosynthesis by O₂ was dependent on pH. At pH values above 8, rates of O₂ evolution were strongly inhibited by O₂ and only a fraction of the added bicarbonate was reduced before O₂ evolution ceased. The extent of O₂ evolution declined with increasing O₂ concentration and decreasing initial bicarbonate concentration. At pH 7.2, the initial photosynthetic rate was inhibited about 30% at high O₂ levels, but the extent of O₂ evolution was unaffected and most of the added bicarbonate was reduced. Photosynthetic O₂ evolution with 3-phosphoglycerate as substrate was similarly dependent on pH and O₂ concentration. In contrast, there was little effect of O₂ and pH on oxaloacetate-dependent oxygen evolution. Acid-base shifts experiments with osmotically shocked chloroplasts showed that ATP formation was not affected by O₂. The results are discussed in terms of a balance between photosynthetic O₂ evolution and O₂ consumption by the ribulose diphosphate oxygenase reaction.

Photosynthesis of plants whose primary product of CO₂ fixation is PGA (C₃-plants) is inhibited by O₂ (the Warburg effect, 26). Inhibition generally amounts to about 30% in normal air and is independent of light intensity (6). It can be relieved by increasing the CO₂ pressure. Recent studies have shown that CO₂ and O₂ compete for the same substrate (RuDP) and, moreover, the carboxylase and oxygenase reactions are catalyzed by the same protein (1-3, 5, 7). The primary products of the oxygenase reaction are 1 molecule of PGA and 1 molecule of phosphoglycolate, whereas the carboxylase reaction gives 2 molecules of PGA. In intact cells, phosphoglycolate is thought to be converted to glycolate and then metabolized in peroxisomes via the glycolate pathway, to eventually form CO₂ and glycerate, which can re-enter the chloroplast and be reduced there (12, 23). Oxygenation of RuDP and CO₂ evolution from the reaction products would decrease the yield of photosynthesis. Glycolate formation related to the O₂ inhibition of photosynthesis has also been explained by oxidative interactions with C₅ or C₇-sugar phosphates (9, 16, 22).

It has been shown that photosynthetic CO₂ reduction by isolated chloroplasts is also inhibited by O₂ (8, 9, 21). In the present work, we have studied the effect of O₂ on photosynthesis by intact chloroplasts, capable of very high rates of photosynthesis. The aim of our experiments was to gain further insight into the problem of O₂ inhibition of photosynthesis in a system less complex than the intact leaf and more sophisticated than a simple enzyme system.

MATERIALS AND METHODS

Spinach Hybrid 102 (Spinacia oleracea L.) was purchased from Arthur Yates and Co., Sydney, who obtained it from Alf. Christianson Seed Co., Mount Vernon, Wash. Seeds were germinated in vermiculite and either transferred to hydroponic culture or grown in vermiculite. The nutrient solution was the same as used by Tsui (24), except that sequestrene iron chelate (15.4 mg/l) replaced iron tartrate. The plants were grown for 3 to 4 weeks before the leaves (length 9-10 cm) were harvested. The detached leaves were preilluminated for 5 to 10 min at 4 C in white light (11) and chloroplasts were isolated by a modification (11) of the method of Jensen and Bassham (18).

Photosynthetic O₂ evolution was measured polarographically at 20 C. using CO₂, PGA, or OAA as substrate. Chloroplasts (100-200 μg Chl) were suspended in 2.5 ml of a buffer containing 300 mM sorbitol, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, 10 mM NaCl, 0.8 mM inorganic phosphate (KH₂PO₄), and 50 mM HEPES, pH 6.8 to 8.5. CO₂ was removed from the buffer by bubbling with N₂ for 30 min at pH 3.8, and the pH readjusted with bicarbonate-free KOH. The O₂ concentration was adjusted to desired level by bubbling with N₂ or O₂ prior to addition of substrate and chloroplasts. Concentration of substrate (NaHCO₃, PGA, or OAA) varied between 0.125 mM and 4 mM. Dithiothreitol (3 mM) was added to the reaction mixture when NaHCO₃ was the substrate, and it was sometimes present during reduction of PGA. In the presence of dithiothreitol, CO₂/O₂ ratios are close to unity (11). Tight sealing of the reaction vessels prevented the escape of O₂. Illumination was provided by a 250-w incandescent lamp, and passed through a 4-cm layer of water. The light intensity at the surface of the reaction vessel was 120 kerg cm⁻² sec⁻¹ (a Calflex B filter was used to block far red radiation). When indicated, catalase (specific activity, 50,000 IU/mg) was added at a concentration of 40 μg/ml. In some experiments aldolase (40 μg/ml, specific activity, 15 IU/mg) and triosephosphate isomerase (Boehringer, Mannheim, 15 μg/ml, specific activity, 24,000 IU/mg) served to convert FDP to DHAP. The enzymes were freed of ammonium problem. 

1 This research was supported by CSIRO and the Deutsche Forschungsgemeinschaft (U.H.) and by a Queen’s Fellowship in Marine Science (T.J.A.).

2 Present address: Institute of Botany, University of Düsseldorf, Germany.

3 Abbreviations: PGA: 3-phosphoglycerate; DHAP: dihydroxyacetone phosphate; FDP: fructose diphosphate; GAP: 3-phosphoglyceraldehyde; OAA: oxaloacetate; Ru-5-P: ribose 5-phosphate; Ru: ribulose diphosphate.
sulfate by passage through a column of Sephadex G-25. Acid-base phosphorylation was measured with osmotically shocked chloroplasts, as described by Jagendorf and Uribe (17).

RESULTS

The chloroplasts used in the present work showed rates of photosynthetic O2 evolution of 100 to 350 μmole mg Chl^{-1} hr^{-1} with CO2 as substrate at pH 7.6 and with light intensity and substrate concentration near saturating. After an induction period, the photosynthetic rate was not influenced by addition of intermediates of the carbon reduction cycle. With PGA as substrate, rates of O2 evolution varied from 100 to 225 μmole mg Chl^{-1} hr^{-1}, and with OAA as substrate (in the absence of an uncoupler of phosphorylation) from 30 to 80 μmole mg Chl^{-1} hr^{-1}. The chloroplasts were very stable; even after 24 hr at 0 °C, rates of CO2 reduction sometimes were 50% of the initial rates with freshly prepared chloroplasts.

Effect of pH on Rate and Extent of O2 Evolution with CO2 as Substrate. Figure 1 shows polarograph traces of intact chloroplasts on addition of 0.25 mM NaHCO3. At pH 7.2, there was the characteristic lag phase, attributed to the accumulation of endogenous intermediates (25), followed by a linear rate of O2 evolution. When the substrate was depleted the rate of O2 evolution declined. In contrast, there was no net O2 evolution at pH 8.3 in the experiment reported in Figure 1. Instead, a considerable O2 uptake was observed due mainly to a Mehler-type reaction, since it could be largely but not completely inhibited by catalase. The extent of O2 evolution at high pH values varied somewhat with different chloroplast preparations. It is estimated that at a bicarbonate concentration of 0.25 mM, the available CO2 was largely reduced at pH 7.2, if the assumption is made that one mole of O2 evolved corresponds to one mole of CO2 reduced. At higher pH values and 0.25 mM bicarbonate it would seem that only a fraction of the added CO2 was reduced. The fraction of bicarbonate which was reduced at higher pH values was influenced by the initial bicarbonate concentration. The initial rate of O2 evolution was also increased as bicarbonate concentrations were increased and, in fact, at bicarbonate concentrations close to saturation, higher rates of O2 evolution were observed at pH 8.3 than at pH 7.2 (Table I). The pH optimum of photosynthesis is dependent on bicarbonate concentration; it was pH 7.3 at a bicarbonate concentration of 0.5 mM, but pH 7.7 with 1 mM bicarbonate.

Table I. Apparent Michaelis Constants of Intact Chloroplasts for CO2 and Bicarbonate and Maximal Reaction Rates as a Function of pH

<table>
<thead>
<tr>
<th>pH</th>
<th>Km[CO2] (μM)</th>
<th>Km[HCO3−] (μM)</th>
<th>V_max (μM O2 min−1 mg Chl−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>39</td>
<td>0.2</td>
<td>100</td>
</tr>
<tr>
<td>7.2</td>
<td>20</td>
<td>0.15</td>
<td>240</td>
</tr>
<tr>
<td>7.4</td>
<td>39</td>
<td>0.45</td>
<td>200</td>
</tr>
<tr>
<td>7.9</td>
<td>19</td>
<td>0.6</td>
<td>300</td>
</tr>
<tr>
<td>8.1</td>
<td>19</td>
<td>1</td>
<td>313</td>
</tr>
<tr>
<td>8.5</td>
<td>12</td>
<td>1.45</td>
<td>250</td>
</tr>
</tbody>
</table>

1 Data were obtained in a different experiment with another chloroplast preparation.

The lower rate and incomplete extent of CO2 reduction at higher pH values under limiting substrate concentration was not caused by faster aging of the chloroplasts at higher pH was shown by two observations. At pH 8.3 and an initial O2 concentration of 0.27 mM an insignificant amount of O2 was evolved with 0.25 mM bicarbonate added (Fig. 2A). After titration of the sample to pH 7.2, a second illumination resulted in O2 evolution corresponding to 95% of the added bicarbonate. In another preparation of chloroplasts (Fig. 2B) some O2 was evolved at pH 8.5 with 0.19 mM bicarbonate and, the extent of O2 evolution corresponding to the reduction of about 30% of the added bicarbonate. Addition of 0.5 mM bicarbonate resulted in some more O2 evolution and led to a new steady state. Further addition of 3 mM bicarbonate produced a higher rate of O2 evolution.

If the failure to reduce most of the available bicarbonate at pH 8.5 is due to inhibition of the carboxylation reaction, then it would seem that an increased bicarbonate concentration can relieve the inhibition in a competitive manner, reminiscent of the relief of O2 inhibition of photosynthesis by CO2 in intact leaves. Figure 3 shows O2 evolution by chloroplasts at pH 8.5 in the presence of a high concentration of bicarbonate. After the steady state was reached, the light was turned off, the stopper sealing the reaction vessel was removed, and O2 was allowed to escape. A second illumination caused further O2 evolution, showing that a high O2 concentration is at least one of the factors responsible for the incomplete reduction of bicarbonate at high pH. The apparent extent of bicarbonate reduction (as calculated from the amount of O2 evolved) decreased with increasing O2 concentration and with increasing pH as shown in Figure 4. Most of the added bicarbonate was reduced at pH 7.2 even at high O2 concentrations. Inhibition of net photosynthetic O2 evolution by O2 increased with increasing pH, suggesting that little of the added bicarbonate was reduced at pH 8.5 and 1 mM O2. Figure 5 shows the effect of O2 concentration on the
OXYGEN INHIBITION OF CHLOROPLASTS

Fig. 2. A: Effect of pH and bicarbonate concentration on CO$_2$-dependent O$_2$ evolution by intact chloroplasts. Insignificant O$_2$ evolution occurred at pH 8.3 in the presence of 0.25 mM HCO$_3^-$, After titration of the sample to pH 7.2 a second illumination almost completely reduced the added CO$_2$. Extent of CO$_2$ reduction at the second illumination was estimated as 97% after proper correction was made for slow O$_2$ uptake after completion of the reaction. Catalase was added to the sample. B: Incomplete reduction of 0.19 mM HCO$_3^-$ in a different experiment at pH 8.5. After O$_2$ evolution ceased, it is estimated that 30% of the HCO$_3^-$ was reduced. End of reaction was not caused by chloroplast aging as shown by resumption of some O$_2$ evolution after addition of 0.5 mM HCO$_3^-$. Further addition of 3 mM HCO$_3^-$ again resulted in O$_2$ evolution.

Fig. 3. Reduction of 2 mM HCO$_3^-$ by intact chloroplasts at pH 8.5. Reaction leveled off after 37% of the substrate was reduced. After O$_2$ had escaped from the opened cuvette a second illumination produced, at the reduced O$_2$ concentration, more reduction of HCO$_3^-$ bringing the total amount of CO$_2$ reduced to 44%.

rate of photosynthetic O$_2$ evolution. There was some inhibition of O$_2$ evolution by O$_2$ at pH 7.2, and the degree of inhibition increased with increasing pH. Increasing the bicarbonate concentration relieved the inhibition, resulting in higher rates of O$_2$ evolution and an increased apparent extent of reduction of bicarbonate at the steady state.

Effect of pH on Rate and Extent of O$_2$ Evolution with Phosphoglycerate as Substrate. Intact chloroplasts have uptake mechanism for some phosphate esters and some dicarboxylic acids (13, 14, 25). In the presence of PGA, intact chloroplasts evolve O$_2$ in the light, and the main reaction product is DHAP. At a saturating concentration of substrate (2 mM) the pH optimum of the reaction was 7.3. At this or a lower pH most of the PGA was reduced in the light, as estimated from the corresponding O$_2$ evolution. At high pH the O$_2$ released was only a fraction of that expected from an assumed stoichiometry of 1 mole of O$_2$ evolved for 2 moles of PGA reduced (Fig. 6). Subsequent adjustment of the pH to 7.2 after reaction at pH 8.3 caused more
O₂ evolution, but the reaction did not go to completion until the pH was lowered further to 6.8. This clearly shows that the poor O₂ evolution at high pH was not caused by consumption of substrate in a side reaction such as that caused by phosphatase action.

Oxygen evolution by chloroplasts which had incompletely reduced added PGA could be restarted by adding either bicarbonate or more PGA. The total percentage of reduction soon after ‘equilibrium’ had been reached was somewhat lower after two consecutive additions of substrate than after addition of the combined amount. Similar observations were also made during CO₂ reduction.

Figure 7 shows the effect of O₂ on the extent of PGA reduction, as calculated from the corresponding O₂ evolution. At pH 7.2 and a final O₂ concentration of about 0.5 mM, most of the added PGA was reduced. Oxygen had only a small influence on the extent of PGA reduction at pH 7.2, but at pH 8.4 high O₂ concentrations had a very marked inhibitory effect on the amount of O₂ evolved.

Oxaloacetate Reduction. In contrast to CO₂ and PGA reductions which also require ATP, OAA reduction only needs reducing equivalents. Comparison of OAA and PGA reductions at three pH values and a high initial O₂ tension is shown in Figure 8. It is apparent that the extent of PGA reduction as estimated
from the amount of O₂ evolved was smaller at high pH than was the extent of OAA reduction. The apparent decrease of OAA reduction with increasing pH was probably due to some decomposition of OAA, which yields CO₂. Only a fraction of such CO₂ would be reduced at high pH.

Ribose 5-Phosphate- and Triosephosphate-dependent O₂ Uptake. In the absence of bicarbonate, addition of 1 mM Ri-5-P or 1 mM FDP together with aldolase (40 μg/ml) and triosephosphate isomerase (15 μg/ml) to chloroplasts suspended in a medium containing catalase caused slow O₂ uptake. This rate was increased by increasing the O₂ level. Ri-5-P-dependent O₂ uptake was somewhat more pronounced at pH 7.2 than at pH 8.3 (Table II). When chloroplasts were uncoupled by 10 mM NH₄Cl or 5 μM carbonyl cyanide-p-trifluorophenylhydrazone, addition of Ri-5-P, or FDP together with aldolase and triosephosphate isomerase, did not produce an extra O₂ uptake in the light, indicating that light-induced oxidation of the phosphate esters was blocking by uncoupling.

Acid-base Shift Experiments. Light-induced shrinkage experiments have indicated that O₂ influences the phosphorylation potential of leaves (10, 19). It is possible, therefore, that the O₂ inhibition of CO₂ and PGA reductions by chloroplasts might be caused by an inhibition of ATP formation by O₂. Indeed, Jagendorf and Uribe (17) performed their elegant acid-base shift experiments, which were designed to test a chemo-osmotic hypothesis of ATP formation, under N₂. Figure 9 shows the effect of O₂ tension on acid-base phosphorylation by osmotically shocked chloroplasts. ATP formation was independent of the O₂ tension, which rules out a direct effect of O₂ on ATP synthesis by thylakoid membranes.

**DISCUSSION**

Our experiments show that the extent to which highly active spinach chloroplasts were able to reduce a given quantity of bicarbonate of PGA decreased as either the pH or the O₂ concentration of the suspending medium was raised. In contrast, OAA reduction was relatively unaffected by comparable changes of pH or O₂ concentration. Since photophosphorylation is not required for OAA reduction, as is the case for the other two substrates, it might appear at first sight that the results would be consistent with deleterious effects of high pH and O₂ concentration on this process. However, the observation that ATP synthesis during acid-base shifts was unimpaired by high O₂ pressure does not support this hypothesis (Fig. 9).

**Table II. Ribose 5-phosphate-dependent O₂ Uptake by Intact Chloroplasts in Light**

<table>
<thead>
<tr>
<th>O₂ Concentration</th>
<th>O₂ Uptake at pH 7.2</th>
<th>O₂ Uptake at pH 8.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>μmol mg⁻¹ Chl h⁻¹</td>
<td>μmol mg⁻¹ Chl h⁻¹</td>
</tr>
<tr>
<td>0.27</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>0.80</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>1.10</td>
<td></td>
<td>7.5</td>
</tr>
</tbody>
</table>

![Fig. 7](image1.png)

**Fig. 7.** Apparent extent of reduction of added 0.5 mM PGA as a function of pH and of the final O₂ concentration reached at the endpoint of the reaction. Different initial O₂ concentrations were produced by gassing with O₂ before illumination.

![Fig. 8](image2.png)

**Fig. 8.** Reduction of 0.28 mM OAA at different pH values as compared with reduction of 0.28 mM PGA. Numbers on the curves indicate apparent extents of reduction. O₂ concentration at beginning of illumination was 0.8 mM. The upward arrows indicate when light was turned on.
dimethylurea, transition as a
mM medium containing
initiated after and
all substrate
point. This is defined
had been slowed
identical
oxygenase
with
characteristics of
showing
activities
oxygenase exists in
concentration
of
pressure
concentration should
reaction is
of
consumption
was
chloroplast's
PGP-reducing
ability. However, a
pronounced effect both on the initial rate as well as on the extent of
O2 evolution with PGA as substrate. With saturating substrate
concentrations, the pH optimum for PGA reduction occurred at
about pH 7.3, whereas for CO2-saturated O2 reduction it was
about 7.9 (Table I). While the pH versus activity curve for PGA
reduction was found to be broad when PGA was available in
large excess, at low PGA concentrations there was a much
steeper decrease in PGA-reduction rate when the pH was
increased (Fig. 8). This may reflect a mass action effect of the
decreasing proton concentration since protons participate in
the PGA-reducing reaction

\[
\text{PGA}^2^- + \text{ATP}^3^- + \text{NADPH} + H^+ \rightarrow \\
\text{GAP}^2^- + \text{ADP}^3^- + \text{Pi}^- + \text{NADP}^+. \tag{1}
\]

Although the pH of the stroma will be higher than that of
the medium (15), it will reflect the varying pH of the medium, as
shown by the varying Km values for HCO3- (Table I). Thus
PGA reduction is adversely affected by increasing pH in a
manner similar to CO2-reduction, although for a different
reason. Since the O2-consuming reaction, RuDP oxygenase, is
largely unaffected by pH in this range (3), the O2-compensation
point for PGA reduction will also occur at progressively lower
O2 concentrations as the pH rises, thus decreasing the extent of
PGA utilization at equilibrium (Fig. 7). Artificially increasing the
O2 concentration of the reaction mixture will stimulate
oxygenase activity (1, 2) and perhaps even inhibit the
PGP-reducing enzyme (8) thus greatly enhancing the effect (Fig. 7).
As for CO2 reduction, increasing the rate of PGA reduction by
lowering the pH will restart a reaction which had stopped at
this O2-compensation point and allow it to proceed to the higher
compensation point appropriate to the lower pH (Fig. 6).

When a reaction which had proceeded to its equilibrium point
was restarted by a second addition of substrate, either bicarbon-
ate or PGA, the second equilibrium point occurred at a lower O2
concentration than if a single addition of the same total amount
of substrate had been made. This observation is consistent with
the O2-compensation point hypothesis since it suggests that at
the first equilibrium point in the former case, substrate
continued to be consumed, albeit at a slow rate, without any net
production of O2.

The products of the reduction of both CO2 and PGA enter the
Calvin cycle and thus may be used to regenerate RuDP, the
substrate of the proposed O2-consuming reaction. This does not
apply for OAA reduction since, in C3 plants, C from malate
enters the Calvin cycle only very slowly, if at all. Therefore, in
the case of OAA reduction, the O2-consuming reaction would
be severely inhibited through lack of substrate and thus the
concept of an O2-compensation point would not apply. This is
in agreement with the data showing that OAA was almost
completely consumed even at high pH and high O2 concentration
(Fig. 8). The slight decrease in extent of OAA reduction with
pH may be more apparent than real as has already been
discussed.

The proposed O2-consuming reaction catalyzed by RuDP
oxygenase has been shown to be quite active in spinach leaf
extracts with a rate of about 35 \( \mu \)moles mg Chl\(^{-1}\) hr\(^{-1}\) at
air levels of CO2 and O2 (2). This rate would be easily adequate
to account for its presently postulated role. Our attempts to
measure this activity in intact chloroplasts as Ri-S-P-dependent O2
uptake (Table II) were probably hindered by a rate-limiting
permeability for Ri-S-P. These highly active chloroplasts may
not be as permeable to Ri-S-P as are other preparations (21, 25).
The demonstration that the slow rate of oxidation of Ru-5-P or other sugar phosphates was completely inhibited by uncouplers supports the contention that RuDP is the substrate for the oxidation reaction. The increased activity at higher than ambient O₂ concentrations (Table II) is also reminiscent of RuDP oxygenase (1, 2).

Although it appears that most of our data can be satisfactorily explained by the presence of an O₂-consuming reaction (RuDP oxygenase) and the idea of an O₂-compensation point, we need to consider the quantitative aspects. With bicarbonate as substrate, the O₂-compensation point (no net O₂ evolution) represents a situation where the rate of the oxygenase reaction is double that of the carboxylase reaction. This should lead to a gradual depletion of RuDP, the substrate for both the oxygenase and carboxylase reactions, since some C is channeled into P-glycolate and insufficient DHAP is formed to sustain the pool of RuDP. The deficiency in RuDP could be overcome, at least for a time, by the utilization of DHAP formed during the initial phase of O₂ evolution and by breakdown of endogenous sugars in the chloroplast. When all sugar phosphates are oxidized, however, the chloroplasts will be incapable of reacting with CO₂ even though sufficient bicarbonate may still be available.

It is obviously complicated to use net O₂ evolution as a measure of substrate reduction, since depending on the nature of the products CO₂ reduction will be underestimated by the extent of O₂ uptake. At saturating CO₂ concentrations, which are more easily achieved at lower pH, oxygenase activity is repressed and O₂ evolution is a good measure of substrate reduction. But at higher pH under nonsaturating CO₂-concentrations the apparent percentage of substrate reduction would be underestimated.

The present results clearly demonstrate an O₂ effect on photosynthesis by isolated chloroplasts. To clarify the mechanisms proposed further studies are needed, particularly time-course measurements of some carbon compounds such as PGA, DHAP, and P-glycolate.

It should be emphasized that our data are not in conflict with the observations that photosynthesis is in part activated by light by alkalization of the chloroplast stroma (14, 15). The decrease in CO₂ concentration with increase in pH which we believe is responsible for our CO₂-reduction data does not apply for leaves, whose obvious adaptations enabling efficient exchange with atmospheric CO₂ ensure that the concentration of dissolved CO₂ remains constant regardless of pH fluctuations.

LITERATURE CITED