Effect of Oxygen on the Light-enhanced Dark Carbon Dioxide Fixation in *Chlorella* Cells

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ABSTRACT

With *Chlorella ellipsoidea* cells, the effect of oxygen was investigated on the products of enhanced dark \(^{14}\)CO\(_2\) fixation immediately following preillumination in the absence of CO\(_2\). When the reaction mixture was made aerobic by bubbling air (CO\(_2\)-free) throughout preillumination and the following dark \(^{14}\)CO\(_2\) fixation periods, the initial fixation product was mainly 3-phosphoglyceric acid. When nitrogen gas had been used instead of air, only about one-half of the total radioactivity in the initial fixation products was in 3-phosphoglyceric acid and the rest in aspartic, phosphoenolpyruvic, and malic acids. The percentage distribution of radioactivity incorporated in these initial products was rapidly decreased during the rest of the dark period. Concurrent with the decrease in the initial \(^{14}\)CO\(_2\) fixation products, some increase was observed in the radioactivities of the sugar phosphates. The maximal radioactivity incorporated in sugar mono- and diphosphates accounted for only 10% of total \(^{14}\)C, under either the aerobic or anaerobic conditions. Under anaerobic conditions most of the \(^{14}\)C incorporated was eventually transferred to alanine, whereas the main end products under aerobic conditions were aspartate and glutamate. The pattern of \(^{14}\)CO\(_2\) fixation products was unaffected by the atmospheric condition during the period of preillumination. The preferential flow of the fixed carbon atom to alanine or aspartate depended on the presence or absence of oxygen during the period of dark \(^{14}\)CO\(_2\) fixation.

Benson and Calvin (3, 4) found that preillumination of green algae in the absence of CO\(_2\) greatly enhanced the ability to fix CO\(_2\) in a dark period immediately following. From an analysis of the products of light-enhanced dark \(^{14}\)CO\(_2\) fixation, which included labeled sugars, they concluded that "reducing power" is formed during preillumination, enabling CO\(_2\) to be reduced to the carbohydrate level in the subsequent dark period. However, Gaffron and his associates (6) reported that 95% of \(^{14}\)C fixed in the dark subsequent to the preillumination was located in PGA. They, therefore, concluded that the photogenic agent responsible for the observed enhancement of dark fixation is a CO\(_2\) acceptor.

Recently, Bassham and Kirk (1) reported a rapid incorporation of \(^{14}\)C into PGA and sugar monophosphates during the enhanced dark \(^{14}\)CO\(_2\) fixation following steady state photosynthesis. The results were interpreted to be a result of formation of RuDP and "reducing power" during the period of illumination and conversion of \(^{14}\)CO\(_2\) into sugar phosphates in the following darkness through the reactions of photosynthetic carbon reduction cycle. We have previously studied the effects of various oxidants and other inhibitors on the light-enhanced dark \(^{14}\)CO\(_2\) fixation, and we have discovered inhibitory effects of oxygen and other Hill oxidants (8, 9). These findings led us to the inference that the agent responsible for the light-enhanced dark CO\(_2\) fixation must be a reducing substance(s) \((R)\) formed during preillumination. The possibility of \(R\) acting as the mediator of reduction of PGA to triose phosphate, however, appeared unlikely because the changes in levels of NADPH and NADH in *Chlorella* cells observed during preillumination and the subsequent dark period of CO\(_2\) fixation did not parallel the changes in \(R\) level measured simultaneously (10).

Detailed analysis of the labeled products of the light-enhanced dark \(^{14}\)CO\(_2\) fixation in green algae was carried out by Togasaki and Gibbs (14). They found in an experiment in helium that the total amounts of \(^{14}\)C in PGA, PEP, alanine, and aspartate accounted for the bulk of the \(^{14}\)C fixed in the dark after preillumination. Since the amounts of \(^{14}\)C incorporated in sugar phosphates during the light-enhanced dark CO\(_2\) fixation were very small, they concluded that the photosynthetic carbon reduction cycle had a limited role in the dark fixation subsequent to preillumination.

Thus, different conclusions had been proposed by different investigators concerning the nature of the light-enhanced dark CO\(_2\) fixation in preilluminated algae. In the present study, the time courses of \(^{14}\)C incorporation into various products during the light-enhanced dark \(^{14}\)CO\(_2\) fixation were investigated under various atmospheric conditions. It was found that the pattern of the labeled products is dependent on the presence or absence of O\(_2\) during the dark fixation period. It was also confirmed that the radioactivity incorporated in sugar phosphates during the enhanced dark \(^{14}\)CO\(_2\) fixation in preilluminated algae amounted to about 10% of the total \(^{14}\)C fixed.

MATERIALS AND METHODS

Algal Cells. *Chlorella ellipsoidea* was grown in an oblong flat vessel (12) containing 500 ml of inorganic medium with a light-dark rhythm of 16 to 8 hr. The culture medium contained 5 g of KN\(_2\)O\(_4\), 1.2 g of NaH\(_2\)PO\(_4\), 1.0 g of Na\(_2\)HPO\(_4\), 1.2 g of MgSO\(_4\)-7H\(_2\)O, 7.5 mg of CaCl\(_2\)-2H\(_2\)O, 2.8 mg of FeSO\(_4\)-7H\(_2\)O, and 1 ml of Arnon's "A5" solution per liter. During the light period, the vessel was illuminated with 10,000 lux of incandescent light. Air enriched with CO\(_2\) was constantly bubbled through the algal suspension, which was kept at 23°. The cells were harvested 2 hr after the start of a light period, washed twice with 2 × 10\(^{-4}\) M K\(_2\)SO\(_4\).
and suspended in 3.3 × 10^{-4} \text{ M} \text{ phosphate buffer (pH 7.0). The final cell density was 10 ml PCV per liter.}

**Experimental Procedures.** The cell suspension was placed in a "lollipop" and bubbled with CO_{2}-free air or nitrogen gas. The light intensity for preillumination was 23,000 lux; the temperature was 23 to 26°C. Immediately after the light was turned off, NaH_{14}CO_{3} solution (final concentration, 0.7 mM) was injected into the lollipop and, at intervals, portions of algal suspension were quickly transferred to vessels containing acetic acid (final concentration, 10%). The acidified suspension was extracted successively with 80% (v/v) ethanol and 20% (v/v) ethanol. Each extraction lasted for 20 min in a water bath kept at 60°C. A part of the combined extracts was put on a planchet and dried under an infra red lamp to determine the total \(^{14}C\) fixed, while the rest was subjected to chromatographic analysis of \(^{14}C\) products according to the following methods. Residues after the ethanol treatments were discarded, since it was found in a preliminary experiment that at least 98% of the incorporated \(^{14}C\) was in the extracts obtained by the above mentioned treatments.

The volume of the ethanol extracts was reduced in vacuo and chromatographed two-dimensionally on Whatman No. 1 filter paper, first with phenol–water (100:40, w/w) and then with n-butanol–propionic acid–water (142:71:100, v/v/v). After radioautograms were prepared, the radioactivity of each radioactive spot was determined. The individual compounds were identified on paper by cochromatography and coelectrophoresis with the authentic compounds.

**Determination of Radioactivities in C-4 of Aspartate and in C-1 of Glutamate.** The radioactivities in C-4 of aspartate and in C-1 of glutamate were determined with aspartic decarboxylase and glutamic decarboxylase, respectively (11).

**RESULTS**

**Time Courses of Dark \(^{14}C\) Fixation by Preilluminated and Non-preilluminated Cells and in the Presence or Absence of Oxygen.** The \(^{14}C\) incorporation in the non-preilluminated cells was quite slow and proceeded almost linearly with time of contact of the cells with \(^{14}CO_{2}\) (Fig. 1). The rate of \(^{14}C\) incorporation in the air was 5 to 10 times as high as that in nitrogen. In the preilluminated cells the incorporation of \(^{14}C\) proceeded rapidly at first, then slowed down gradually to attain finally a steady rate, which was almost the same as that found in the non-preilluminated cells. As was reported in our previous paper (9), the magnitude of light-enhanced dark \(^{14}CO_{2}\) fixation was greater in N_{2}-atmosphere than in air. The initial rapid increase in the nitrogen-atmosphere was complete within 2 min; afterwards, there was a rapid decrease to a steady rate. On the other hand, the initial rapid \(^{14}CO_{2}\) fixation was followed by a less rapid \(^{14}CO_{2}\) fixation before it eventually slowed down to a steady rate in air.

**Effect of Oxygen on the Products of Dark \(^{14}CO_{2}\) Fixation without Preillumination.** Table I shows that O_{2} markedly increased the dark incorporation of \(^{14}C\) into aspartate and glutamate in the non-preilluminated Chlorella cells.

**Time Courses of \(^{14}C\) Incorporation in the Products Formed during Light-enhanced Dark \(^{14}CO_{2}\) Fixation under the Nitrogen Atmosphere.** Figure 2 shows that most of \(^{14}C\) fixed during the first 10 sec of the light-enhanced dark fixation resided in PGA, aspartate, malate, and PEP. The radioactivities in these compounds did not continue to increase for longer than 30 sec, and after attaining maxima, there followed a rapid decrease in levels of radioactivity in these compounds. In contrast, radioactivity in alanine increased steadily to attain a stationary level after 5 min of incubation with \(^{14}CO_{2}\) in the dark. In the experiment shown in Figure 2, the tracer uptake was calculated with the aid of the radioactivity in alanine and PGA as shown in the following equation:

\[
\text{Tracer uptake} = (\text{Radioactivity in alanine} + \text{Radioactivity in PGA}) - \text{Radioactivity in PGA at the beginning of the experiment}
\]

**Table I. Effects of Oxygen on Dark Incorporation of \(^{14}C\) into Various Compounds in Non-preilluminated Cells of Chlorella ellipsoidea**

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>Radioactivity in Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insolubles</td>
</tr>
<tr>
<td>Air</td>
<td>10</td>
</tr>
<tr>
<td>N_{2}</td>
<td>0</td>
</tr>
</tbody>
</table>

**FIG. 1. Time courses of dark \(^{14}CO_{2}\) fixation in the presence or absence of oxygen. Solid lines: Enhanced dark \(^{14}CO_{2}\) fixation after preillumination; dotted lines: dark \(^{14}CO_{2}\) fixation without preillumination; closed circles: \(^{14}CO_{2}\) fixation under nitrogen gas; open circles: \(^{14}CO_{2}\) fixation under CO_{2}-free air. NaH_{14}CO_{3} solution was added in the dark immediately after turning off the light or after 25 min of continuous darkness.**

**FIG. 2. Time courses of \(^{14}C\) incorporation into products during light-enhanced dark \(^{14}CO_{2}\) fixation under an atmosphere of nitrogen. Ala: alanine; Asp: aspartate; Di-P: sugar diphosphates; Glu: glutamate; Mal: malate.**
content in malate showed a gradual small increase after the initial decrease. In some experiments which are not reproduced here, the increase was not observed, but there was instead a continuous decrease of the radioactivity. Since the total radioactivity incorporated was practically constant after 2 min of the light-enhanced dark $^{14}CO_2$ fixation (Fig. 1), the steady increase of $^{14}C$ in alanine must be due to the transfer of $^{14}C$ from other intermediate compounds such as PGA. No radioactivity was detected in sugar mono- and diphosphates during the initial 10 sec of the enhanced $^{14}CO_2$ fixation. Radioactivity then appeared in both fractions, increased to reach each maximum level in 2 min, and then gradually decreased to the stationary level.

When the percentage incorporation of $^{14}C$ into individual compounds was plotted against time of $^{14}CO_2$ fixation, the curves for PGA, aspartate, and PEP each showed a steady rapid dip, whereas the curve for alanine showed a steady rapid rise. Also the maximum level of the radioactivity incorporated in sugar mono- and diphosphates accounted for only 10% of total $^{14}C$ incorporated. These sugar phosphates are probably formed through reduction of the initial $^{14}CO_2$ fixation product, PGA. The limited amounts of radioactivity incorporated in sugar phosphates during the course of the light-enhanced dark $^{14}CO_2$ fixation suggest that most of the $^{14}C$-PGA may be converted to alanine without being reduced to sugar phosphates.

Table II. Distribution of Radioactivity in $C\_4$ of Aspartate and in $C\_1$ of Glutamate Formed During Light-enhanced $^{14}CO_2$ Fixation Under Aerobic Conditions

<table>
<thead>
<tr>
<th>Radioactivity after Dark Fixation for:</th>
<th>10 sec</th>
<th>20 sec</th>
<th>60 sec</th>
<th>160 sec</th>
<th>300 sec</th>
<th>600 sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-4 of aspartate</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>C-1 of glutamate</td>
<td>49.1</td>
<td>49.5</td>
<td>54.8</td>
<td>78.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Time Course of $^{14}C$ Incorporation into Products during Light-enhanced Dark $^{14}CO_2$ Fixation Under Aerobic Conditions. When NaH$^{14}CO_3$ was added to the cells which had been preilluminated under CO$_2$-free air, $^{14}C$ was almost exclusively incorporated in PGA during the initial 10 sec of dark $^{14}CO_2$ fixation. The radioactivity in PGA attained the maximum level after 1 min, followed by a continuous decrease during the rest of the experiment (Fig. 3). The maximum level in $^{14}C$-PGA was about half as high as that attained under the atmosphere of nitrogen (cf. Fig. 2). The increase of radioactivity in alanine during the dark $^{14}CO_2$ fixation was not so great as under anaerobic conditions. On the other hand, $^{14}C$ in aspartate increased steeply during 5 min of dark fixation. The secondary increase of total $^{14}C$ fixed subsequent to the initial rapid $^{14}CO_2$ fixation (Fig. 1) was roughly accounted for by the increase of $^{14}C$-aspartate observed in the present experiment, thus indicating the occurrence of C$_3$-C$_4$ carboxylation reaction lasting about 5 min after the addition of $^{14}CO_2$ in the dark. Also the radioactivity in glutamate showed a steady increase during the experimental period. The time courses of distribution of $^{14}C$ into sugar mono- and diphosphates under aerobic conditions were similar to those observed under anaerobic conditions, the maximum amount being about 10% of the total fixed $^{14}C$. Also the percentage incorporation of $^{14}C$ into PGA showed a rapid dip while those in aspartate and glutamate rapidly rose during the enhanced dark $^{14}CO_2$ fixation. The curve for aspartate attained a stationary level after 5 min, whereas that for glutamate showed a continuous rise during the experimental period.

Table II shows the distribution of radioactivity in the C-4 of aspartate and in the C-1 of glutamate formed during the enhanced $^{14}CO_2$ fixation under aerobic conditions. About 70% of the total radioactivity in aspartate molecules was in the C-4 and about one-half of the total radioactivity in glutamate molecules was in C-1.

Effects of Changing Atmospheric Conditions From N$_2$ to CO$_2$-free Air and Vice Versa on Light-enhanced Dark $^{14}CO_2$ Fixation. The following experiments were carried out in order to test whether the path of $^{14}C$ leading to alanine or aspartate is determined by the nature of the gas atmosphere during the enhanced dark fixation or during preillumination. Chlorella cells were preilluminated under continuous bubbling of N$_2$ or CO$_2$-free air. During the last 3 min of preillumination in nitrogen gas or CO$_2$-free air, the lollipop was evacuated and then filled with CO$_2$-free air or nitrogen gas to change the gas atmosphere. Immediately after the gas exchange was completed, the light was turned off and NaH$^{14}CO_3$ solution was introduced into the algal suspension.

From the comparison of the time courses of total $^{14}CO_2$ fixation shown in Figure 4 with the corresponding ones in Figure 1, it will be seen that the composition of the gas atmosphere during the preillumination period (N$_2$ or CO$_2$-free air) did not essentially alter the course of CO$_2$ fixation in the subsequent dark period. In
the present set of experiments with the gas transfers, the dark $^{14}\text{CO}_2$ fixation in $N_2$ reached a stationary state in less than 1 min, while in $CO_2$-free air the fixation continued during the whole period of experiment (5 min).

A similar finding occurs with respect to the patterns of $^{14}$C incorporation in various fixation products. A comparison of Figure 5 with Figures 2 and 3 shows that the paths of $^{14}$C incorporated in the dark in $N_2$ or $CO_2$-free air were not influenced by whether the preillumination had been carried out under the aerobic or anaerobic conditions. The features which most characterized the aerobic and anaerobic $^{14}$C fixation in the former experiments (Figs. 2 and 3), namely, the preferential formation of aspartate under aerobic conditions and of alanine under anaerobic conditions, were also confirmed in the present set of experiments. When $CO_2$-free air was replaced by nitrogen gas at the end of preillumination, most of the $^{14}$C incorporated immediately after turning off the light resided in PGA, aspartate, and PEP, and then the radioactivities in these compounds decreased in the later periods. On the other hand, the radioactivity in alanine, which was insignificant immediately after the light was turned off, continued to increase during the experimental periods. When $N_2$ was replaced by $CO_2$-free air at the end of preillumination, $^{14}$C fixed immediately after the light was turned off resided mostly in PGA. The amounts of $^{14}$C in aspartate and alanine, which were very small at the beginning, increased steadily during the subsequent periods. The amount of $^{14}$C in alanine attained a stationary level after 2 min of dark $^{14}$CO$_2$ fixation, whereas the radioactivity in aspartate continued to increase throughout the experimental periods. It was concluded from these experimental results that the pathway of carbon in the light-enhanced dark $^{14}$CO$_2$ fixation is regulated by the presence or absence of oxygen during the period of dark $^{14}$CO$_2$ fixation.

**DISCUSSION**

About 80% of $^{14}$C incorporated during 10 sec of enhanced dark $^{14}$CO$_2$ fixation following preillumination under aerobic conditions resided in PGA, whereas about 50% resided in PGA and the rest in aspartate, PEP, and malate under anaerobic conditions. On the other hand, the amount of radioactivity found in PGA under the aerobic condition was about half as much as that found under the anaerobic condition. The initial $^{14}$C incorporation into PGA will be due to carboxylation of RuDP, which accumulated in _Chlorella_ cells during the preillumination (14). Gibbs _et al._ (7) reported that the activity of phosphoribulokinase and the amounts of RuDP in spinach chloroplasts photosynthesizing in an oxygen atmosphere were both smaller than observed in $N_2$. The effect of $O_2$ on the incorporation of $^{14}$C into PGA encountered in the present experiment may be accounted for by such a decrease in amount of RuDP as reported by these investigators.

Distribution of radioactivity incorporated in the initial $^{14}$C fixation products continued to decrease rapidly during the rest of the experimental periods. Some increase in radioactivity was observed with respect to sugar phosphates. The increase, however, stopped 2 min after the light was turned off, when 10% of total incorporated radioactivity appeared in these compounds. It has been shown in our laboratory (10) that the amount of NADPH in _Chlorella_ cells increased rapidly during the initial periods of enhanced dark CO$_2$ fixation. It was also found (Hogetsu and Miyachi, unpublished data) that the addition of ATP at the end of the preillumination period did not increase the distribution of $^{14}$C in sugar phosphates, although the total amount of $^{14}$C fixed during the enhanced dark $^{14}$CO$_2$ fixation under aerobic conditions was increased significantly by this treatment. It may, therefore, be difficult to explain the limited labeling in sugar phosphates in terms of the limited supply of NADPH and ATP which are required as cofactors in the step of PGA reduction. In this connection, Bassham and Kirk (2) reported that FDP diphosphatase in _Chlorella_ cells was activated in a reversible way by light. It was also reported by Ziegler _et al._ (15) that NADP-dependent glyceraldehyde 3-phosphate dehydrogenase is activated by light. The limited accumulation of radioactivity in the sugar phosphate fraction observed in the present study might be attributable to the inactivation of FDP diphosphatase and other enzymes participating to the reductive pentose cycle in darkness.

Incorporation of $^{14}$C in aspartate observed immediately after the addition of $^{14}$CO$_2$ under anaerobic conditions most probably results from transamination of the C$_3$-C$_4$ carboxylation product, OAA. The C$_3$ compound from which OAA was formed by carboxylation might have been derived from the compound endogenous to the cells, or it might be converted from 1-C-PGA. If the former took place, the radioactivity in the 1-C-aspartate molecules will be localized exclusively in C-4. On the other hand, two-thirds of the total radioactivity will be in C-4 and the rest in C-1 if the latter occurred. From the analysis of the distribution of radioactivity in aspartate formed during light-enhanced dark $^{14}$CO$_2$ fixation under anaerobic conditions, it was found that 77.6% of total $^{14}$C in aspartate molecules labeled during 18 sec resided in C-4 and the values decreased with increasing time of $^{14}$CO$_2$ fixation (71.6 and 67.8% after 40 and 120 sec, respectively). It may, therefore, be assumed that more than half of the C$_3$ compounds which were converted to aspartate during the initial 18 sec of the enhanced $^{14}$CO$_2$ fixation had been derived from 1-C-PGA, and the rest, from other endogenous source(s). The latter sources of C$_3$ compound seem to have been exhausted rather rapidly, so that in a later period (120 sec after the addition of $^{14}$CO$_2$) practically all the C$_3$ compound was supplied from 1-C-PGA. The fairly rapid drop observed in the curve for aspartate could result from the aspartate-pyruvate transaminase reaction to form alanine and OAA. The OAA thus formed may either be converted to other compounds within the cells or decomposed during the procedures of extraction and the subsequent analysis.

During the enhanced CO$_2$ fixation under anaerobic conditions, most of $^{14}$C incorporated was transferred to alanine, whereas the main end products under aerobic conditions were aspartate and glutamate. These C$_3$ compounds are probably derived from OAA.
formed as a result of C₁-C₄ carboxylation reaction. The distribution of radioactivity in the C-4 of aspartate formed during the enhanced ¹⁴CO₂ fixation under aerobic conditions (Table II) supports the inference that the C₄ compound participating in the C₁-C₄ carboxylation reaction had been derived from ¹³C-PGA. Glutamate labeling probably resulted from transamination of α-KG which had been derived from OAA. If OAA was converted to α-KG by means of the tricarboxylic acid cycle, the radioactivity in glutamate would be contained exclusively in C-1. On the other hand, the radioactivity will be distributed in C-2, C-5, and C-1 if OAA was converted to α-KG by means of the reversed tricarboxylic acid cycle as proposed by Arnon et al. (5). The tracer content in C-4 would be 40% of the total radioactivity if OAA derived from ¹³C-PGA was converted to α-KG by the reversal of the tricarboxylic acid cycle. The results shown in Table II, therefore, may be taken as an indication that during enhanced ¹⁴CO₂ fixation under aerobic conditions, most of the glutamate is formed by means of the reductive dicarboxylic acid pathway. The simplest explanation for the incorporation of ¹⁴C into alanine would be that PEP derived from PGA is converted to pyruvate, which is then transformed to alanine by the reaction of transaminase. One may also assume that alanine is formed from aspartate as a result of decarboxylation by aspartic decarboxylase, which might be activated under anaerobic conditions. It was, however, not possible to detect the activity of aspartic decarboxylase in intact Chlorella cells or extracts of Chlorella under either the aerobic or anaerobic condition. We would assume, therefore, that aerobic conditions favor the production of OAA from PEP, whereas anaerobic conditions favor the conversion of PEP to pyruvate. Since pyruvate kinase isolated from rat tissues is inhibited by ATP (13), it is tempting to infer that ATP is playing a key role in the control of carbon metabolism by O₂. Under anaerobic conditions, PEP is converted to alanine by a series of reactions mentioned above. Under aerobic conditions, ATP produced by the tricarboxylic acid cycle may inhibit pyruvate kinase, and then the resulting accumulation of PEP leads to an enhanced production of OAA. The inference, however, was not supported by our experiment mentioned above, which showed that the addition of ATP at the end of preillumination did not alter the pattern of distribution of radioactivity in various compounds incorporated in the subsequent enhanced dark ¹⁴CO₂ fixation.

With the purpose of elucidating the regulatory mechanism of carbon metabolism induced by O₂, studies on the effects of adenine nucleotides on the activities of enzymes pertaining to the above mentioned reactions, and on changes in the intracellular levels of adenine nucleotides during preillumination and the subsequent dark CO₂ fixation, are currently in progress in this laboratory.

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LITERATURE CITED


