Effect of Triacontanol on *Chlamydomonas*1

II. SPECIFIC ACTIVITY OF RIBULOSE-BISPHOSPHATE CARBOXYLASE/OXYGENASE, RIBULOSE-BISPHOSPHATE CONCENTRATION, AND CHARACTERISTICS OF PHOTORESPiration

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ABSTRACT

Increased photosynthetic CO₂ assimilation by *Chlamydomonas reinhardii* cells treated with triacontanol (TRIA) was not due to changes in glycolate excretion, CO₂ compensation point, or the sensitivity of photosynthetic CO₂ assimilation to O₂. Kinetic analysis of TRIA-treated cells showed that the increase in photosynthetic CO₂ assimilation was a result of an increase in the apparent *Vₐ₅₉₅* for intact cells. The total activity of ribulose-P₂ carboxylase/oxygenase was higher in cell lysates from TRIA-treated cells. However quantification of this enzyme concentration by binding of [¹⁴C]glycolate to [¹⁴C]glycolate-carboxylase/oxygenase was not shown in TRIA-treated cells. Thus, there was an increase in the specific activity of ribulose-P₂ carboxylase/oxygenase extracted from *Chlamydomonas* cells treated with TRIA. TRIA alone yielded no effect on the activity of the enzyme in cell lysates from *Chlamydomonas* or purified from spinach (*Spinacia oleracea* L.) leaves.

The ribulose-P₂ pool was 50 to 60% higher in cells grown with TRIA that were assayed for photosynthetic CO₂ assimilation at high- and low- CO₂. TRIA also increased ribulose-P₂ levels in the absence of CO₂ in the light with atmospheres of N₂ or N₂ with 21% O₂.

CO₂ (5%). Some of the changes in photosynthetic CO₂ assimilation by *Chlamydomonas* cells that possess this C₅ pump, with respect to cells that do not, are: (a) the rate of photosynthetic CO₂ assimilation is insensitive to O₂; (b) the apparent *Kₐ* (CO₂) for photosynthesis is substantially decreased; and (c) glycolate excretion during photosynthesis at low-CO₂ does not occur (1, 3, 14, 22). These changes contribute to an increase in photosynthetic CO₂ efficiency at low levels of external CO₂ by algae cells cultured at low-CO₂ compared to cells cultured at high CO₂. We find that TRIA-treated *Chlamydomonas* cells grown at high-CO₂ exhibit some properties that could be interpreted as effecting the C₅ accumulation mechanism (10). Other researchers have also reported that TRIA treatment reduced the O₂ inhibition of photosynthetic CO₂ assimilation in *Chlamydomonas* cells (7). The objective of this research was to investigate the TRIA stimulation of CO₂ fixation by *Chlamydomonas* grown at high-CO₂ and the possibility that the C₅ accumulation system was affected.

MATERIALS AND METHODS

Algae Culture and Treatment. *Chlamydomonas reinhardii* Dangeard (− strain (N. 90) were cultured, treated with TRIA, and photosynthetic CO₂ assimilation assayed as described in the accompanying paper. Chl was determined spectrophotometrically and glycolate excretion by the Calkins assay as described in (23). *Chlamydomonas* cells were harvested by centrifugation and washed twice with cold (4°C) 50 mM Bicine-KOH (pH 8.2) or 50 mM HEPES-KOH (pH 7.5) buffer, and resuspended in the same buffer at a final Chl concentration of 50 to 100 μg/ml.

Ribulose-P₂ Carboxylase Extraction and Assay. Harvested cells, suspended in Bicine-KOH buffer, were passed twice through a chilled Yeda press (Linca Science Instruments, Tel-Aviv, Israel) with compressed N₂ (10,400 kPa). The cell extract or the supernatant obtained after centrifugation for 2 min at about 2000g was used as the source for ribulose-P₂ carboxylase.

The extract was assayed with and without further activation with 1 or 10 mM KHCO₃ and 20 mM MgCl₂ as described previously (16). Activation time at 30°C was for 30 min and assay time was 15 s.

The level of ribulose-P₂ carboxylase in lysates of *Chlamydomonas* cells was also quantified by measuring the binding of [¹⁴C]CABP (16). An aliquot (1 to 3 ml) of the extract from *Chlamydomonas* cells after activation was incubated with 5 μM [¹⁴C]CABP. After incubation at 30°C for 45 min, a large excess of [¹⁴C]CABP was added and any exchange with [¹⁴C]CABP allowed to proceed for 1 h. The [¹⁴C]CABP-ribulose-P₂-carboxylase complex was precipitated with 20% PEG 4000, 20 mM MgCl₂, and the precipitate collected by centrifugation at 30,000g for 20 min. The pellet was washed twice with 20% PEG 4000.

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3 Abbreviations: TRIA, triacontanol (CH₃(C₇H₁₅CH₂OH); C₅, inorganic carbon (HCO₃⁻ and CO₂); CABP, carboxyarabinitol bisphosphate; PEP, phosphoenol pyruvate.

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that contained 20 mM MgCl₂, resuspended in 0.5 ml H₂O, and mixed with 4.5 ml of scintillation cocktail for ¹⁴C determination by liquid scintillation counting. The specific activity of ribulose-P₂ carboxylase was calculated from the bound [¹⁴C]CABP on the basis of a mol wt of 550,000 D with eight catalytic sites, each of which bound one molecule of CABP.

Ribulose-P₂ Determinations. Ribulose-P₂ was determined by modification of the procedure described previously (11). Aliquots (430 µl) of illuminated *Chlamydomonas* cells suspended in 50 mM Hepes-KOH (pH 7.5) buffer were quickly removed and added (5 s) to 70 µl of iced-cold 70% HClO₄ in 1.5-ml plastic microfuge tubes on ice. After 20 min, the samples were centrifuged for 1 min with an Eppendorf centrifuge and 450 µl of the supernatant removed. The supernatant samples were placed in 1.5-ml microfuge tubes on ice and 50 µl of 1 M Bicine-KOH (pH 8.2) and 85 µl of 10 N KOH added to each tube. Following incubation for 5 min with occasional mixing, the tubes were centrifuged to remove the insoluble KClO₄ and samples of the supernatants (250 µl, pH 8.0-8.2) removed for determination of ribulose-P₂. These samples were incubated with activated ribulose-P₂ carboxylase purified from spinach (*Spinacia oleracea L.*), leaves and the incorporation of H¹⁴CO₃⁻ into acid-stable H¹⁴C measured. The assay media contained 100 mM Bicine-KOH buffer (pH 8.0-8.2), 0.2 mM Na₂EDTA, 0.5 M DTT, 20 mM MgCl₂, 10 mM KH₂PO₄ (1.0 µCi/µmol), and 50 µg of activated ribulose-P₂ carboxylase in a final volume of 0.5 ml in serum-stoppered 8-ml glass scintillation vials. After incubation at 30°C for 1 h the assay was terminated with 200 µl of 2 N HCl and the samples dried at 80°C. After cooling, 0.5 ml of H₂O and 4.5 ml of scintillation cocktail were added and radioactivity determined. This method gave reliable recoveries (95%) of ribulose-P₂ added to *Chlamydomonas* cell extracts.

PEP Carboxylase Activity. The *Chlamydomonas* cell extracts that were used for ribulose-P₂ carboxylase determinations were also used to assay PEP-carboxylase activity by modification of a published procedure (4). Aliquots of the cell extract (20-50 µl) were added to assay media in serum-stoppered 8-ml glass scintillation vials held in a water bath at 30°C. The assay medium contained 5 mM PEP, 10 mM KH₂PO₄ (1.0 µCi/µmol), 1 mM NADH, 2000 units of malic dehydrogenase, 100 mM Bicine-KOH (pH 8.0) buffer, and enzyme in a final volume of 0.5 ml. The reaction was initiated by the addition of cell extracts and terminated after 30 s with 200 µl of 2 N HCl for blanks, PEP was omitted. The samples were left at 25°C for 12 h for removal of excess H¹⁴CO₂. After adjusting the volume to 0.5 ml with H₂O, 4.5 ml of scintillation cocktail were added and fixed H¹⁴C determined.

CO₂ Compensation Point. *Chlamydomonas* cells suspended in 50 mM Hepes-KOH (pH 7.5) buffer (50 ml, 20-40 µg Chl/ml), were placed in a glass chamber (150 ml) fitted with inlet and outlet ports. The inlet port extended to the bottom of the chamber so that gas entering the chamber bubbled through the cell suspension. The chamber was held in a water bath at 21°C and PAR (600 µmol/s/m²) provided by a metal halide lamp. Air that contained CO₂ (51 µl/L) was circulated with a piston pump (200 ml/min) through the chamber until the level of CO₂ in the gas exiting the chamber was stable at 51 µl/L (approximately 10 min). The system was closed and the internal atmosphere circulated (200 ml/min) through a Beckman model 865 IR gas analyzer. The IR gas analyzer was calibrated with air containing known levels of CO₂ such that the output voltage was linear in response to CO₂ levels from 0 to 100 µl/L. After closing the system, the CO₂ compensation point of the cell suspension was reached in the next 20 to 30 min.

Effect of O₂ on Photosynthetic CO₂ Assimilation. Photosynthetic CO₂ assimilation by *Chlamydomonas* cells was measured in an atmosphere of air (21% O₂) and an atmosphere of air diluted 1:9 v/v with N₂ (approximately 2% O₂). The CO₂ in the air was removed with a column (2 x 18 cm) of Ascarite (Arthur H. Thomas Co.). *Chlamydomonas* cells suspended in 20 ml of 50 mM Hepes-KOH (pH 7.5) buffer were placed in a water-jacketed (25°C) photosynthesis lollipop tube (75 ml) and the tube sealed with a rubber serum stopper fitted with two Teflon tubes. The inlet tube extended to the bottom of the lollipop tube so that inlet gas bubbled through the *Chlamydomonas* cell suspension. After sealing the tube, the cell suspension was flushed with the 2 or 21% O₂ atmosphere (100 ml/min) for 10 min in the light (1000 µmol/s/m²) prior to the initiation of photosynthetic CO₂ assimilation with 1 mM KH₂CO₃ (1.0 µCi/µmol). After addition of KH₂CO₃, the inlet and outlet tubes were pinched closed and samples removed (100 µl) by opening the inlet tube and withdrawing a sample with a 250-µl Hamilton syringe. Acid stable H¹⁴C was determined as described previously. Prior to sampling, 100 µl of the same gas that had been used to flush the cell suspension, were injected into the lollipop tube to maintain constant pressure.

Chemicals. Ribulose-P₂ (2-¹⁴C)CABP (1.0 µCi/µmol), and ribulose-P₂ carboxylase were prepared by Dr. R. Michael Mulligan as described in references (9, 13, 15), respectively. NaH¹⁴CO₃ (40-60 µCi/µmol) was from New England Nuclear. TRIA and sodium tallow alkyl sulfate were from the same source as described previously (10). PEP (tri-mono-cyclohexyl-ammonium salt), NADH (disodium salt, grade III), malic dehydrogenase (porcine, mitochondrial), and PEG 4000 were obtained from Sigma Chemical Co.

Statistical Procedures. All experiments were replicated at least once. Variation between replicates was removed in the analysis of variance as blocks. The null hypothesis, that the treatment variance was equal to the error variance, was tested in each investigation with an F-ratio. When appropriate, an F-ratio was also determined for treatment variance with trend analysis of nonorthogonal comparisons. Treatment means were also compared with an LSD value in some tests.

RESULTS

Characteristics of the Oxidative Photosynthetic Carbon Cycle. The rate of photosynthetic CO₂ assimilation by control and TRIA-treated *Chlamydomonas* cells grown on high-CO₂ under atmospheres containing 2 and 21% O₂ was measured (Table I). Low (1 mm) KHCO₃ was used so that the CO₂ concentration would be limiting. When the partial pressure of O₂ was reduced, photosynthetic CO₂ assimilation increased approximately 20% in both control and TRIA-treated cells. Regardless of the O₂ concentration, TRIA-treated cells had higher rates of photosynthetic CO₂ assimilation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Photosynthetic CO₂ Assimilation</th>
<th>CO₂ Compensation Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2% O₂  21% O₂</td>
<td>Inhibition by 21% O₂</td>
</tr>
<tr>
<td>Control</td>
<td>54.5  45.5</td>
<td>17</td>
</tr>
<tr>
<td>TRIA</td>
<td>76.6*  65.4*</td>
<td>15</td>
</tr>
</tbody>
</table>

* F-ratio for difference between treatments is significant at the 1% level.
The CO₂ compensation point represents the balance between the reductive photosynthetic carbon cycle and the oxidative photosynthetic carbon cycle. Changes in the internal steady-state level of CO₂ in *Chlamydomonas* cells should result in change in the CO₂ compensation point. In *Chlamydomonas* cells grown on air, the presence of the Ci accumulation system results in low CO₂ compensation points (<5 μL/L), since the internal CO₂ level is high and the inhibition of ribulose-P₂ carboxylase by O₂ is reduced (14). There was no effect of TRIA on the compensation point of *Chlamydomonas* cells grown on high-CO₂. From these results, it appears that TRIA does not affect the sensitivity of photosynthetic CO₂ assimilation to inhibition by O₂ in *Chlamydomonas* cells cultured at high-CO₂. Thus, the TRIA stimulation of photosynthetic CO₂ assimilation would not appear to be associated with stimulation of the Ci pump.

**Photosynthetic CO₂ Assimilation Kinetics.** *Chlamydomonas* cells adapted to low-CO₂ have a whole-cell apparent *Kₘ* (CO₂) for photosynthetic CO₂ assimilation of 3 μM (14) which is below the *Kₘ* (CO₂) of 46 μM for the isolated ribulose-P₂ carboxylase enzyme. Cells cultured with high-CO₂ have an apparent photosynthetic *Kₘ* (CO₂) that approximates that for the isolated carboxylase (3). Kinetic analysis of control and TRIA-treated high-CO₂ grown *Chlamydomonas* cells showed that the increase in photosynthetic CO₂ assimilation by TRIA-treated cells was due to an increase in the whole cell *Vₘₐₓ* (Fig. 1). There was no change in the apparent *Kₘ* (CO₂) with TRIA treatment. This result is also consistent with the hypothesis that TRIA treatment does not affect the internal level of Ci in *Chlamydomonas*, i.e. the Ci pump.

**In Vivo and in Vitro Ribulose-P₂ Carboxylase Activity.** Three-d-old control and TRIA-treated *Chlamydomonas* cells were ruptured and ribulose-P₂ carboxylase assayed before and after activation with CO₂ and MgCl₂. The ribulose-P₂ carboxylase activity from TRIA-treated cells before activation was approximately twice that from the control cells (Table II). The increase in carboxylase activity in the lysates after incubation with excess KHCO₃ and MgCl₂ was about 10-fold, and there was a further increase (20%) in the activity with TRIA treatment. The large difference in carboxylase activities between activating and non-activating conditions is probably the result of deactivation during lysis of the cells. With a N₂ pressure of 10,400 kPa, the cells would be under nearly anaerobic conditions and this would facilitate the loss of activator CO₂ from the carboxylase enzyme. However, the amount of deactivation should be the same for control and TRIA-treated cells since both lysates were prepared within 20 min. There was no difference in PEP-carboxylase activity in the lysates from control and TRIA-treated *Chlamydomonas* cells.

The possibility that the extract from TRIA-treated *Chlamydomonas* cells contained a promoter of ribulose-P₂ carboxylase activity, possibly synthesized in response to TRIA treatment, was excluded by assaying combinations of the extracts from

Table II. Effect of TRIA on Photosynthetic CO₂ Fixation by Intact Cells and the Activity of Ribulose-P₂ Carboxylase and Phosphoenolpyruvate Carboxylase in Lysates from *Chlamydomonas* Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Whole Cells</th>
<th>Ribulose-P₂ Carboxylase</th>
<th>PEP-Carboxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.3</td>
<td>19.5</td>
<td>275.1</td>
</tr>
<tr>
<td>TRIA</td>
<td>62.3**</td>
<td>29.6**</td>
<td>329.2*</td>
</tr>
</tbody>
</table>

* F-ratio for difference between treatments is significant at the 5% level. ** F-ratio for difference between treatments is significant at the 1% level.

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**Fig. 2.** Ribulose-P₂ carboxylase activity in extracts from *Chlamydomonas* cells. The lysate from 3-d-old control (sodium tallow alkyl sulfate, 1 μg/L) and TRIA (100 μg/L)-treated cells cultured at high-CO₂ were mixed at varying proportions and ribulose-P₂ carboxylase activity assayed without activation. The r value from linear regression analysis is significant at the 1% level. The rate of photosynthetic CO₂ assimilation by intact TRIA-treated cells was significantly higher (1% level) than that for control cells (data not shown). Each observation is the mean of three experiments with triplicate determinations.
Table III. Absence of a TRIA Effect on Ribulose-P2 Carboxylase from Cell Lysates of Chlamydomonas or Purified from Spinach

The enzyme source, 400 µl cell extract or 50 µg spinach carboxylase, was treated with TRIA or sodium tallow alkyl sulfate during activation. Activation was with 1 or 10 mM KH4CO3 and 20 mM Mg2+ at 30°C for 30 min. Activity was measured with 10 mM KH4CO3. Each observation is the mean of three experiments with triplicate determinations. There were no significant differences among treatments.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Carboxylase from Spinach</th>
<th>Carboxylase from Chlamydomonas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activation level of H4CO3-</td>
<td></td>
</tr>
<tr>
<td>µg/L</td>
<td>µmol CO2/1·mg Chl</td>
<td>µmol CO2/min·mg Chl</td>
</tr>
<tr>
<td>None</td>
<td>100.8</td>
<td>195.6</td>
</tr>
<tr>
<td>TASP</td>
<td>98.7</td>
<td>201.3</td>
</tr>
<tr>
<td>TRIA</td>
<td>102.5</td>
<td>199.6</td>
</tr>
<tr>
<td>TRIA</td>
<td>100.7</td>
<td>189.2</td>
</tr>
<tr>
<td>TRIA</td>
<td>100</td>
<td>199.0</td>
</tr>
<tr>
<td>TRIA</td>
<td>1000</td>
<td>201.8</td>
</tr>
</tbody>
</table>

* TAS, sodium tallow alkyl sulfate.

Table IV. Specific Activity and Level of Ribulose-P2 Carboxylase in Lysates from Chlamydomonas Cells

Lysates from Chlamydomonas cells cultured for 2 d with high-CO2 and 100 µg/L TRIA or 1 µg/L sodium tallow alkyl sulfate as control were assayed for ribulose-P2 carboxylase activity, and for total enzyme levels as determined by 14CJCABP binding. Each observation is the mean of three experiments with triplicate determinations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Active Site Concentration</th>
<th>Activity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/ mg Chl</td>
<td>µmol CO2/h·mg Chl</td>
<td>µmol CO2/min·mg protein</td>
</tr>
<tr>
<td>Control</td>
<td>13.4</td>
<td>261.3</td>
<td>4.73</td>
</tr>
<tr>
<td>TRIA</td>
<td>12.9</td>
<td>351.8*</td>
<td>6.61*</td>
</tr>
</tbody>
</table>

* F-ratio for difference between treatments is significant at the 1% level.

Table V. Concentration of Ribulose-P2 in Control and TRIA-Treated Chlamydomonas Cells

Chlamydomonas were grown with high-CO2 for 2 or 3 d with 100 µg/L TRIA or 1 µg/L sodium tallow alkyl sulfate as control, and harvested cells assayed for ribulose-P2 during photosynthetic CO2 assimilation with 10 mM KHCO3. Each mean is the average of two experiments with duplicate determinations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Test 1</th>
<th>Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIA</td>
<td>11.7</td>
<td>10.3</td>
</tr>
<tr>
<td>Control</td>
<td>18.2*</td>
<td>15.8*</td>
</tr>
</tbody>
</table>

* F-ratio for difference between treatments is significant at the 1% level.
EFFECT OF TRIACONTANOL ON CHLAMYDOMONAS

Fig. 3. Photosynthetic CO₂ assimilation and the levels of ribulose-P₃ in TRIA-treated Chlamydomonas cells. Chlamydomonas cells cultured at high-CO₂ were treated with 1 mg/L TRIA or 10 μg/L sodium tallow alkyl sulfate as control. After 3 d of growth, harvested cells were suspended in 50 mM Hepes-KOH (pH 7.5) buffer and aerated for 15 min with N₂ (50 ml/min) in the light (1000 μmol·m⁻²·s⁻¹) prior to the initiation of the experiment. At zero time and at 1- or 2-min intervals, samples were removed for determination of ribulose-P₂ (*). Levels remained between 2 min after zero time, 1 ml KHCO₃ was added and the N₂ gassing stopped. Photosynthetic CO₂ assimilation (C) by another portion was determined with 1 ml KHCO₃ under identical conditions beginning with the addition of KH₁⁴CO₃. The F-ratio for the effect of TRIA on RuBP levels is significant at the 1% level. The F-ratio for the interaction of TRIA with linear time for ¹⁴CO₂ fixation is significant at the 1% level. Each observation is the mean of two experiments with duplicate determinations.

oxygenase (21-23). This probably reflects an inability to metabolize glycerol fast enough, because of inadequate glycolate dehydrogenase activity (20, 21). Thus, glycolate formation and excretion reflects a loss of photosynthetic carbon metabolites from Chlamydomonas chloroplasts. After ribulose-P₂ accumulation in a N₂ atmosphere for 15 min, the addition of 20% O₂ as CO₂ free air (Fig. 4) resulted in ribulose-P₂ utilization and excretion of glycolate. The levels of excreted glycolate by Chlamydomonas were not statistically different. However, both the initial and final rates of glycolate synthesis after addition of O₂ appear to be different for control and TRIA-treated cells. The control cells showed an initial increase in glycolate excretion for 13 min while excretion by the TRIA-treated cells did not increase until after 13 min in O₂. A higher rate of glycolate excretion with TRIA treatment would be consistent with an increase in the rate of photosynthetic CO₂ assimilation by TRIA-treated cells due to a higher ribulose-P₂ carboxylase/oxygenase activity. For the experiments in Figure 4 with the same cells, in duplicate tests with 10 mM KHCO₃, average CO₂ fixation was increased by TRIA to 96.1 from 60.5 μmol CO₂/mg Chl·h in the control.

DISCUSSION

In the preceding paper (10), evidence was presented that the addition of a small amount (10-1000 μg/L) of TRIA to the culture media promotes a long-lasting stimulation of photosynthetic CO₂ fixation and growth of Chlamydomonas cells cultured with 5% CO₂. This stimulation persisted through many cell divisions. The purpose of this investigation has been to evaluate how TRIA could promote algal growth, and a mechanism for increasing photosynthetic CO₂ fixation, even with limiting CO₂.

Stimulation by trace levels of TRIA suggest that it might target a specific enzyme, and the hydrophobicity of TRIA indicates that the target might be in a membrane or the hydrophobic region of an enzyme.

For several reasons, the effect of TRIA on glycolate biosynthesis and metabolism by Chlamydomonas has been examined. A previous report indicated that TRIA treatment of Chlamydomonas lowered the O₂ inhibition of photosynthesis (7), but we could not confirm this. In fact TRIA did not alter the CO₂ compensation point, the amount of glycolate excretion, or O₂ inhibition of photosynthesis. TRIA also did not alter the distribution of ¹⁴CO₂ among the soluble products of ¹⁴CO₂ photosynthetic fixation, as examined by paper chromatography. The fact that TRIA stimulated CO₂ fixation even at high CO₂, when there should be reduced glycolate synthesis, is consistent with the conclusion that TRIA stimulation is not associated with the oxidative photosynthetic carbon cycle and glycolate excretion.

The TRIA stimulation of photosynthesis occurred only with high-CO₂ grown cells and was lost when the cells were switched to low-CO₂ (air) in an adaptation time similar to the development of carbonic anhydrase and the C₄ pump (10). These facts suggest that TRIA stimulation might be related or similar to the unknown mechanisms for the CO₂ accumulation in Chlamydomonas. The nature of this association is vague, because the loss of TRIA stimulation of CO₂ fixation with air-grown Chlamydomonas was also associated with reduction (over 50%) of TRIA binding to the cells. TRIA altered Vₘₐₓ for CO₂ fixation by cells grown on high-CO₂, but it did not alter the Kₘ (CO₂), which would have occurred if the C₄ pump and carbonic anhydrase had increased in cells grown with TRIA.

The activity of ribulose-P₂ carboxylase was significantly higher (40%) in extracts from TRIA-treated high-CO₂-grown Chlamydomonas cells. This increase was not a result of an increase in the level of ribulose-P₂ carboxylase, as measured by [¹⁴C]CABP binding sites, but was due to an increase in the specific activity.
of the enzyme as present in the cell lysate. Changes in the levels of chloroplast metabolites, some of which can affect ribulose-2-P carboxylase activity (12), may influence the specific activity of the enzyme by maintaining the enzyme in the active ternary complex. Since ribulose-2-P and 6-P-gluconate increase the amount of ribulose-2-P carboxylase in the active state under conditions of low CO2 and the level of ribulose-2-P in TRIA-treated \textit{Chlamydomonas} cells was increased about 40 to 60%, perhaps this increase resulted in a higher specific activity of the carboxylase from these cells. However, the extract from TRIA-treated \textit{Chlamydomonas} cells did not influence the ribulose-2-P carboxylase activity in extracts from control cells in an anomalous manner. Thus, there did not appear to be an excess of positive effectors of ribulose-2-P carboxylase activity in the extract from TRIA-treated \textit{Chlamydomonas} cells.

For \textit{Chlamydomonas} cells cultured at 5% CO2, the in situ rate of photosynthetic CO2 assimilation should be limited by the rate of ribulose-2-P regeneration (5). TRIA-treated \textit{Chlamydomonas} cells, when assayed for photosynthetic CO2 assimilation with saturating levels of CO2, had 40 to 60% higher levels of ribulose-2-P. Therefore, the increased growth of \textit{Chlamydomonas} cells cultured at 5% CO2 (10) may be associated with increased levels of ribulose-2-P. At less saturating levels of CO2, photosynthetic CO2 assimilation may be limited by the specific and total activity of ribulose-2-P carboxylase rather than by the ribulose-2-P concentration (5). Under these conditions, TRIA-treated \textit{Chlamydomonas} cells grown on high-CO2 still had increased photosynthetic CO2 assimilation rates perhaps because the specific activity of carboxylase was higher than that in control cells. However, ribulose-2-P levels were also significantly increased in TRIA-treated \textit{Chlamydomonas} cells at less saturating levels of CO2 where the level of ribulose-2-P was in considerable excess of the active site concentration of ribulose-2-P-carboxylase (Table IV, Figs. 3 and 4). This increase in ribulose-2-P concentration was equal or similar to the per cent increase in photosynthetic CO2 assimilation with TRIA treatment. A search for similar observations by other researchers indicated that ribulose-2-P levels may influence the rate of photosynthetic CO2 assimilation even when in excess of the ribulose-2-P-carboxylase active site concentration. Observations on the levels of ribulose-2-P and photosynthetic CO2 assimilation in bean (\textit{Phaseolus vulgaris} L.) leaves, suggested that ribulose-2-P limitation of photosynthetic CO2 assimilation can occur at ribulose-2-P levels that are in considerable excess of ribulose-2-P carboxylase active site concentrations (2).

An increase in ribulose-2-P levels in TRIA-treated, high-CO2-cultured \textit{Chlamydomonas} cells, in the absence of CO2 (Figs. 3 and 4) suggests that the level of chloroplastic metabolites available for ribulose-2-P synthesis increased over the control cells. TRIA-treated \textit{Chlamydomonas} cells may have an increased ability to mobilize carbon reserves within the chloroplast for synthesis of ribulose-2-P. In rice (\textit{Oryza sativa} L.) and soybean (\textit{Glycine max} L.) leaves, TRIA increased the activity of starch phosphorylase and decreased levels of starch (R. L. Houtz, S. K. Ries 1982 Effect of triacontanol on starch phosphorylase and PEP carboxylase activities. 21st International Horticultural Congress, Vol II, No. 2087). A similar effect in \textit{Chlamydomonas} might account for increased pool sizes of sugar-phosphate esters, thus increasing ribulose-2-P. This could occur in \textit{Chlamydomonas} cells grown with high-CO2 because of excess photosynthese as carbohydrates, but not in air-grown cells without significant storage reserves.

If TRIA causes changes in higher plants similar to those described here for \textit{Chlamydomonas}, these observations may explain the increase in dry weight associated with TRIA treatment. However, photosynthetic CO2 assimilation by higher plants is controlled by other mechanisms not present in algae, such as stomatal conductance. Therefore, changes in ribulose-2-P levels and specific activity of ribulose-2-P-carboxylase similar to those observed in \textit{Chlamydomonas} cells, may not lead to as large an increase in photosynthetic CO2 assimilation in higher plants treated with TRIA. Furthermore, the dry weight of higher plants treated with TRIA increases in the dark (18).

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