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STIMULATION OF GROWTH AND PHOTOSYNTHETIC CARBON METABOLISM IN CHLAMYDOMONAS REINHARDTII WITH TRIACONTANOL

presented by

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Major professor

Stanley K. Ries

Date 2/7/85

STIMULATION OF GROWTH AND PHOTOSYNTHETIC CARBON METABOLISM IN *CHLAMYDOMONAS REINHARDTII* WITH TRIACONTANOL

By

Robert L. Houtz

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DEDICATION

This dissertation is dedicated in memory of Alice W. Price and Harry James Houtz.

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ABSTRACT

STIMULATION OF GROWTH AND PHOTOSYNTHETIC CARBON METABOLISM IN *CHLAMYDOMONAS REINHARDTII* WITH TRIACONTANOL

By

Robert L. Houtz

Treatment of *Chlamydomonas reinhardtii* Dangeard cells (-, strain N. 90), cultured at 5% CO_2, with 1 to 1000 ug/L triacontanol (TRIA) resulted in a 21% to 35% increase in cell density, 7% to 31% increase in total chlorophyll, and 20% to 100% increase in photosynthetic CO_2 assimilation. The increase in photosynthetic CO_2 assimilation with TRIA treatment occurred before, and was independent of the increase in total chlorophyll or cell number. *Chlamydomonas* cells responded to a broad range of TRIA concentrations that were at least 10-fold above the optimum concentration for higher plants. The necessity for higher concentrations of TRIA may be due to destabilizing effects of Ca^{++} and K^{+} present in the *Chlamydomonas* growth medium on the TRIA formulation. TRIA particles were bound to *Chlamydomonas* cells treated with colloidally dispersed [^{14}C]TRIA. Octacosanol inhibited the effect of TRIA on photosynthetic CO_2 assimilation. TRIA treatment did not alter the distribution of ^{14}C-label among photosynthetic products. The effect of TRIA on photosynthetic CO_2 assimilation increased with time up to 3 days after treatment. *Chlamydomonas* cells cultured in low CO_2 (air) did not respond to TRIA. Transfer of high CO_2 (5%) cultured cells that had responded to TRIA to a low CO_2 atmosphere resulted in a

loss of the effect of TRIA. The effect of pH on photosynthetic CO_2 assimilation indicated that the maximum increase by TRIA treated Chlamydomonas cells was between pH 5.0 and 7.0.

TRIA did not alter glycolate excretion, the CO_2 compensation point or sensitivity of photosynthetic CO_2 assimilation to O_2 in Chlamydomonas. Kinetic analysis of TRIA-treated cells showed that the increase in photosynthetic CO_2 assimilation was a result of an increase in the whole-cell apparent Vmax. The activity of RuBP carboxylase/oxygenase was significantly higher in cell lysates from TRIA-treated cells than those from control cells. However, quantification of RuBP carboxylase/oxygenase levels by ^{14}CABP binding did not show increased enzyme levels in TRIA-treated cells. Therefore, there was an increase in the specific activity of RuBP carboxylase/oxygenase extracted from Chlamydomonas cells treated with TRIA. TRIA alone had no effect in vitro on the activity of RuBP carboxylase/oxygenase purified from spinach (Spinacia oleracea) leaves or from cell lysates of Chlamydomonas.

RuBP levels were significantly higher in TRIA-treated cells at high and low CO_2. Increased RuBP levels in TRIA-treated Chlamydomonas cells were also observed in the absence of CO_2 with atmospheres of N_2 and 21% O_2.

The increase in photosynthetic CO_2 assimilation by TRIA-treated Chlamydomonas cells was associated with an increase in the specific activity of RuBP carboxylase/oxygenase and RuBP levels.

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Bicine, N,N'-bis(2-hydroxyethyl(glycine)

CABP, 2-carboxyarabinitol 1,5-bisphosphate

CHES, 2-(N-cyclohexylamino)ethanesulfonic acid

C_i, inorganic carbon (HCO_3^- + CO_2)

CV, coefficient of variation

DTT, dithiothreitol

EDTA, ethylene diaminetetraacetate

HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

IRGA, infrared gas analyzer

LSD, least significant difference

MES, 2-(N-morpholino)ethanesulfonic acid

NADH, nicotinamide adenine dinucleotide

PEP, phosphoenol pyruvate

PGA, 3-phosphoglycerate

P_i, inorganic orthophosphate

POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene

PPO, 2,5-diphenyloxazole

RuBP, ribulose 1,5-bisphosphate

RuBP carboxylase, ribulose 1,5-bisphosphate carboxylase/oxygenase

TAS, sodium tallow alkyl sulfate

TRIA, triacontanol [$CH_3(CH)_{28}CH_2OH$]

INTRODUCTION

During this century there have been considerable increases in crop productivity in the United States. This has not been due to increases in tillable land, but increases in cropping efficiency per hectare (5, 11, 12). The introduction of new cultivars and mechanization have contributed greatly to these increases. However, during the past few decades increases in crop yields per hectare have leveled off (28). This may reflect the limitations of the techniques, and knowledge of plant biology that facilitated those increases. Substantial increases in crop productivity are presently envisioned by many plant scientists because of advances in molecular biology.

Although the use of plant growth regulators and hormones for increasing crop yields has not met with much success in the past, there is still interest in discovering and developing yield-enhancing chemicals. Triacontanol (TRIA), a compound with plant growth regulator properties, has potential for increasing the biomass (total dry weight) of plants (25). Although increases in plant biomass do not necessarily translate into increases in harvested yield (10, 11), there have been significant increases in the yield of several crop species when treated with TRIA (23). However, inconsistencies under field conditions limit the recommendation of TRIA for use in commercial agriculture.

Laboratory results with TRIA are more encouraging; corn (Zea mays L.) and rice (Oryza sativa L.) plants respond well to TRIA under controlled environmental conditions, with up to 15 to 20 percent increases in dry weight evident 24 h after application (22, 23). Although rice plants respond to TRIA in the absence of light, the percent increase in dry weight is considerably less than that in the presence of light (1, 24). Therefore, even though TRIA affects some processes in plants that are independent of light, there is also a substantial interaction with light dependent processes. TRIA has been shown to affect the growth of many plant species from basidiomycetes and blue-green algae to higher plants. A review of the effects of TRIA on plants was recently published (23).

TRIA stimulation of dry matter accumulation in plants has lead several researchers to investigate the effects of TRIA on photosynthesis and photoassimilate partitioning (4, 7, 14). The results suggest that TRIA may affect photoassimilate partioning and the photosynthetic carbon oxidation cycle in plants. These two processes have been thoroughly investigated in plants with the goal of increasing crop productivity. Understanding the potential of TRIA for increasing crop productivity requires a critical evaluation of the effects of TRIA on plant processes fundamental to crop yield. One of these processes is photosynthetic CO_2 assimilation, initiated by the enzyme RuBP carboxylase/oxygenase. This enzyme is responsible for the annual input of approximately 5.0×10^{16} g of atmospheric CO_2 into the global biomass (29), and is probably the most abundant protein in the world (6). The oxygenase reaction catalyzed by RuBP carboxylase is regarded by many plant scientists as the most important metabolic constraint on

plant productivity (10, 13). The objective of these studies was to investigate the effects of TRIA on photosynthetic CO_2 assimilation, photorespiration, and factors affecting these processes. Chlamydomonas reinhardtii, a unicellular green algal species, was chosen as the test organism, since it has been shown to respond to TRIA, and possesses a biochemical pathway of photosynthetic CO_2 assimilation similar to that in higher plants.

CHAPTER 1

GROWTH AND PHOTOSYNTHETIC CO_2 ASSIMILATION IN *CHLAMYDOMONAS REINHARDTII* AS AFFECTED BY TRIACONTANOL

ABSTRACT

Treatment of *Chlamydomonas reinhardtii* cells, cultured at 5% CO_2, with 1 to 1000 ug/L triacontanol (TRIA) resulted in a 21% to 35% increase in cell density, 7% to 31% increase in total chlorophyll, and 20% to 100% increase in photosynthetic CO_2 assimilation. The increase in photosynthetic CO_2 assimilation with TRIA treatment occurred before, and was independent of, increases in total chlorophyll or cell number. *Chlamydomonas* cells responded to a broad range of TRIA concentrations that were at least 10-fold above the optimum concentration established for higher plants. The necessity for larger concentrations of TRIA may be due to destabilizing effects of Ca^{++} and K^{+} present in the *Chlamydomonas* growth medium on the TRIA formulation. TRIA particles were bound to *Chlamydomonas* cells treated with colloidally dispersed [^{14}C]TRIA. Octacosanol inhibited the effect of TRIA on photosynthetic CO_2 assimilation. TRIA treatment did not alter the distribution of ^{14}C-label among photosynthetic products. The effect of TRIA on photosynthetic CO_2 assimilation increased with time up to 3 days after treatment. *Chlamydomonas* cells cultured in low levels of CO_2 (air) did not respond to TRIA. Transfer of high CO_2 (5%) cultured

cells that had responded to TRIA to a low CO_2 atmosphere resulted in a loss of the effect of TRIA. The effect of pH on photosynthetic CO_2 assimilation indicated that the maximum increase by TRIA treated Chlamydomonas cells occurred between pH 5.0 and 7.0.

TRIA is a 30-carbon, straight-chain primary alcohol, possessing plant growth regulator properties discovered in 1977 (19). The effects of TRIA on plant growth, development, and metabolism have been summarized recently (17). Some of the inconsistencies in reproducing the effects of TRIA have been attributed to inadequate formulation and/or inhibition by traces of aliphatic hydrocarbons and phthalate-esters (13, 14, 17, 20). Acceptable procedures for formulation and application of TRIA are now available (14, 20).

The most profound effect of TRIA on plants is an increase in dry weight (10, 13, 14, 18). Therefore, it follows that photosynthetic CO_2 assimilation may be a factor involved in the response of plants to TRIA. With tomato (Lycopersicon esculentum), and a unicellular green alga (Chlamydomonas reinhardtii), treatment with TRIA resulted in a decrease in the O_2 inhibition of photosynthetic CO_2 assimilation (7, 11). At atmospheric levels of CO_2 and O_2 the carboxylase reaction catalyzed by RuBP carboxylase in C-3 plants and Chlamydomonas cells cultured at high-CO_2, is inhibited by about 15 to 30% by O_2 (4, 8, 9), which is similar to the maximum percent increase in dry weight induced by TRIA (17). Therefore, it was postulated that an alleviation of the O_2 inhibition of photosynthetic CO_2 assimilation could be a mechanism of action of TRIA. However, an increase in photosynthetic CO_2 assimilation in tomato or Chlamydomonas in response to TRIA treatment was not observed (7, 11). The following investigations on the effects of TRIA on Chlamydomonas showed a stimulation of photosynthetic CO_2 assimilation, and confirmed an increase in cell number, which has been observed with Chlamydomonas, Anacystis nidulans, and Scenedesmus acutus (6, 11) as well as with tissue cultures of several higher plant species (10).

MATERIALS AND METHODS

Algae Culture and Treatment. Axenic cultures of Chlamydomonas reinhardtii Dangeard, (-) strain (N. 90) from the algal collection at the University of Texas (R.C. Starr), were cultured in 3-L Fernbach flasks containing 1.0 L of growth media (21) or in 0.25-L Erlenmeyer flasks with 0.1 L of growth medium. The cultures were continuously mixed with a reciprocating shaker and aerated with 50 to 100 ml/min of CO_2-enriched air (5% CO_2) or air alone. PAR from fluorescent lamps was 100 umol/s·m^2, and the temperature was maintained between 21 and 23°C with fans to move room air over the flasks. Cell densities were determined with a hemacytometer after suspending the cells in 5% glycerol. Starting cell density after inoculation was typically 100 to 500 cells/ul. The density reached approximately $1x10^4$ cells/ul after 3 d growth. Chlorophyll was determined by the method of Arnon (1). TRIA was applied as a sterile aqueous colloidal dispersion (800 to 1000 ug/ml stock concentration) containing sodium tallow alkyl sulfate (TAS) as the dispersive agent, present at 1% of the level of TRIA (14). TAS alone, or distilled water were used as controls. As reported previously for higher plants (14), TAS lacked biological activity at the concentrations used on Chlamydomonas.

Photosynthetic CO_2 Assimilation Assays. Cells were harvested by centrifugation at 1000g, washed once with an equal volume of 50mM Hepes-KOH buffer (pH 7.5), suspended in the same buffer to give a

chlorophyll concentration of 10 to 50 ug/ml, and placed on ice. In experiments where the pH was varied the cells were resuspended in 3 mM Hepes-KOH (pH 7.5) and later added to a buffer consisting of 20 mM Hepes, 20 mM Ches and 20 mM Mes adjusted to the desired pH with HCl or KOH. Aliquots of resuspended cells were placed in 1.5 cm x 5 cm flat bottom glass vials which were held in a circulating water bath at 25°C. The cell suspension was stirred with a small magnetic stirring bar. Illumination from a light projector was filtered through 6.0 cm of a 0.1% solution of $CuSO_4$ to remove heat and provided 1200 umol/s·m^2 of PAR at the top of the cell suspension.

After addition of 1 to 10 mM $KH^{14}CO_3$ (0.14 to 0.54 uCi/umol) to the cell suspensions, photosynthetic CO_2 assimilation was initiated with light. Aliquots (0.1 to 0.5 ml) of the cell suspensions were removed at various intervals and mixed with an equal volume of 2N HCl. These samples were evaporated to dryness at 85°C. After cooling, 0.5 ml H_2O and 4.5 ml of scintillation cocktail (2 L toluene, 1 L Triton X-100, 12 g PPO, 0.15 g POPOP) were added for measurement of acid-stable ^{14}C. When photosynthetic CO_2 assimilation was measured at different pH values, the vials with the cell suspensions were capped with rubber serum stoppers to prevent loss of $^{14}CO_2$. Under these conditions the cell suspensions were illuminated from the side and a water bath was not used. Instead, aliquots of the cell suspensions on ice were removed and acclimated in a water bath to room temperature (23°C) for 5 min prior to use.

[^{14}C]TRIA Binding Measurements. Centrifugal silicone oil filtration (2) was used to measure binding of [^{14}C]TRIA to Chlamydomonas cells. The incubations were carried out in 400 ul plastic microfuge tubes in

the light (100 umol/s·m^2) at 25°C. The tubes contained from bottom to top 20 ul of 1 M glycine, 65 ul silicone oil (1:1,v/v, Wacker AR 20 and AR 200), and 250 ul cell suspension.

Incubations were initiated by the addition of colloidally dispersed [^{14}C]TRIA and terminated by centrifugation for 1 min with an Eppendorf model 5414 centrifuge. After centrifugation the cell pellet was removed by cutting the centrifuge tube with a razor blade. The pellet was placed into a scintillation vial containing 0.5 ml of distilled H_2O, and resuspended with 4.5 ml of scintillation cocktail.

Flocculation of Colloidally Dispersed TRIA. Flocculation of the TRIA formulation was measured by determining the amount of colloidally dispersed [^{14}C]TRIA that would pass through a Milipore AP 25 filter with an 8 um pore size. Aliquots of $CaCl_2$ or KCl solutions (0.1 to 1.0 M) were added to colloidally dispersed [^{14}C]TRIA (450 ul), and after incubation at 25°C for 4 min, the dispersion was filtered and samples (150 ul) were removed from the filtrate for determination of [^{14}C]TRIA. The stability of the TRIA formulation in the presence of the Chlamydomonas growth medium was determined in a similar manner. Colloidally dispersed [^{14}C]TRIA was added to growth media (20 ml) and samples (1.2 ml) were removed for filtration and determination of [^{14}C]TRIA in the filtrate.

^{14}C-label Distribution. The distribution of ^{14}C-label incorporated during photosynthetic CO_2 assimilation was measured by adding aliquots (0.5 ml) of cells which were actively assimilating $^{14}CO_2$, to an equal volume of methanol. After centrifugation the supernatant was removed

(soluble fraction) and the pellet (insoluble fraction) was resuspended in 200 ul of distilled H_2O. Both fractions were counted for acid-stable ^{14}C. Measurement of excreted ^{14}C and analysis of ^{14}C-labeled products by 2-dimensional paper chromatography was conducted as previously described (21).

Chemicals. Octacosanol, TRIA, TAS, and [15,16-^{14}C]TRIA (23.2 uCi/umol) were provided by the Proctor and Gamble Company (Miami Valley Laboratories, Cincinnati, Ohio 45247). The octacosanol and TRIA were provided as aqueous colloidal dispersions (800 to 1000 ug/ml) with approximately $3x10^{11}$ particles per ml (0.1-0.6 um diameter). $NaH^{14}CO_3$(40 to 60 uCi/umol) was obtained from New England Nuclear and Wacker AR 20 and AR 200 silicone oils from Wacker Chemie GmBH, Munich, West Germany.

Statistical Procedures. All experiments were replicated at least once. Variation among 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 or non-orthogonal comparisons. In some tests treatment means were also separated with an LSD value.

RESULTS

TRIA Stimulation. TRIA treatment resulted in significant increases in cell density, total chlorophyll, and photosynthetic CO_2 assimilation of Chlamydomonas cultures (Tables I and II). The cell density was not

Table I. The Effect of TRIA (100 ug/L) on Cell Density, Chlorophyll and Photosynthetic CO_2 Assimilation by High CO_2 Grown Chlamydomonas Cells.

The total growth medium for control and TRIA-treated cultures was inoculated with Chlamydomonas cells at approximately 575 cells/ul and 0.1 to 1.0 L aliquots of inoculated media were added to 3-L Fernbach or 0.25-L Erlenmeyer flasks respectively. The cells in one flask were treated with TRIA and the cells in the control flask with TAS (1 ug/L) or distilled H_2O. Cultures were aerated for 3 d with an atmosphere of air supplemented with 5% CO_2. Photosynthetic CO_2 assimilation was determined with 10 mM $KH^{14}CO_3$. Each observation is the mean of 7 experiments with duplicate determinations.

Treatment	Cell Density	Chlorophyll		Photosynthetic CO_2 Assimilation
	cells/ul x 10^4	ug/ml	pg/cell	umol/hmg chlorophyll
Control	1.13	10.0	0.88	51.5
TRIA	1.37	10.7*	0.78	72.8**

*,**F ratio for comparison of treatments was significant at 5% and 1% level respectively.

Table II. Chlorophyll, Cell Density, and Photosynthetic CO_2 Assimilation in Chlamydomonas Cells 3 Days After Transfer of an Aliquot of TRIA-Treated (100 ug/L) and Control (TAS, 1 ug/L) Cells to Fresh Growth Medium.

Aliquots of 3-d-old control (500 ul) and TRIA-treated (400 ul) cultures, that were equivalent in total chlorophyll content, were transferred to fresh growth medium (100 ml) without TRIA and incubated with an atmosphere supplemented with 5% CO_2. After 3 d growth, cell density, chlorophyll and photosynthetic CO_2 assimilation (10 mM $KH^{14}CO_3$) were determined as described in Materials and Methods. Each observation is the mean of 3 experiments with duplicate determinations.

Treatment	Cell Density	Chlorophyll		Photosynthetic CO_2 Assimilation
	Cells/ul x 10^4	ug/ml	pg/cell	umol/h·mg Chlorophyll
Control	1.20	10.5	0.88	40.5
TRIA	1.62*	13.8*	0.85	55.8*

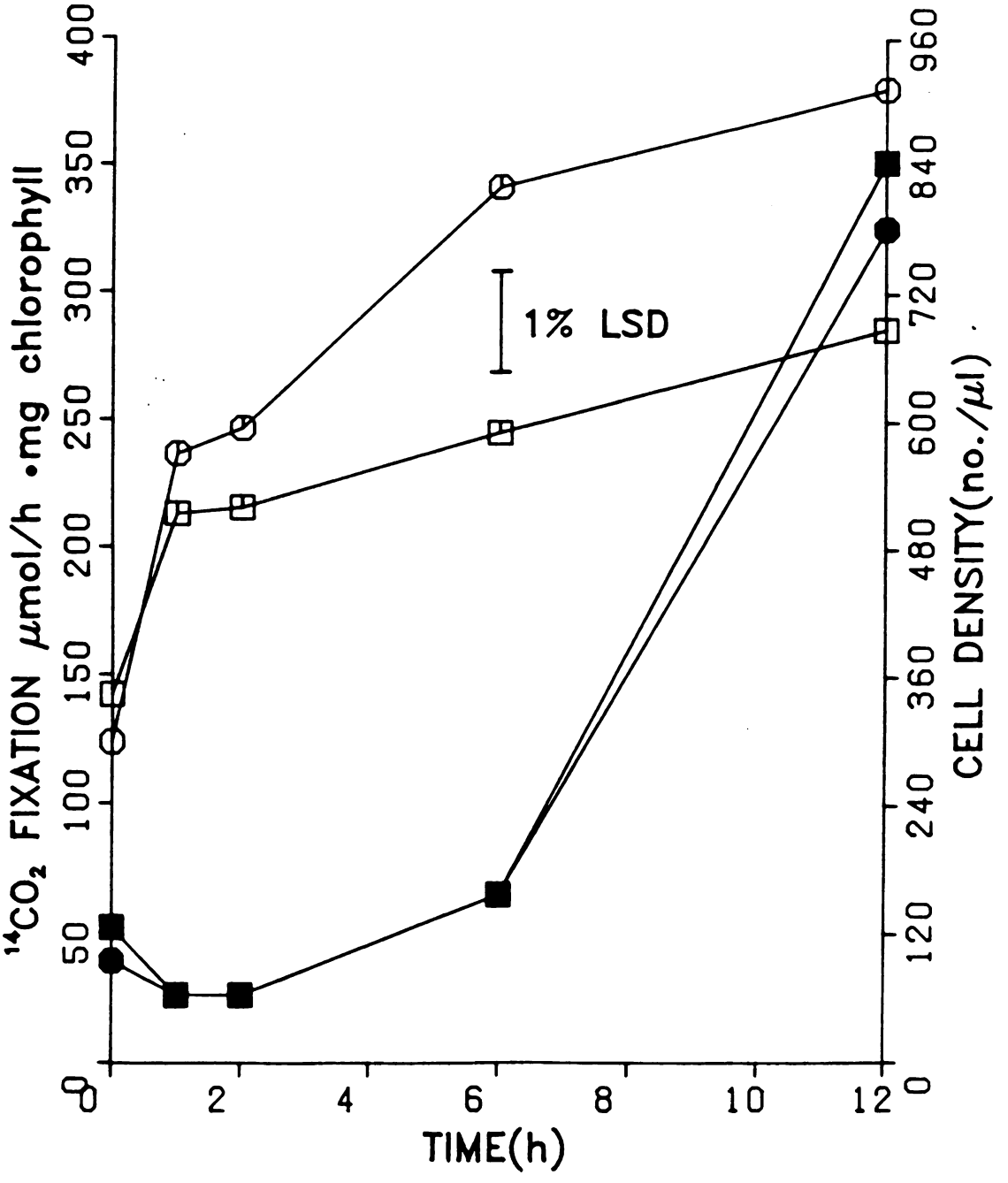
*F ratio for comparison of treatments was significant at 5% level.

higher in the TRIA-treated cultures after growth for 3 d (Table I), but the error variance in these tests was sufficiently high (CV of 37%) to eliminate any statistical significance. The hemacytometric method used to determine cell number probably contributed to this variation. Chlamydomonas cells were present in different states of division (diad, tetrad, octad) and thus the total cell number could not be determined accurately using a hemacytometer. Under these culture conditions Chlamydomonas cells began to enter the stationary phase of growth after about 48 h, when nutrients and other factors may limit the potential for further growth that would be expected with increased photosynthetic CO_2 assimilation. When aliquots of 3-d-old TRIA-treated and control cells were transferred to new media, the stimulation of cell growth with TRIA was greater (Table II).

The increase in chlorophyll was small (7%) but significant during the first culture (Table I) and, in a manner similar to cell density, became greater after a second culture of the cells (Table II). The increases in chlorophyll were a result of increased cell number since the amount of chlorophyll per cell did not change (Tables I and II).

The largest and earliest effect of TRIA was on photosynthetic CO_2 assimilation. The increase in photosynthetic CO_2 assimilation in Chlamydomonas cells treated with TRIA could be measured after 1 h of treatment, before any change in cell density was demonstrable, and was evident on a chlorophyll basis (Fig. 1). After treatment of newly inoculated cultures there was a rapid increase in the rate of photosynthetic CO_2 assimilation by cells in control and TRIA cultures. However, this increase was larger in TRIA treated cells than in the control. In the majority of the following studies this increase

Figure 1. Effect of TRIA on Cell Density and Photosynthetic CO_2 Assimilation in Chlamydomonas With Time. After treatment 100 ml samples were removed for determination of cell density (closed symbols) and photosynthetic CO_2 assimilation with 10 mM $KH^{14}CO_3$ (open symbols) with time in control (TAS, 1 ug/L) (□) and TRIA-treated (100 ug/L) (0) cultures. Each observation is the mean of two experiments with duplicate determinations. The F ratio is significant at the 1% level for the interaction of TRIA with linear time. There was no significant difference in cell number between control and TRIA-treated cultures. The LSD shown is for comparison of any two data points for photosynthetic CO_2 assimilation.


$^{14}CO_2$ FIXATION µmol/h •mg chlorophyll
CELL DENSITY(no./µl)
TIME(h)
1% LSD
0
50
100
150
200
250
300
350
400
120
240
360
480
600
720
840
960
2
4
6
8
10
12

in photosynthetic CO_2 assimilation was used as an indicator of a response to TRIA.

TRIA Formulation and Dose Response. Dose response studies showed that the response of Chlamydomonas cells to TRIA leveled off at 10 ug/L without further increases in photosynthetic CO_2 assimilation (Table III). The lowest concentration of TRIA that resulted in an increase in photosynthetic CO_2 assimilation (10 ug/L), was above the optimum dose of TRIA established for higher plants by two orders of magnitude. The necessity for this relatively large concentration of TRIA led to the investigation of the stability of the TRIA formulation in the presence of the Chlamydomonas growth medium, and the binding of TRIA to Chlamydomonas cells.

Laughlin et al. (14) reported that the optimum dose for the TRIA-elicited increase in dry weight in corn (Zea mays) plants, was two orders of magnitude lower when the particle size of the TRIA formulation was decreased. The colloidal dispersion of TRIA used in these experiments was not stable in the presence of the Chlamydomonas growth medium. When TRIA at a concentration of 1.0 mg/L or higher was added to the culture medium, the small particles (mean diameter 0.1 to 0.8 um) originally present in the colloidal dispersion aggregated to form larger particles which were visible to the unaided eye. Chlamydomonas cells did not show increased photosynthetic CO_2 assimilation when treated with flocculated TRIA (Table IV). Therefore, it was postulated that the necessity for high concentrations of TRIA, and the ineffectiveness of the concentrations of TRIA greater

Table III. Dose Response for TRIA and Photosynthetic CO_2 Assimilation by Chlamydomonas Cells.

Cultures were inoculated, then treated and after 3 d growth, 100 ml samples were removed for determination of photosynthetic CO_2 assimilation (10 mM $KH^{14}CO_3$). Each observation is the mean of five experiments with duplicate determinations.

Treatment		Photosynthetic CO_2 Assimilation
Chemical	(ug/L)	umol/h·mg Chlorophyll
TAS	10	31.9[1]
TRIA	1	46.0
TRIA	10	48.8
TRIA	100	48.5
TRIA	1000	52.4
5% LSD		14.8

[1] The F ratio was significant at the 5% level for the linear trend of increasing photosynthetic CO_2 assimilation with increasing concentration of TRIA.

Table IV. Absence of an effect of Flocculated TRIA Dispersions on Photosynthetic CO_2 Assimilation by Chlamydomonas.

TRIA dispersions were added to 100 ml of growth media in 0.1 L Erlenmeyer flasks, and after incubation for 6 h at 25°C inoculated with Chlamydomonas. After 2 d growth samples were removed for determination of photosynthetic CO_2 assimilation (10 mM $KH^{14}CO_3$). Each observation is the mean of 2 experiments with duplicate determinations. There were no significant differences in photosynthetic CO_2 assimilation as a result of treatments.

Treatment		Photosynthetic CO_2 Assimilation
Chemical	(ug/L)	umol/h·mg Chlorophyll
TAS	10	61.6
TRIA	1	59.8
TRIA	10	61.6
TRIA	100	64.3
TRIA	1000	53.0

than 10 ug/L at eliciting further significant increases in photosynthetic CO_2 assimilation, may be a result of an increase in the particle size of the TRIA dispersion upon addition to the Chlamydomonas growth medium. Experiments designed to test this hypothesis showed that the concentration of colloidally dispersed [^{14}C]TRIA capable of passing through an 8 um filter, decreased with time in a logarithmic manner after addition to the Chlamydomonas growth medium (Fig. 2). This increase in particle size was dependent on the starting concentration of TRIA. Half of the colloidally dispersed [^{14}C]TRIA in the Chlamydomonas growth medium was present as particles larger than 8 um in diameter after 165 min and after 50 min with 100 ug/L and 1000 ug/L TRIA respectively. With the lower concentration of TRIA (10 ug/L) the percentage of TRIA present as particles larger than 8 um in diameter did not fall below 50% even after 256 min of exposure to the algae growth medium. Although the TRIA dispersions were not completely flocculated by the Chlamydomonas growth media (Fig. 2) apparently the increase in particle size was sufficient to decrease the effectiveness of the formulation (Table IV). Since particle size was not quantitated in these experiments, it is possible that the TRIA remaining as particles less than 8um in diameter (Fig. 2) are still considerably larger than the initial particle size (0.1-0.6 um diameter) and are inactive. K^+ and Ca^{++} in the growth medium could be responsible for the instability of the colloidally dispersed TRIA. When colloidally dispersed [^{14}C]TRIA was exposed to K^+ or Ca^{++} there was a rapid increase in the particle size (Fig. 3). The slopes of the two regression lines showed that calcium ions were approximately 12 times

Figure 2. The Effect of Chlamydomonas Growth Medium on the Particle Size of Colloidally Dispersed [^{14}C]TRIA. [^{14}C]TRIA was added to 20 ml of Chlamydomonas growth medium at time zero, and samples (1.2 ml) removed after different times for determination of the amount of TRIA capable of passing through a filter with an 8 um pore size. Each observation is the mean ±SE of duplicate determinations.

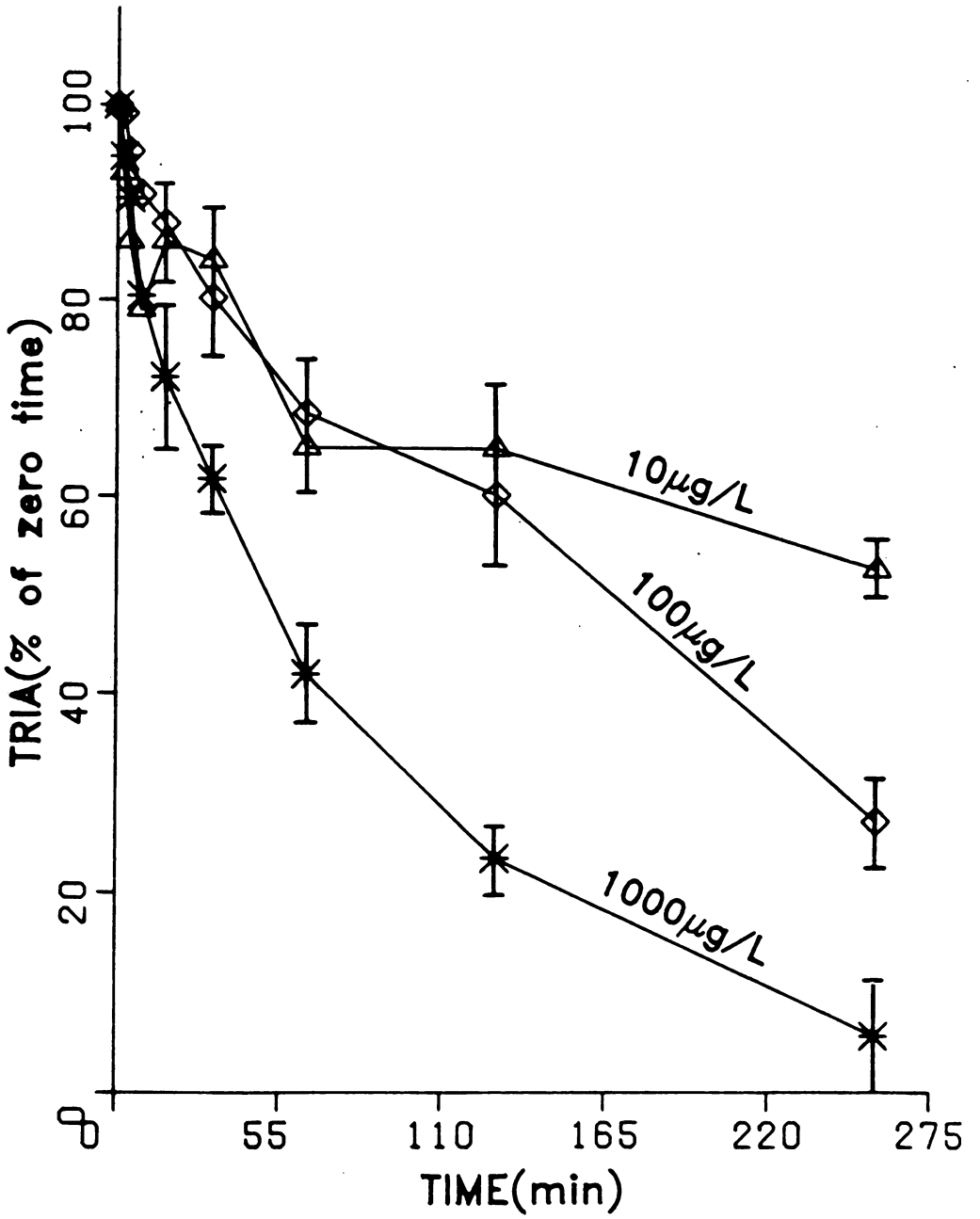
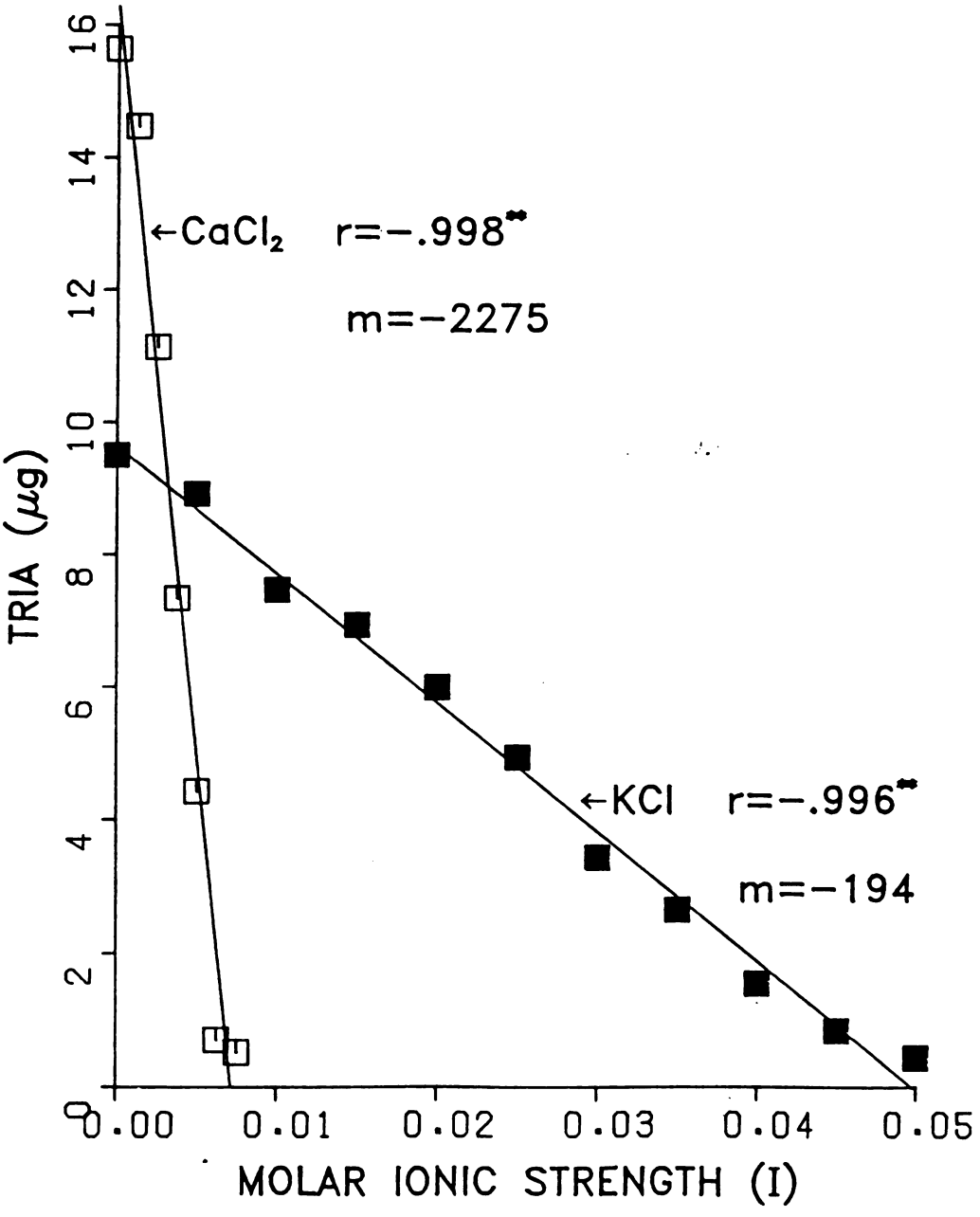

TRIA(% of zero time)
100
80
60
40
20
0
0
55
110
165
220
275
TIME(min)
10μg/L
100μg/L
1000μg/L

Figure 3. Calcium (□) and Potassium (■) Induced Flocculation of Colloidally Dispersed [^{14}C]TRIA. Aliquots (0.5ml) of the TRIA dispersion (210 ug/ml for Ca^{++} and 180 ug/ml for K^{+}) were incubated with $CaCl_2$ or KCl at 25°C for 4 min, filtered, and the [^{14}C]TRIA remaining in the filtrate determined as described in Materials and Methods. Each observation is the mean of three determinations. The range in the SE measurments was 0.001 to 0.140. The r values from linear regression analyses for the loss of TRIA in the filtrate with increasing ionic strength are significant at the 1% level. The slopes (m, ug TRIA/I) of the two lines are shown for comparison of the effectiveness of the two cations.


TRIA (μg)
0
2
4
6
8
10
12
14
16
0.00
0.01
0.02
0.03
0.04
0.05
MOLAR IONIC STRENGTH (I)
←CaCl₂ r=-.998**
m=-2275
←KCl r=-.996**
m=-194

more effective at initiating this flocculation than potassium ions. The level of these two cations in the growth medium (0.2 mM Ca^{++} and 29.4 mM K^{+}) are within the range of Ca^{++} and K^{+} levels used in these studies to initiate flocculation. This increase in particle size is probably the result of an interaction of cations with the negatively charged surface of the TRIA particles.

Since the Chlamydomonas growth medium can initiate flocculation of the colloidally dispersed TRIA, and flocculated TRIA fails to elicit an increase in photosynthetic CO_2 assimilation, it might seem that Chlamydomonas cells should not respond at all to TRIA. However, when Chlamydomonas cells are treated with [^{14}C]TRIA under conditions favorable for flocculation (i.e. cells suspended in growth medium) TRIA particles became bound to the cells (Fig. 4). The binding of TRIA particles to Chlamydomonas cells reached saturation in 20 min (Fig. 4) with approximately 62% bound in the first 10 min of incubation.

Chlamydomonas cells cultured at low CO_2 (air) did not bind as much [^{14}C]TRIA as cells cultured at high CO_2 (5%) (Fig. 4). This difference in ability to bind [^{14}C]TRIA may be related to the absence of an effect of TRIA on photosynthetic CO_2 assimilation, by Chlamydomonas cells cultured with air, as discussed later. The relationship between the number of TRIA particles bound to Chlamydomonas cells grown at high CO_2 and the concentration of TRIA is shown in Fig. 5. The binding of TRIA to the cells was linear with increasing concentrations of TRIA and did not exhibit saturation. The binding of TRIA to Chlamydomonas cells was measured using a silicone oil filtration technique (2), since flocculated TRIA did not migrate through the silicone oil layer. This technique is adapted for small volumes of cell suspensions and the

Figure 4. The Binding of [^{14}C] TRIA to Chlamydomonas Cells. [^{14}C] TRIA (66.7 ug/ml) was added to cells suspended in growth medium (2.9 x 10^4 cells/ul) and the latter incubated at 25°C for various periods of time as indicated. The incubations were terminated by centrifugation, and the bound [^{14}C] TRIA determined as described in Materials and Methods. Blanks consisted of [^{14}C] TRIA added to growth medium without cells. For zero time determinations the cells were centrifuged within 3 s after addition of [^{14}C] TRIA. Each observation is the mean of two experiments with determinations. The F ratio for the interaction of TRIA with linear time was significant at the 1% level. The LSD shown is for comparison of any two data points.

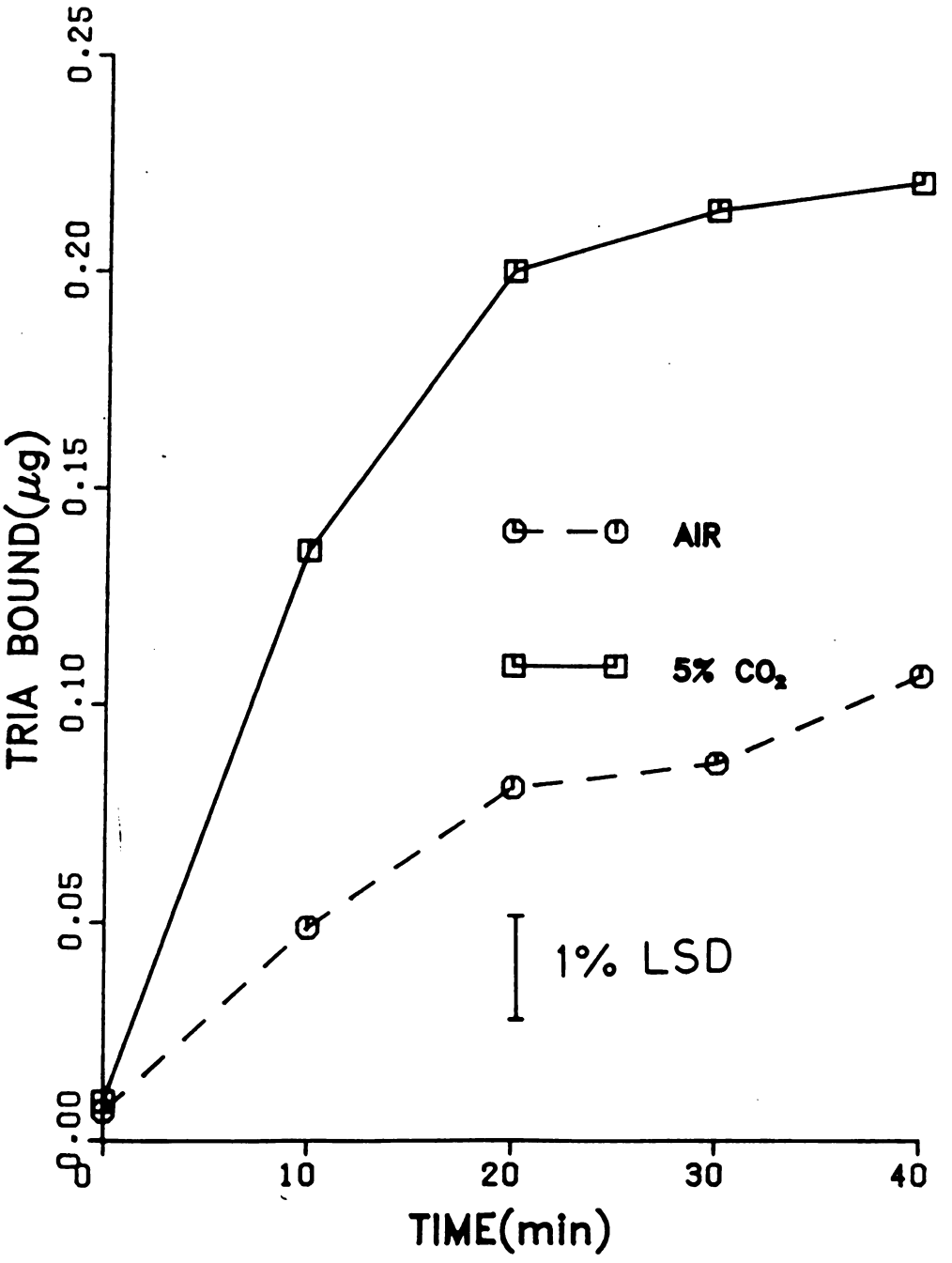
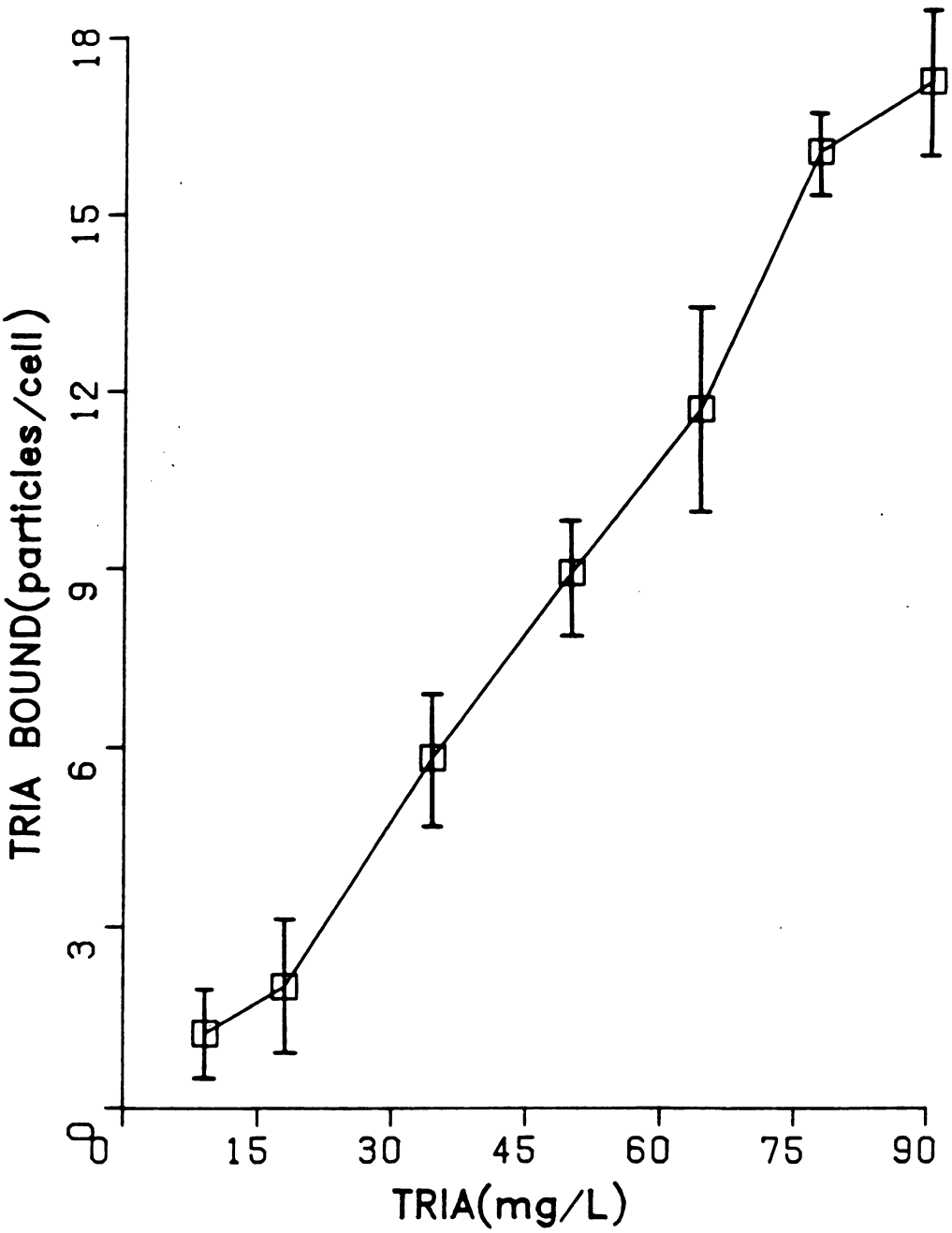

TRIA BOUND(μg)
0.00
0.05
0.10
0.15
0.20
0.25
10
20
30
40
TIME(min)
AIR
5% CO₂
1% LSD

Figure 5. Effect of TRIA Concentration on the Number of TRIA Particles Bound to Chlamydomonas Cells. Cell suspensions (3.0×10^4 cells/ul) were incubated with [^{14}C]TRIA for 30 min and then centrifuged through silicone oil. Bound [^{14}C]TRIA was determined as described in Materials and Methods. Blanks consisted of [^{14}C]TRIA added to growth medium that did not contain Chlamydomonas cells. Each observation is the mean $\pm$SE of duplicate determinations.


TRIA BOUND(particles/cell)
0
3
6
9
12
15
18
0
15
30
45
60
75
90
TRIA(mg/L)

specific activity of the [^{14}C]TRIA was low. This necessitated the use of higher concentrations of TRIA than those used in the dose-response studies. The higher concentrations of TRIA were partially compensated for by maintaining the ratio of the number of particles of TRIA to the number of cells, at levels similar or equal to those in the dose-response studies. The range of ratios for the dose-response experiments was 0.6 to 3000 particles of TRIA per cell and in the binding experiment was 150 to 1800 particles of TRIA per cell. Kinetic analysis of this binding was precluded because the TRIA was not in solution.

Effect of Culture Age on Photosynthetic CO_2 Assimilation in Control and TRIA Treated Cells. The rate of photosynthetic CO_2 assimilation in Chlamydomonas cells decreased with culture age both in control and TRIA-treated cells. However, this decrease was less in cells treated with TRIA (Fig. 6); thus, the effect of TRIA on photosynthetic CO_2 assimilation increased with culture age. After treatment with TRIA (100 ug/L) for 1 d photosynthetic CO_2 assimilation increased 13%; by 3-d it had increased by approximately 100%. The decrease in CO_2 assimilation in algal cultures is a common observation as they approach the stationary phase of growth. Limited nutrients and other factors controlling photosynthetic CO_2 assimilation may be causes of reduced CO_2 assimilation in cells from stationary phase growth.

Octacosanol Inhibition. Octacosanol, a 28-carbon straight-chain primary alcohol, which inhibits the effects of TRIA on higher plants (13), also inhibited the increase in photosynthetic CO_2 assimilation in

Figure 6. The Effect of TRIA (1 or 100 ug/L) on Photosynthetic CO_2 Assimilation by Chlamydomonas as Affected by Culture Age. After inoculation and treatment 100 ml samples were removed after 1, 2, and 3 d of growth for determination of photosynthetic CO_2 assimilation (10 mM $KH^{14}CO_3$). Photosynthetic CO_2 assimilation is plotted as a function of log TRIA concentration with controls equal to zero. Each observation is the mean of two experiments with duplicate determinations. Photosynthetic CO_2 assimilation decreased linearly with culture age and increased linearly with TRIA treatment. The F ratio for the interaction of linear TRIA with culture age was significant at the 5% level. The LSD shown is for comparison of any two data points.

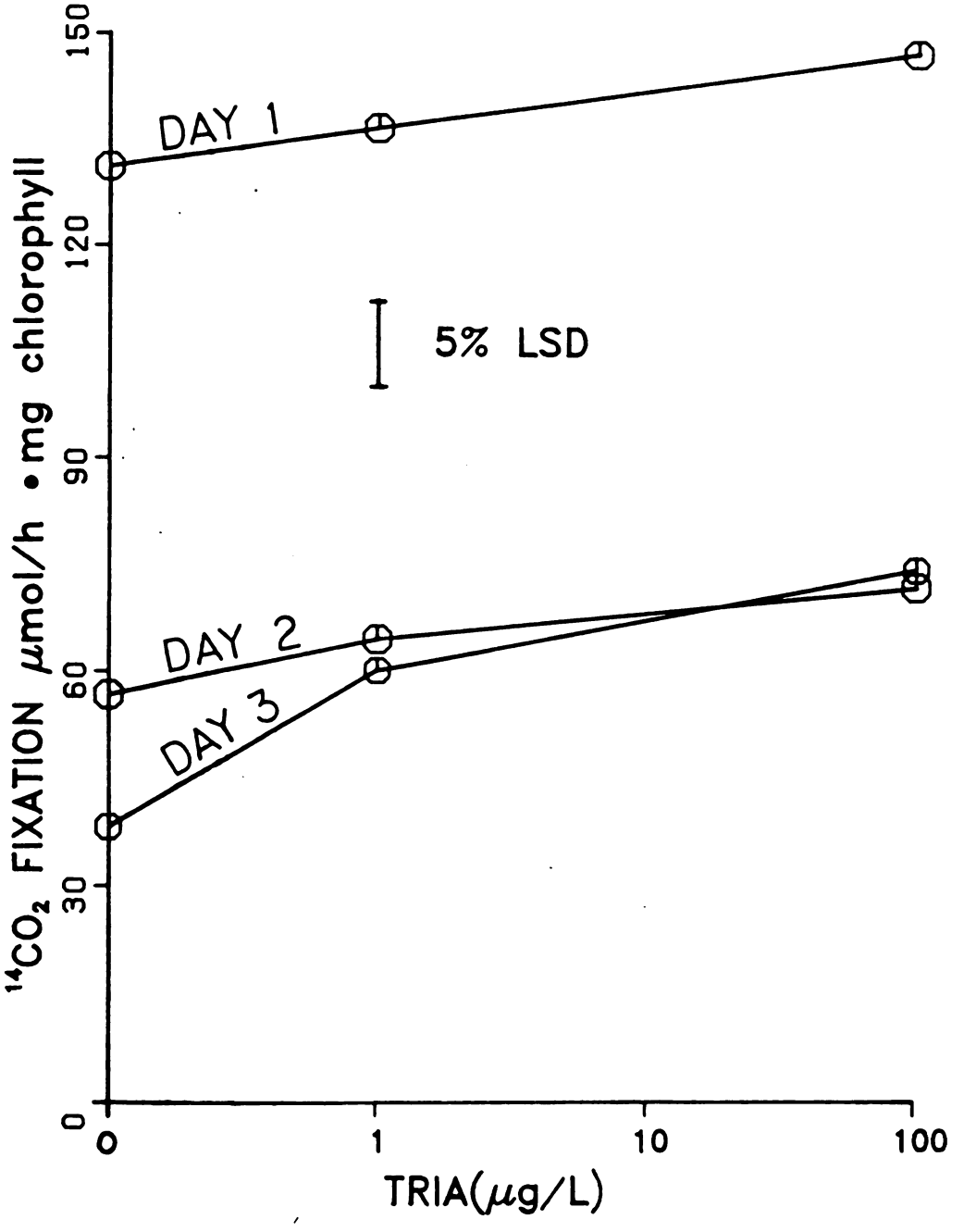

150
120
90
60
30
0
DAY 1
5% LSD
DAY 2
DAY 3
$^{14}CO_2$ FIXATION μmol/h • mg chlorophyll
0
1
10
100
TRIA(μg/L)

Table V. The Effect of Octacosanol (100 ug/L) on the Increase in Photosynthetic CO_2 Assimilation by TRIA-treated (100 ug/L) Chamydomonas Cells.

Procedures are the same as those given in Table I. Each observation is the mean of 2 experiments with duplicate determinations.

Treatment	Photosynthetic CO_2 Assimilation
	umol/h·mg Chlorophyll
Control	38.9
TRIA	52.1
TRIA + octacosanol	44.2
Octacosanol	39.4
5% LSD	9.1

Table VI. The Distribution With Time of ^{14}C Incorporated From Photosynthetic $^{14}CO_2$ Assimilation in Control and TRIA-Treated Chlamydomonas cells.

Cultures were treated with TRIA (1.0 mg/L) or distilled H_2O (control) and assayed after 2 d growth. Cultures were grown as indicated in Table I. Tests were conducted begining with 1 mM $KH^{14}CO_3$. At 20 min intervals $KH^{14}CO_3$ was added to maintain 1 mM $KH^{14}CO_3$. Each observation is the mean of 2 experiments with duplicate determinations.

Time (min)	Control[2]	TRIA	Excreted		Soluble		Insoluble	
			Control	TRIA	Control	TRIA	Control	TRIA
	---- nBq -----		---------- % of Total counts[1] ----------					
10	3.8	4.4	1.3	1.4	41	40	58	58
20	7.6	8.9	3.4	4.1	34	35	63	61
30	13.0	15.6	3.9	4.6	34	34	63	62
40	18.2	21.4	4.8	5.8	31	31	64	63
50	24.6	29.2	4.7	5.9	30	30	66	64
60	28.6	36.3	5.2	5.4	30	29	65	65

[1] The F ratio for the comparison of control with TRIA for the excreted, soluble, and insoluble fractions was not significant.

[2] The rate of photosynthetic CO_2 assimilation with 10 mM $KH^{14}CO_3$ in TRIA-treated cells (133 umol/h·mg chlorophyll) was significantly higher (5% level) than the control rate (109 umol/h·mg chlorophyll).

TRIA treated Chlamydomonas cells (Table V). Octacosanol alone had no effect on photosynthetic CO_2 assimilation; therefore, the effect of TRIA on this process was specific for TRIA.

Effect of TRIA on Photosynthetic $^{14}CO_2$ Fixation Products. The distribution of ^{14}C-label among the photosynthetic products formed by control and TRIA treated Chlamydomonas cells was investigated. Chlamydomonas cells excrete a percentage of photosynthetically fixed $^{14}CO_2$ as glycolate under non-saturating levels of CO_2 (21). TRIA has been shown to affect plasma membrane function and integrity in isolated barley root vesicles (15). Therefore, it was postulated that TRIA might alter the excretion of glycolate in Chlamydomonas cells. However, the distribution of ^{14}C incorporated from photosynthetic CO_2 assimilation between soluble, insoluble, and excreted fractions was not altered by treatment with TRIA (Table VI). TRIA treated Chlamydomonas cells had increased ^{14}C in all of these fractions up to 1 hr after addition of $KH^{14}CO_3$ due to the increased rate of photosynthetic CO_2 fixation. Total ^{14}C distribution among the products was also determined by two dimensional paper chromatography followed by development on X-ray film. There was no visual difference in the ^{14}C labeling pattern between control and TRIA treated cells (data not shown).

Effect of pH and Low CO_2 on TRIA Stimulated Photosynthetic CO_2 Assimilation. Badger et al. (2) showed that Chlamydomonas cells cultured at low CO_2 develop a mechanism for concentrating inorganic carbon ($HCO_3^- + CO_2$) within the cells. The system for accumulating C_i is induced when the cells are transferred from a high-CO_2 to low-CO_2 environment. Since TRIA stimulated photosynthetic CO_2 assimilation by

Chlamydomonas cells, the effects of TRIA on cells cultured with low CO_2 (air), and the effect of transferring high-CO_2-grown cells treated with TRIA to low-CO_2 was investigated. Chlamydomonas cells cultured at low CO_2 levels did not show increased photosynthetic CO_2 assimilation in response to treatment with TRIA (Table VII). Transfer of high CO_2-grown cells that had responded to TRIA treatment, to an atmosphere with low CO_2, resulted in a loss of the TRIA effect after 6 h (Fig. 7). The time course of this loss is similar to that for induction of the C_i accumulation system (5). This indicates that TRIA treatment may bear some relationship to the mechanism for accumulating C_i normally associated with cells grown at low CO_2.

The identity of the actively accumulated species (HCO_3^- or CO_2) in the chloroplasts of Chlamydomonas cells grown at low CO_2 is under investigation by many researchers. CO_2 is probably the species of inorganic carbon that diffuses across the plasma membrane of Chlamydomonas cells grown at low or high CO_2 (16). The equilibrium between HCO_3^- and CO_2 is shifted by changes in pH. At equal total C_i levels photosynthetic CO_2 assimilation by air-grown Chlamydomonas at different external pH is constant up to pH 8.0 (3, 16). For high CO_2-grown cells without the C_i pump, photosynthetic CO_2 assimilation is reduced at pH 8.0 and above due to limiting CO_2. This difference was used to investigate which species of C_i was utilized by TRIA-treated Chlamydomonas. The rate of photosynthetic CO_2 assimilation by control and TRIA treated cells as a function of pH was determined (Fig. 8). As the pH was increased from pH 5.0, photosynthetic CO_2 assimilation was constant in both control and TRIA-treated Chlamydomonas cells up to pH 7.0. Above pH 7.0 photosynthetic CO_2 assimilation was severely

Table VII. The Effect of TRIA on Photosynthetic CO_2 Assimilation by Chlamydomonas Cells Grown at High or Low CO_2.

Chlamydomonas cells were treated and cultured for 3 d as described in Materials and Methods. Cultures were aerated with high CO_2 (5%) or low CO_2 (air) at approximately 100 ml/min. Photosynthetic CO_2 assimilation was determined with 1 mM $KHCO_3$. Each observation is the mean of 2 experiments with duplicate determinations.

	Atmosphere[1]	
	High CO_2	Low CO_2
Treatment	Photosynthetic CO_2 Assimilation umol/h·mg chlorophyll	
TAS (1 ug/L)	20.3	79.4
TRIA (100 ug/L)	30.2	78.2

[1]The F ratios for the effect of atmosphere on photosynthetic CO_2 assimilation and the interaction of TRIA with atmosphere were significant at the 1% level.

Figure 7. Photosynthetic CO_2 Assimilation in Control (TAS) (.01 ug/L) and TRIA-Treated (1.0 ug/L) Chamydomonas Cells Before and After Transfer to Low CO_2 (air). Cultures were inoculated then treated, and after 3 d growth at 5% CO_2 the cells were harvested (900 ml), resuspended in fresh media, and samples were removed at zero time and 3 h intervals for determination of photosynthetic CO_2 assimilation with 1 mM $KH^{14}CO_3$ as described in Materials and Methods. Each observation is the mean of two experiments with duplicate determinations. The F-ratio for the interaction of TRIA with linear time was significant at the 1% level. The LSD shown is for comparison of any two data points.

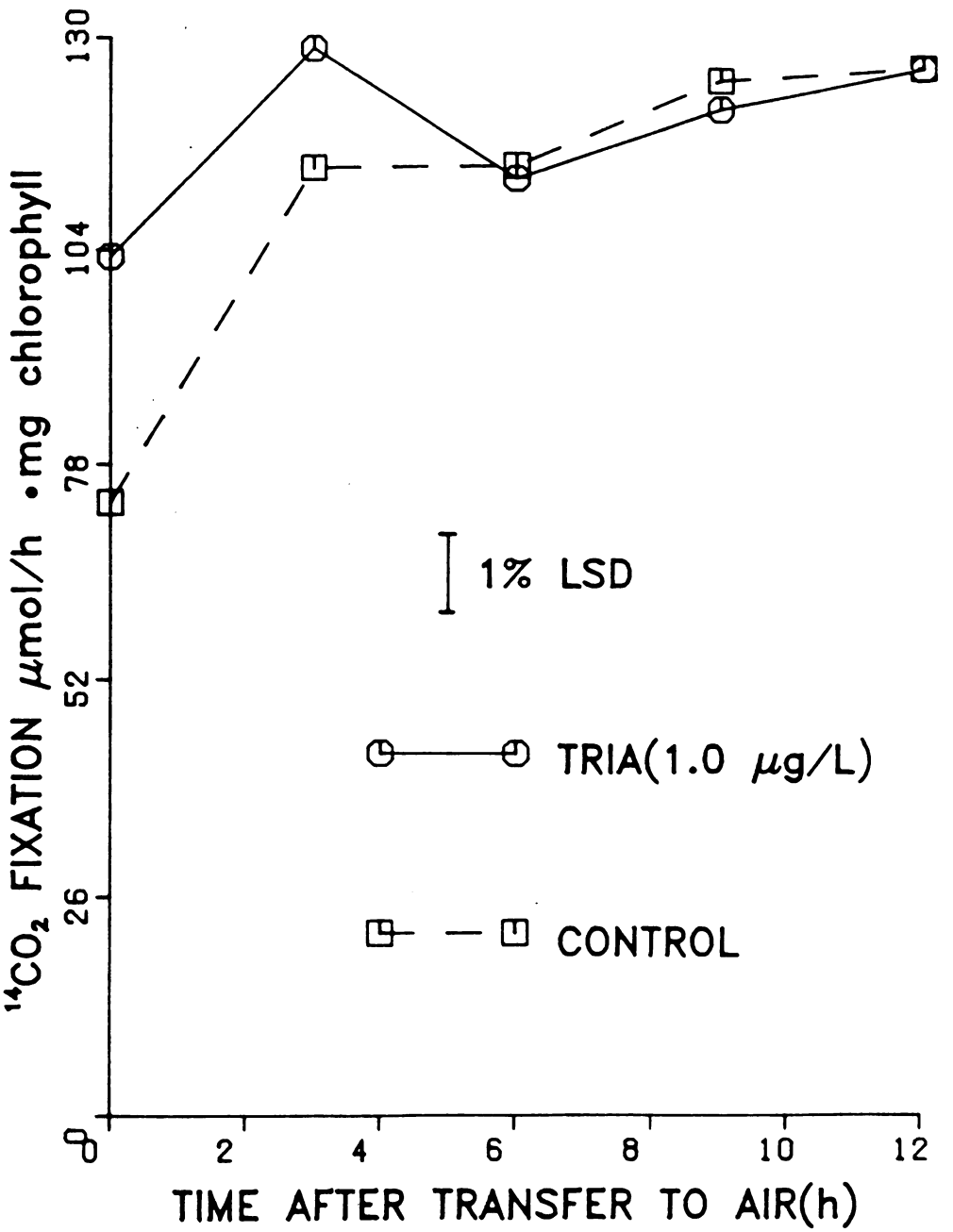
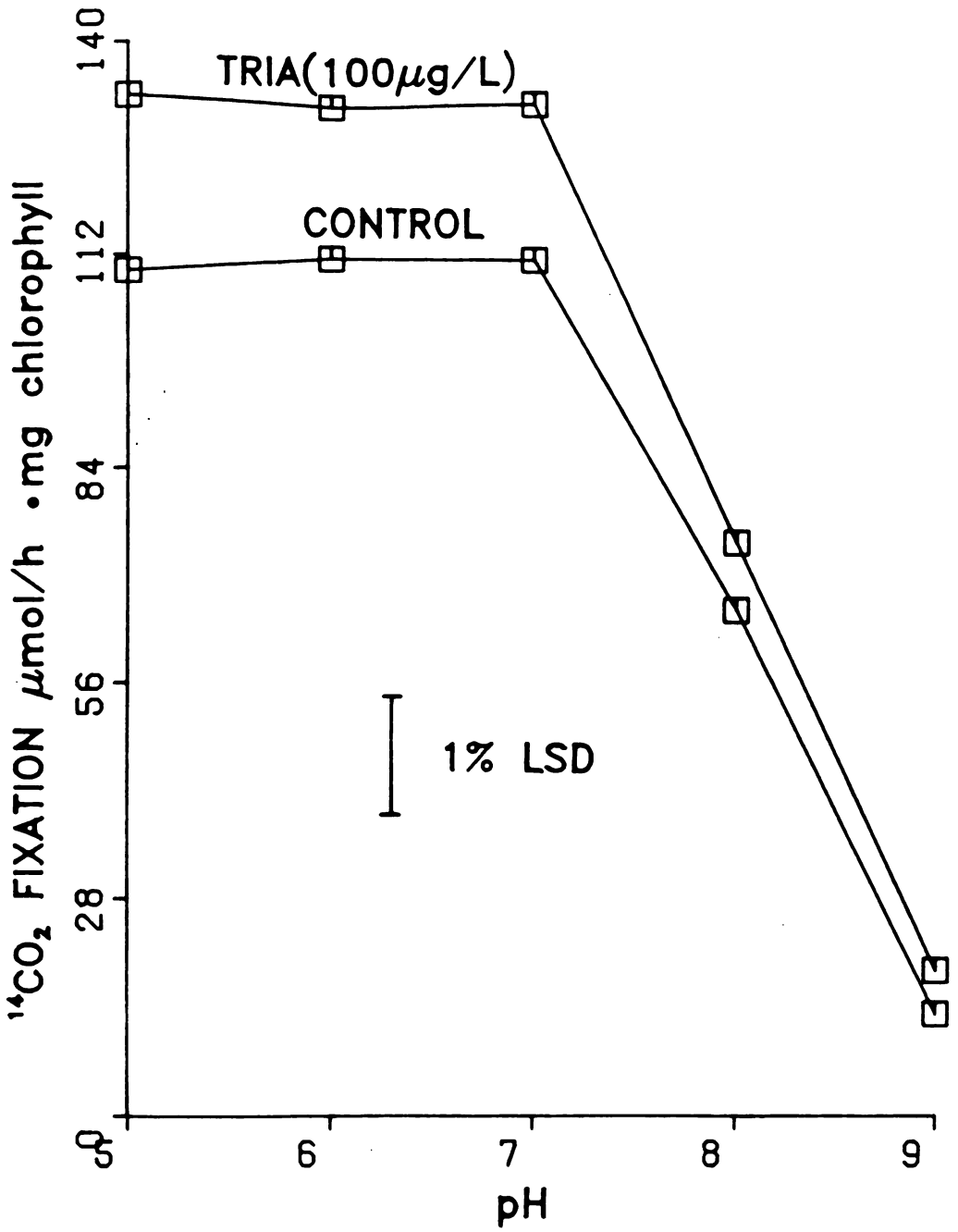

$^{14}CO_2$ FIXATION µmol/h • mg chlorophyll
130
104
78
52
26
0
2
4
6
8
10
12
TIME AFTER TRANSFER TO AIR(h)
1% LSD
TRIA(1.0 µg/L)
CONTROL

Figure 8. Photosynthetic CO_2 Assimilation in Control (TAS, 1 ug/L) and TRIA-treated (100 ug/L) Chlamydomonas Cells. Cultures were inoculated, then treated, and after 2 d samples (500 ml) were removed for determination of photosynthetic CO_2 assimilation with 1 mM $KH^{14}CO_3$ at several pH levels. Each observation is the mean of two experiments with duplicate determinations. The F ratio for the interaction of TRIA with linear pH was significant at the 1% level. The LSD shown is for comparison of any two data points.


TRIA(100μg/L)
CONTROL
1% LSD
$^{14}CO_2$ FIXATION μmol/h • mg chlorophyll
0
28
56
84
112
140
5
6
7
8
9
pH

inhibited in both control and TRIA-treated Chlamydomonas cells and the effect of TRIA decreased.

DISCUSSION

Treatment of Chlamydomonas cultures with 1 to 1000 ug/L TRIA resulted in significant increases in cell density, total chlorophyll, and photosynthetic CO_2 assimilation. The increase in chlorophyll was dependent upon increases in cell density since there was no change in the amount of chlorophyll per cell. During the first 3-d culture period cell density was not significantly increased, but upon transfer of an aliquot of TRIA-treated cells to fresh medium, a significant increase in cell density occurred. The earliest and largest response of Chlamydomonas cells to TRIA was an increase in photosynthetic CO_2 assimilation which was independent of increases in chlorophyll or cell density. TRIA treatment reduced the decrease in photosynthetic CO_2 assimilation in Chlamydomonas cells entering stationary phase growth. The increase in photosynthetic CO_2 assimilation was specific for TRIA and was inhibited by octacosanol. TRIA treatment did not cause a change in the distribution of fixed ^{14}C label between soluble, insoluble, and excreted ^{14}C-products. Chlamydomonas cells cultured at low CO_2 levels did not respond to TRIA, possibly due to changes in the binding affinity for TRIA and/or decreased adsorption. Transfer of cells grown at high CO_2 that had responded to TRIA treatment, to a low CO_2 atmosphere, resulted in a loss of the effect of TRIA after 6 h. The effect of pH on photosynthetic CO_2 assimilation by control and TRIA-treated Chlamydomonas cells suggests that CO_2 is the species of inorganic carbon utilized, and that this species is also utilized by

TRIA treated cells.

The colloidally dispersed formulations of TRIA, which are probably the best formulations of TRIA available for experimental purposes (14, 17), were not stable when added to the Chlamydomonas culture medium. The instability is probably the result of an increase in particle size due to an interaction of cations present in the growth medium with the negatively charged surface of the TRIA particles. Calcium ions were more effective than potassium ions at initiating flocculation of TRIA dispersions. Since this increase in particle size and/or flocculation is detrimental to the effects of TRIA on higher plants and Chlamydomonas cells, the presence of contaminating cations in the water used for preparing and diluting colloidally dispersed TRIA may be a crucial factor in achieving consistent and reproducible results. Although the TRIA dispersions were not stable in Chlamydomonas growth medium Chlamydomonas cells apparently bound sufficient TRIA during treatment to elicit a lasting response. The binding is fairly rapid with the majority of [^{14}C]TRIA becoming bound within the first 10 min of treatment. Since the specific activity of the [^{14}C]TRIA was low, it was not feasible to conduct the binding experiments within the range of TRIA concentrations and Chlamydomonas cell densities used in the dose-response and other studies. Therefore, it is not possible based on these data to accurately describe the relationship between bound TRIA and the response of Chlamydomonas cells to TRIA. However, the evidence presented suggest that the response of Chlamydomonas cells to TRIA is influenced by the instability of the TRIA formulation in the presence of Chlamydomonas growth medium and the binding of TRIA to the cells. Since these two processes occur simultaneously during treatment of

Chlamydomonas cultures with colloidally dispersed TRIA, there is in all likelihood competition between the binding of TRIA particles to Chlamydomonas cells and self-aggregation of the particles to form inactive TRIA floccules. The partitioning of TRIA particles between that bound to Chlamydomonas cells and that aggregating to form floccules may determine the magnitude of the response of Chlamydomonas cells to TRIA. These physical factors could also influence the binding of TRIA to the roots or shoots of higher plants, and therefore, play an important role in determining the response of higher plants to TRIA.

TRIA treatment may affect some process associated with the CO_2-concentrating mechanisms in Chlamydomonas cells, but the evidence presented is not conclusive. There may be other biological processes involved in the development of a CO_2-concentrating mechanism and TRIA may have affected one of these and not the CO_2-concentrating mechanism per se. The effect of TRIA on photosynthetic CO_2 assimilation was greatest in Chlamydomonas cells entering stationary phase growth. Under conditions of high CO_2, RuBP levels would be expected to play an important role in determining the maximum rate of photosynthetic CO_2 assimilation in algae and higher plants. RuBP levels are increased in Chlamydomonas cells by treatment with TRIA, which may explain the increased photosynthetic CO_2 assimilation observed in TRIA- treated cells (12).

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CHAPTER 2

THE SPECIFIC ACTIVITY OF RuBP CARBOXYLASE/OXYGENASE, RuBP LEVELS, AND CHARACTERISTICS OF PHOTORESPIRATION IN *CHLAMYDOMONAS REINHARDTII* CELLS TREATED WITH TRIACONTANOL

ABSTRACT

Increased photosynthetic CO_2 assimilation by *Chlamydomonas reinhardtii* cells treated with TRIA was not due to changes in glycolate excretion, CO_2 compensation point, or the sensitivity of photosynthetic CO_2 assimilation to O_2. Kinetic analysis of TRIA-treated cells showed that the increase in photosynthetic CO_2 assimilation was a result of an increase in the whole-cell apparent Vmax. The activity of RuBP carboxylase/oxygenase was higher in cell lysates from TRIA-treated cells than control cells. Quantification of RuBP carboxylase/oxygenase levels by ^{14}CABP binding did not show increased enzyme levels in TRIA-treated cells. Therefore, there was an increase in the specific activity of RuBP carboxylase/oxygenase extracted from *Chlamydomonas* cells treated with TRIA. TRIA alone had no effect *in vitro* on the activity of RuBP carboxylase/oxygenase purified from spinach (*Spinacia oleracea* L.) leaves or in cell lysates from *Chlamydomonas*.

RuBP levels were 50% to 60% higher in cells treated with TRIA at high and low CO_2. TRIA also increased RuBP levels in the absence of CO_2 with atmospheres of N_2 or N_2 with 21% O_2.

Previously it was demonstrated that TRIA stimulated photosynthetic CO_2 fixation by Chlamydomonas cells cultured at 5% CO_2 by 20 to 100% (14). The distribution of ^{14}C label among the products of photosynthetic CO_2 fixation and glycolate excretion were not affected by TRIA. Increased photosynthetic CO_2 fixation was measured 1 h after treatment with TRIA and persisted for 3 d. Transfer of cells cultured at 5% CO_2, that had responded to TRIA, to a low CO_2 environment (air), resulted in a loss of the stimulation from TRIA. Control and TRIA-treated cells responded similarly to changes in external pH, but the largest effect of TRIA on photosynthetic CO_2 assimilation occurred at pH 5 to 7.

Several species of algae, including Chlamydomonas, possess a mechanism for concentrating C_i within the cells when they are cultured at ambient levels of CO_2 (2, 4, 9, 11, 22). In the same species of cells this mechanism does not exist when cultured at high CO_2 (5%). Chlamydomonas cells that possess this C_i pump exhibit three changes in photosynthetic CO_2 assimilation with respect to cells that do not have this mechanism: (a) photosynthetic CO_2 assimilation is insensitive to O_2; (b) the apparent $Km(CO_2)$ of the algae cells is substantially decreased, and (c) the apparent Vmax for photosynthetic CO_2 assimilation occurs at low levels of external C_i (2, 4). Together, these changes contribute to increased photosynthetic CO_2 assimilation and efficiency at low levels of external C_i by algae cells cultured at low CO_2 compared to cells cultured at high CO_2. TRIA-treated Chlamydomonas cells exhibit some properties suggesting that TRIA may effect the C_i accumulation mechanism (14). Other researchers reported that TRIA treatment reduced the O_2 inhibition of photosynthetic CO_2

assimilation in Chlamydomonas cells (10). The object of this research was to investigate the possibility that the C_i accumulation system in Chlamydomonas cells is affected by TRIA treatment.

MATERIALS AND METHODS

Algae Culture and Treatment. Chlamydomonas reinhardtii Dangeard (-) strain (N.90) were cultured, treated and photosynthetic CO_2 assimilation assayed as described earlier (14). Chlorophyll was determined by the method of Arnon (1) and glycolate excretion by the Calkins assay with the procedure described previously (27).

RuBP Carboxylase Extraction and Assay. Chlamydomonas cells were harvested by centrifugation at 1000g. The cell pellet was washed twice with and resuspended in cold (4^oC) 50 mM Bicine-KOH (pH 8.2) buffer, to a final chlorophyll concentration of 50 to 100 ug chlorophyll/ml. The cell suspension was passed twice through a Yeda press (Linca Science Instruments, Tel-Aviv, Israel) with compressed N_2 (10,400 kPa). The crude cell extract or the supernatant solution obtained after centrifugation for 2 min at approximately 2000g with an Eppendorf model 5414 centrifuge, was used as the enzyme source for RuBP carboxylase. The activation and assay of RuBP carboxylase were as described previously (21). Activation time at 30^oC was 30 min and assay incubation time was 15 s.

RuBP Carboxylase Specific Activity and Active Site Concentration. The level of RuBP carboxylase in lysates of Chlamydomonas cells was quantified by measuring the binding of [^{14}C]CABP (21). Aliquots (1 to

3 ml) of the extract from Chlamydomonas cells were incubated with 5 uM [^{14}C]CABP. After incubation at 30°C for 45 min nonradioactive CABP was added and any exchange with [^{14}C]CABP allowed to proceed for 1 h. The [^{14}C]CABP-RuBP carboxylase-complex was precipitated with 20% PEG 4000 containing 20 mM $MgCl_2$ and the precipitate collected by centrifugation at 30,000g for 20 min. The pellet was washed twice with 20% PEG 4000 containing 20 mM $MgCl_2$ and resuspended in 0.5 ml H_2O. The 0.5 ml sample was mixed with 4.5 ml of scintillation cocktail and ^{14}C was determined by liquid scintillation counting. The specific activity of RuBP carboxylase was calculated assuming the enzyme had a molecular weight of 550,000 daltons with 8 catalytic sites per mol, each of which bound one molecule of CABP.

RuBP Determinations. RuBP was determined by a modification of the procedure described previously (15, 16). Aliquots (430 ul) of Chlamydomonas cells suspended in 50 mM HEPES-KOH (pH 7.5) buffer were added to 70 ul of ice cold 70% $HClO_4$ in 1.5 ml plastic microfuge tubes on ice. After 20 min the samples were centrifuged for 1 min with an Eppendorf centrifuge and most of the supernatant (450 ul) was removed. The supernatant samples were placed in 1.5 ml microfuge tubes on ice and 50 ul of 1 M Bicine-KOH (pH 8.2) and 85 ul of 10 N KOH were added to each tube. Following incubation for 5 min with occasional mixing, the tubes were centrifuged to remove the insoluble $KClO_4$ and samples of the supernatants (250 ul, pH 8.0 to 8.2) were removed for determination of RuBP. RuBP was determined by incubating samples with activated RuBP carboxylase purified from spinach (Spinacia oleracea L.) leaves by measuring the incorporation of $H^{14}CO_3^-$ into acid-stable ^{14}C. The RuBP

assay media contained 100 mM Bicine-KOH (pH 8.0 to 8.2) buffer, 0.2 mM Na_2 EDTA, 0.5 mM DTT, 20 mM $MgCl_2$, 10 mM $KH^{14}CO_3$ (1.0 uCi/umol) and 50 ug of activated RuBP 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 ul of 2 N HCl and the samples were dried at 80°C. After cooling 0.5 ml of H_2O and 4.5 ml of scintillation cocktail were added and radioactivity determined by liquid scintillation counting. This method of RuBP determination gave reliable results and good recoveries (95%) of RuBP added to Chlamydomonas cell extracts.

PEP Carboxylase Activity. The Chlamydomonas cell extracts used for RuBP carboxylase determinations were also used to assay PEP carboxylase activity. Aliquots of the cell extract (20 to 50 ul) 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 $KH^{14}CO_3$ (1.0 uCi/umol), 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 with enzyme and terminated after 30 s with 200 ul of 2 N HCl. Blanks consisted of identical samples minus PEP. The samples were allowed to stand at 25°C for 12 h to facilitate the exchange of $^{14}CO_2$ from the samples with atmospheric CO_2. After adjusting the volume to 0.5 ml with H_2O, 4.5 ml of scintillation cocktail were added and ^{14}C determined.

CO_2 Compensation Point Determinations. Chlamydomonas cells suspended in 50 mM HEPES-KOH (pH 7.5) buffer (50 ml, 20 to 40 ug chlorophyll/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 glass chamber was held in a water bath at 21°C and PAR (600 umol/s·m^2) was provided by a metal halide lamp. Air containing CO_2 (51 ul/L) was circulated with a piston pump (200 ml/min) through the chamber until the level of CO_2 in the gas exiting the chamber was stable at 51 ul/L (approximately 10 min). The system was closed and the internal atmosphere was circulated (200 ml/min) through a Beckman Model 865 IRGA. The IRGA was calibrated with air containing known levels of CO_2 such that the output voltage was linear in response to CO_2 levels from 0 to 100 ul/L. After closing the system the CO_2 compensation point of the cell suspension was reached in the next 20 to 30 min.

Effect of O_2 on Photosynthetic CO_2 Assimilation. Photosynthetic CO_2 assimilation by Chlamydomonas cells was measured in an atmosphere of air (21% O_2) and an atmosphere of air diluted 1:9 v/v with N_2 (approximately 2% O_2). The CO_2 in the air was removed with a column (2 cm x 18 cm) of Ascarite (Arthur H. Thomas Co., Philadelphia, PA). Chlamydomonas cells suspended in 20 ml of 50 mM HEPES-KOH (pH 7.5) buffer were placed in a water-jacketted (25°C) lollipop tube (75 ml) and the tube was 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 lollipop tube, the cell suspension was flushed with the appropriate atmosphere (100 ml/min) for 10 min in the light (1000 umol/s·m^2) prior to the initiation of photosynthetic

CO_2 assimilation with 1 mM $KH^{14}CO_3$ (1.0 uCi/umol). After addition of $KH^{14}CO_3$ the inlet and outlet tubes were pinched closed and samples were removed (100 ul) by opening the inlet tube and withdrawing a sample with a 250 ul Hamilton syringe. Acid stable ^{14}C was determined as described previously. Prior to sampling, 100 ul of the same gas that was used to flush the cell suspension was injected into the lollipop tube to maintain constant pressure.

Chemicals. RuBP, [2'-^{14}C]CABP (1.0 uCi/umol) and RuBP carboxylase were prepared by Dr. M. Mulligan as described by (12, 20, 18), respectively, and were available from the laboratory of Professor N. E. Tolbert (Department of Biochemistry, Michigan State University). $NaH^{14}CO_3$ (40 to 60 uCi/umol) was obtained from New England Nuclear. TRIA and TAS were obtained from the Proctor and Gamble Company. Phosphoenol pyruvate (tri-monocyclohexyl-ammonium salt), NADH (disodium salt, grade III), malic dehydrogenase (porcine, mitochondrial), and polyethylene glycol (PEG 4000) were obtained from Sigma Chemical Co. (St. Louis, MO 63178).

Statistical Procedures All experiments were replicated. 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 or non-orthogonal comparisons. Treatment means were also compared with an LSD value in some tests.

RESULTS

Characteristics of the Oxidative Photosynthetic Carbon Cycle. Glycolate excretion, CO_2 compensation point, and the inhibition of photosynthetic CO_2 assimilation by O_2 are all reduced in Chlamydomonas cells cultured under low CO_2(air) as compared to cells grown at high CO_2(5%). These observations are consistent with reports showing that air-grown cells concentrate C_i relative to the external medium. Consequently, measurement of the aforementioned parameters, as well as other parameters, can be indicative of the presence or absence of the C_i accumulation system. In these considerations it is necessary to designate the CO_2 level during growth. Cells grown on air(low CO_2) do not respond to TRIA but have the C_i pump. Cells grown at high CO_2 levels do not have a C_i pump but show increased photosynthetic CO_2 assimilation when treated with TRIA (14).

The rate of photosynthetic CO_2 assimilation by control and TRIA-treated high-CO_2- grown Chlamydomonas cells under atmospheres containing 2% and 21% O_2 was measured (Table I). One milimolar $KHCO_3$ was used so that the CO_2 concentration would be limiting. When the partial pressure of O_2 was reduced, photosynthetic CO_2 assimilation increased approximately 20% in both control and TRIA-treated cells. Regardless of the O_2 concentration, TRIA treated cells had higher rates of photosynthetic CO_2 assimilation.

The CO_2 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 CO_2 in Chlamydomonas cells will result in changes in the CO_2 compensation point. In Chlamydomonas cells grown with air, the presence of the C_i accumulation system results in low-CO_2 compensation points ($\leq$ 5 ul/L), since for any given external level of CO_2 the internal level is higher and the inhibition of RuBP carboxylase by O_2 is reduced (2). There was no effect of TRIA on the compensation point of Chlamydomonas cells grown on high CO_2 (Table I). From these results it appears that TRIA does not affect the sensitivity of photosynthetic CO_2 assimilation to inhibition by O_2 in Chlamydomonas cells cultured at high CO_2. Thus the TRIA stimulation of photosynthetic CO_2 assimilation does not appear to be associated with stimulation of the C_i pump.

Photosynthetic CO_2 Assimilation Kinetics. Chlamydomonas cells adapted to low CO_2 have a whole-cell apparent Km (CO_2) for photosynthetic CO_2 assimilation of 3uM, which is below the Km (CO_2) of 46uM for the isolated RuBP carboxylase enzyme. Cells cultured with high CO_2 have an apparent photosynthetic Km (CO_2) approximating that for isolated RuBP carboxylase (4). Kinetic analysis of control and TRIA-treated high-CO_2-grown Chlamydomonas cells showed that the increase in photosynthetic CO_2 assimilation by TRIA-treated cells was due to an increase in the whole cell apparent Vmax (Fig. 1A, B). There was no change in the apparent Km(CO_2) with TRIA treatment. Therefore, it does not appear that TRIA treatment affects the internal level of C_i in Chlamydomonas, i.e. the C_i pump.

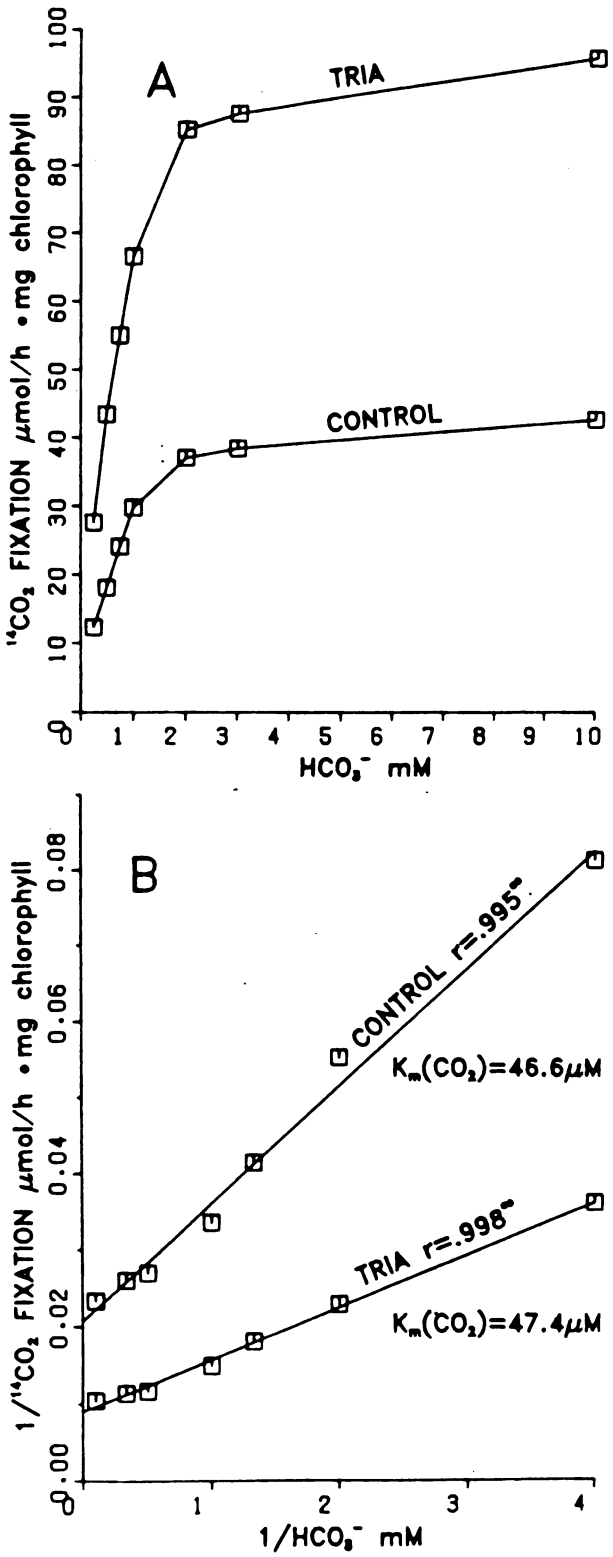
Table I. CO_2 Compensation Levels and Photosynthetic CO_2 Assimilation with 2% and 21% O_2 by Control (TAS, 0.1 ug/L) and TRIA-Treated (10 ug/L) Chlamydomonas cells cultured at 5% CO_2.

Photosynthetic CO_2 assimilation was determined with 1 mM $KH^{14}CO_3$ at pH 7.5. CO_2 compensation levels were determined with an IRGA as described in Materials and Methods. Each observation is the mean of 2 experiments with duplicate determinations.

Treatment	Photosynthetic CO_2 Assimilation		% Increase	CO_2 Compensation Level
	2% O_2	21% O_2		
	umol/h·mg chlorophyll		(2% O_2/21% O_2)-1	ul CO_2/L
Control	54.5	45.5	20	51.3
TRIA	76.6**	65.4**	17	54.8

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

Figure 1. Kinetics of Photosynthetic CO_2 Assimilation by Control (TAS, 1 ug/L) and TRIA-Treated (100 ug/L) Chlamydomonas Cells. (A), Photosynthetic CO_2 assimilation by control and TRIA-treated Chlamydomonas cells cultured at high-CO_2 with increasing concentrations of $KHCO_3$. (B), Lineweaver-Burk plot of data in (A). Two-day-old Chlamydomonas cells were assayed for photosynthetic CO_2 assimilation as described in Materials and Methods. The endogenous level of HCO_3^- in equilibrium with atmospheric CO_2 in the buffer (50 mM HEPES-KOH pH 7.5) was included in the total HCO_3^- concentration. A pK_a value of 6.3 was used for calculating the whole cell $Km(CO_2)$. The Vmax for control and TRIA-treated cells was 48.3 and 111.4 umol/h·mg chlorophyll, respectively. The F ratio for the effect of TRIA on Photosynthetic CO_2 assimilation was significant at the 1% level. Each observation is the mean of 2 experiments with duplicate determinations.


A
TRIA
CONTROL
$^{14}CO_2$ FIXATION µmol/h • mg chlorophyll
HCO_3^- mM
B
CONTROL r=.995**
$K_m(CO_2)$=46.6µM
TRIA r=.998**
$K_m(CO_2)$=47.4µM
$1/^{14}CO_2$ FIXATION µmol/h • mg chlorophyll
$1/HCO_3^-$ mM

In Vivo and In Vitro RuBP Carboxylase Activity. TRIA at several concentrations did not affect the carboxylase activity of RuBP carboxylase activity in extracts from Chlamydomonas cells (Table II). When RuBP carboxylase is activated at less than saturating levels of CO_2, the total activity measured is sensitive to compounds that influence the activation state of the enzyme (17). TRIA did not influence the activity of RuBP carboxylase in extracts from Chlamydomonas cells that were activated at 1 mM $KH^{14}CO_3$. Therefore, it does not appear that TRIA affects the activation reaction of RuBP carboxylase (Table II). A similar experiment with purified RuBP carboxylase from spinach leaves was also negative for TRIA stimulation of activation or activity of the enzyme (Table III).

When 3-d-old control and TRIA-treated Chlamydomonas cells were ruptured and RuBP carboxylase assayed before and after activation, TRIA treated cells showed significantly higher rates of RuBP carboxylase activity (Table IV). There were no differences in PEP carboxylase activities in the lysates from control and TRIA-treated Chlamydomonas cells.

The possibility that the extract from TRIA-treated Chlamydomonas cells contained a promotor of RuBP carboxylase activity, possibly synthesized in response to TRIA treatment, was tested by assaying combinations of the extracts from control and TRIA-treated cells (Fig. 2). Total RuBP carboxylase activity increased linearly with increasing levels of the extract from TRIA-treated cells. This indicates that a negative or positive effector of RuBP carboxylase activity does not exist in the cell extracts from control or TRIA-treated Chlamydomonas cells, respectively.

Table II. The Absence of TRIA and TAS Activity on RuBP Carboxylase from Cell Lysates of Chlamydomonas.

The lysate from 3-d-old Chlamydomonas cells were treated with TRIA or TAS. The enzyme was activated with 1 or 10 mM $KH^{14}CO_3$ at 30°C for 30 min, and the activity of RuBP carboxylase was measured with 10 mM $KH^{14}CO_3$. Each observation is the mean of 3 experiments with triplicate determinations. There were no significant differences in $^{14}CO_2$ fixation rates as a result of treatments.

Treatment		Activation Level of $H^{14}CO_3^-$	
		1 mM	10 mM
Chemical	ug/L	$^{14}CO_2$ Fixation Rate umol/h·mg chlorophyll	
None		100.8	195.6
TAS	10	98.7	201.3
TRIA	1	102.5	199.6
TRIA	10	100.7	189.2
TRIA	100	99.8	199.0
TRIA	1000	103.6	201.8

Table III. Activation and Activity of RuBP Carboxylase Purified from Spinach Leaves With and Without TAS and TRIA.

RuBP carboxylase (50 ug) was activated with 1 or 10 mM $KH^{14}CO_3$ at 30°C for 30 min and then assayed with 10 mM $KH^{14}CO_3$. TRIA or TAS were present during activation. Each observation is the mean of 2 experiments with triplicate determinations. There were no significant differences in $^{14}CO_2$ fixation rates as a result of treatments.

Treatment		Activation Level of $H^{14}CO_3^-$	
		1 mM	10 mM
Chemical	ug/L	$^{14}CO_2$ Fixation Rate umol/min·mg protein	
None		0.82	1.66
TAS	10	0.82	1.65
TRIA	1	0.83	1.67
TRIA	10	0.82	1.58
TRIA	100	0.81	1.59
TRIA	1000	0.82	1.61

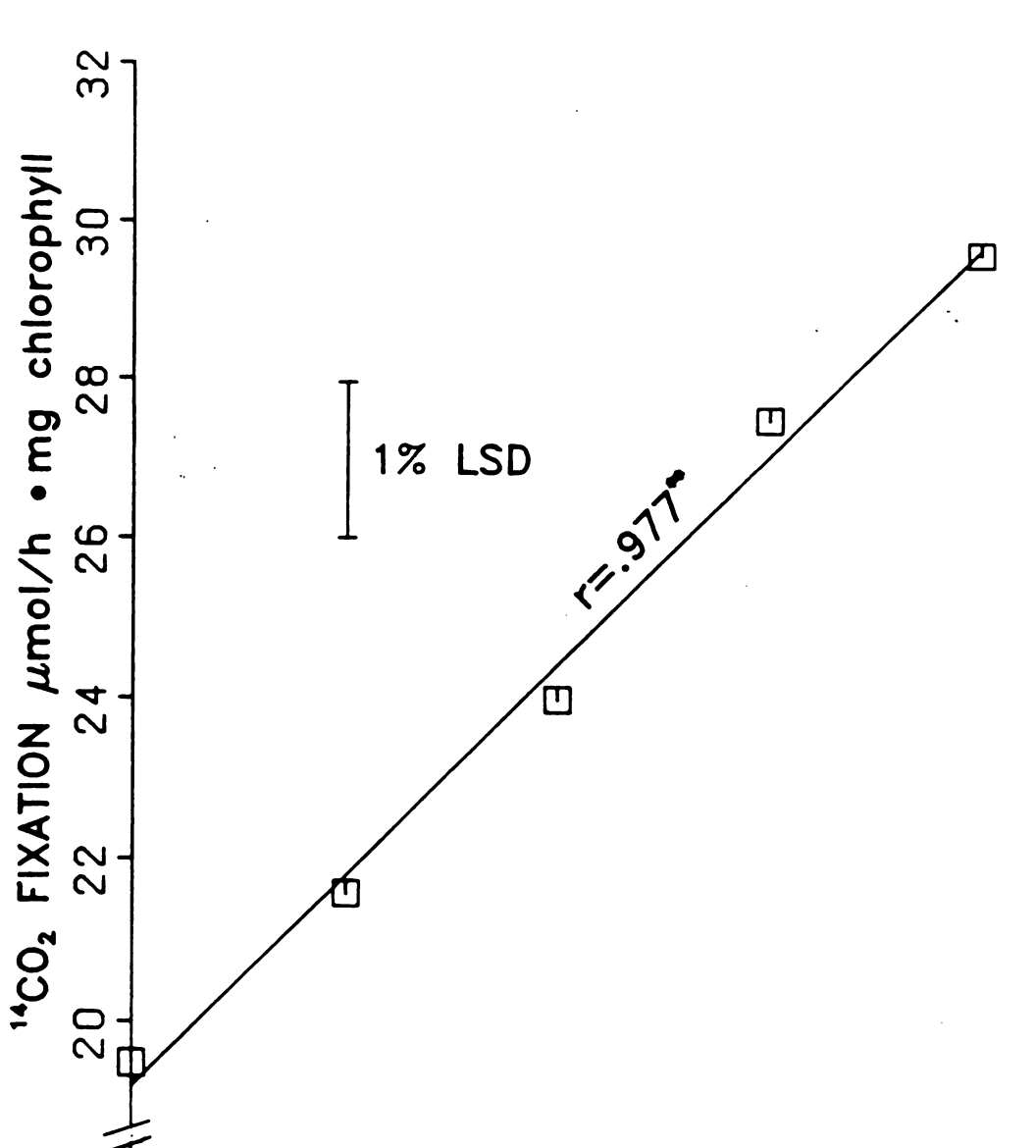
Table IV. Photosynthetic CO_2 Fixation by Intact Cells and the Activity of RuBP Carboxylase and PEP Carboxylase in Lysates from Control (H_2O) and TRIA-Treated (1 mg/L) Chlamydomonas Cells.

High-CO_2-grown Chlamydomonas cells (3-d-old) were lysed and the lysate was assayed for RuBP carboxylase and PEP carboxylase activity, with 10 mM $KH^{14}CO_3$. RuBP carboxylase activity was determined before and after activation. Each observation is the mean of 3 experiments with triplicate determinations.

Treatment	RuBP Carboxylase			PEP-Carboxylase
	Whole Cells	Not Activated	Activated	
	$^{14}CO_2$ Fixation Rate (umol/h·mg Chlorophyll)			
Control	32.3	19.5	277.2	6.9
TRIA	62.3**	29.6**	301.4*	7.5

*,** F ratio for difference between treatments was significant at the 5% and 1% level respectively.

Figure 2. RuBP Carboxylase Activity in Combinations of Extracts From Control (H_2O) and TRIA-Treated (100 ug/L) Chlamydomonas Cells. The lysate from 3-d-old control and TRIA-treated cells cultured at high CO_2 was mixed at varying proportions and RuBP carboxylase activity assayed without prior activation. The r value from linear regression analysis was significant at the 1% level. The rate of photosynthetic CO_2 assimilation by intact TRIA-treated cells was significantly higher (1% level) than that for control cells. Each observation is the mean of 3 experiments with triplicate determinations.


$^{14}CO_2$ FIXATION µmol/h • mg chlorophyll
0
20
22
24
26
28
30
32
1% LSD
r=.977*
100:0
75:25
50:50
25:75
0:100
EXTRACT RATIO (CONTROL:TRIA)

RuBP Carboxylase Levels. Increased RuBP carboxylase activity in extracts from TRIA-treated Chlamydomonas cells suggests that either the amount of RuBP carboxylase or its activation potential could be higher in TRIA-treated cells. Binding of [^{14}C]CABP to activated RuBP carboxylase showed no significant changes in the active site concentration of RuBP carboxylase in TRIA-treated Chlamydomonas cells (Table V). However, there was an increase in the specific activity of RuBP carboxylase from TRIA-treated cells. This increase in specific activity was substantial (40%), but could not entirely account for the 100% percent increase in photosynthetic CO_2 assimilation observed with TRIA-treated intact Chlamydomonas cells (Fig. 1). This led to the investigation of other changes in Chlamydomonas cells treated with TRIA that were stimulating photosynthetic CO_2 assimilation.

RuBP Levels. Chlamydomonas cells cultured at 5% CO_2 should be RuBP-limited with respect to photosynthetic CO_2 assimilation (7, 8). The levels of RuBP in TRIA-treated Chlamydomonas cells at saturating levels (10 mM $KHCO_3$, pH 7.5) of CO_2 were 46% to 55% higher than control cells (Tables V, VI). The levels of RuBP in both treatments were below or similar to the active site concentration of RuBP carboxylase.

At less than saturating levels of CO_2 and high light intensities RuBP levels would not be expected to influence the rate of photosynthetic CO_2 assimilation since RuBP carboxylase should be saturated with RuBP. The increase in photosynthetic CO_2 assimilation by TRIA treated Chlamydomonas cells at less than saturating levels of CO_2 (Fig. 1) could be a result of increased specific activity of RuBP carboxylase (Table V). However, RuBP levels in TRIA-treated

Table V. Specific Activity and Level of RuBP Carboxylase in Lysates from Control (TAS, 1 ug/L) and TRIA-Treated (100 ug/L) Chlamydomonas Cells.

Lysates from 2-d-old control and TRIA-treated Chlamydomonas cells cultured at high CO_2 were assayed for RuBP carboxylase activity, and enzyme levels determined by [^{14}C]CABP binding. The specific activity was calculated assuming a molecular weight for RuBP carboxylase of 550,000 daltons and 8 catalytic sites per mol of enzyme. Each observation is the mean of 3 experiments with triplicate determinations.

	RuBP Carboxylase		
	Active Site Concentration	Activity	Specific Activity
Treatment	nmol/mg chlorophyll	umol CO_2/h· mg chlorophyll	umol CO_2/min· mg protein
Control	13.4	261.3	1.43
TRIA	12.9	351.8**	2.00**

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

Table VI. Levels of RuBP in Control (TAS, 1 ug/L) and TRIA-Treated (100 ug/L) Chlamydomonas Cells.

Two or 3-d-old Chlamydomonas cells grown with high CO_2 that were actively assimilating CO_2 (10 mM $KH^{14}CO_3$, pH 7.5) were assayed for RuBP levels as described in Materials and Methods. In each experiment the effect of TRIA on photosynthetic CO_2 assimilation was significant. Each mean is the average of 2 experiments with duplicate determinations.

Treatment	Test 1	Test 2	Test 3
	RuBP nmol/mg Chlorophyll		
Control	11.7	2.4	10.3
TRIA	18.2**	3.5**	15.8**

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

Chlamydomonas cells were 50% higher than control cells with and without 1 mM $KHCO_3$ (Fig. 3). Since the levels of RuBP that were determined before addition of $KHCO_3$ were from cells under a N_2 atmosphere and high light intensity, these levels may reflect the pool of phosphorylated metabolites in the chloroplast available for RuBP synthesis. Since the increase in RuBP levels with TRIA treatment was maintained under steady state carboxylation conditions, after the addition of $KHCO_3$, the RuBP regeneration rate may also be affected. In a similar experiment, without the addition of $KHCO_3$, RuBP levels were again higher in TRIA-treated Chlamydomonas cells (Fig. 4). Overall, the levels of RuBP were lower in Chlamydomonas cells with an atmosphere of CO_2 free air than with 1 mM $KHCO_3$. This was expected since the oxygenase activity of RuBP carboxylase results in a loss of chloroplast metabolites available for RuBP synthesis. The levels of RuBP increased with time in both control and TRIA-treated cells and may have eventually reached higher levels. The increase in the levels of RuBP in TRIA-treated cells after addition of O_2 was larger than the increase in control cells. Since these conditions (air minus CO_2) could facilitate the loss of chloroplast metabolites in Chlamydomonas cells, the question arises as to the origin of the precursors for the increased RuBP levels in TRIA-treated cells.

Glycolate Levels. Chlamydomonas cells excrete glycolate, a product of the oxygenase activity of RuBP carboxylase (26, 27). This probably reflects an inability to metabolize glycolate, because of inadequate glycolate dehydrogenase activity (25). Glycolate formation and excretion may reflect a loss of photosynthetic carbon metabolites from

Figure 3. Photosynthetic CO_2 Assimilation and the Levels of RuBP in Control (TAS, 10 ug/L) and TRIA-Treated (1 mg/L) Chlamydomonas Cells. Chlamydomonas cells cultured at high CO_2 for 3 d were suspended in 50 mM HEPES-KOH (pH 7.5) buffer and gassed for 15 min with N_2 (50 ml/min) in the light (1000 umol/s·m^2) prior to the initiation of the experiment. At zero time and at 1 or 2 min intervals samples were removed for determination of RuBP (✱) levels. Three min after zero time 1 mM $KHCO_3$ was added and the N_2 gassing stopped. Photosynthetic CO_2 assimilation (□) by another portion was determined with 1 mM $KH^{14}CO_3$ under identical conditions begining with the addition of $KH^{14}CO_3$. 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 $^{14}CO_2$ fixation is significant at the 1% level. Each observation is the mean of 2 experiments with duplicate determinations.

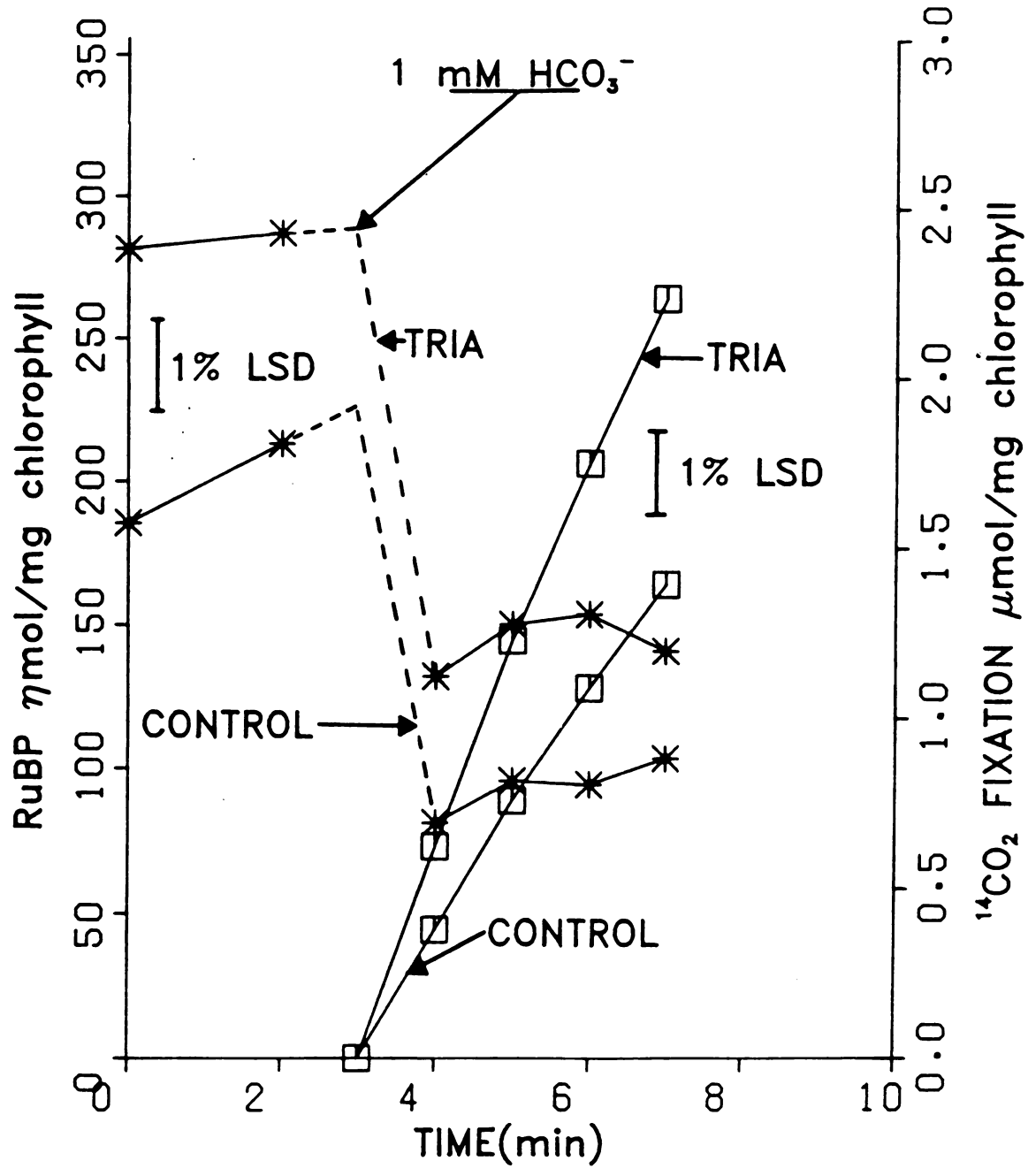
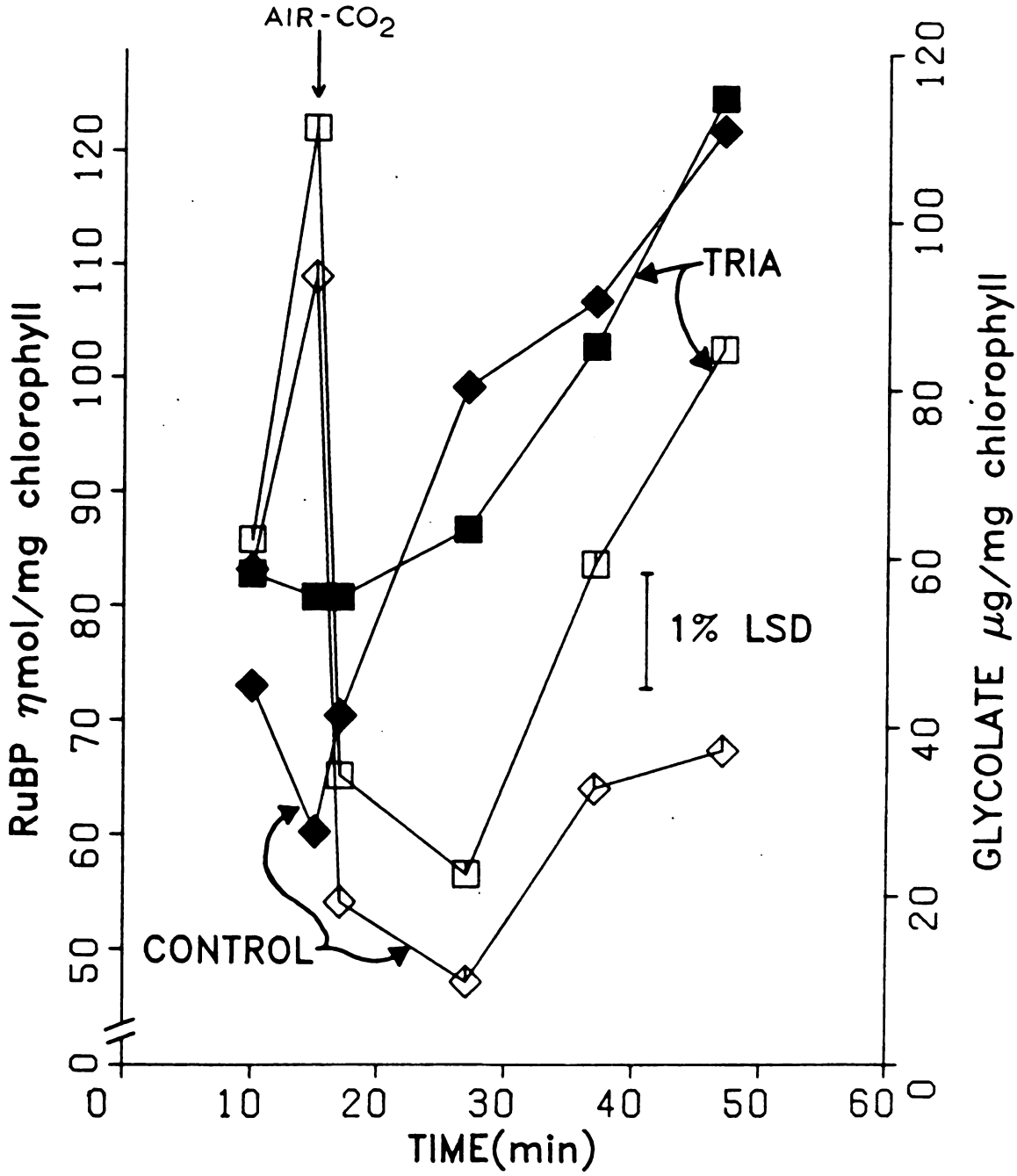

1 mM HCO_3^-
TRIA
1% LSD
CONTROL
TRIA
1% LSD
CONTROL
RuBP ηmol/mg chlorophyll
$^{14}CO_2$ FIXATION μmol/mg chlorophyll
TIME(min)
0
50
100
150
200
250
300
350
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
2
4
6
8
10

Figure 4. RuBP Levels and Glycolate Excretion by Control (TAS, 10 ug/L) and TRIA-Treated (1 mg/L) Chlamydomonas Cells. Initial conditions were the same as described for Figure 3. After gassing with N_2 for 15 min, control (◇) and TRIA (□) treated cells were gassed with CO_2-free air. RuBP (open symbols) and glycolate (closed symbols) determinations were as described in Materials and Methods. Photosynthetic CO_2 assimilation was measured under identical conditions, except 1 mM $KH^{14}CO_3$ was added instead of CO_2-free air after termination of N_2 aeration. The F ratio for the interaction of TRIA with cubic time for RuBP levels is significant at the 1% level. There were no significant differences, in glycolate levels. Each observation is the mean of 2 experiments with duplicate determinations.


AIR - CO_2
TRIA
CONTROL
1% LSD
RuBP ηmol/mg chlorophyll
GLYCOLATE μg/mg chlorophyll
TIME(min)
0
50
60
70
80
90
100
110
120
0
20
40
60
80
100
120
0
10
20
30
40
50
60

Chlamydomonas chloroplasts. Absolute levels of excreted glycolate were not altered in Chlamydomonas cells treated with TRIA (Fig. 4). However, the levels of glycolate should be interpreted with caution because they were low, and the initial determinations (10, 15, 17 min) were at or near the detection limit of the assay. These low levels of glycolate are not unusual considering the initial absence of O_2. The levels of glycolate determined 12, 22, and 32 min after addition of O_2 are more reliable and may reflect increased glycolate synthesis by TRIA-treated cells (2.6 vs 1.5 ug glycolate/min·mg chlorophyll). This would be expected since the rate of photosynthetic CO_2 assimilation in these same cells was increased by TRIA (96.1 vs 60.5 umol CO_2/h·mg chlorophyll, at 10 mM $KHCO_3$) and TRIA does not appear to affect the photorespiratory metabolism of glycolate (Table I) (14).

DISCUSSION

Treatment of Chlamydomonas cells with TRIA resulted in significant increases in photosynthetic CO_2 assimilation (14). Previously it was reported that photosynthetic CO_2 assimilation by TRIA-treated Chlamydomonas cells exhibited reduced sensitivity to inhibition by O_2 (10). This could explain the increased photosynthetic CO_2 assimilation by cells treated with TRIA. However, in this study reduced sensitivity of photosynthetic CO_2 assimilation to O_2, or a lower CO_2 compensation point by TRIA-treated Chlamydomonas cells was not detected. Kinetic analysis of photosynthetic CO_2 assimilation by TRIA-treated Chlamydomonas cells supports these observations since the whole cell apparent $Km(CO_2)$ was not affected. A low apparent $Km(CO_2)$ for

Chlamydomonas cells is associated with the ability to actively accumulate internal inorganic carbon (HCO_3^- + CO_2) (2). Since TRIA-treated Chlamydomonas cells do not show significant decreases in the apparent Km(CO_2) over control cells, the inorganic carbon accumulation process is probably not involved in the response of Chlamydomonas cells to TRIA. When TRIA-treated Chlamydomonas cells are placed in an atmosphere low in CO_2 (air) the increase in photosynthetic CO_2 assimilation over control cells is lost within 6 h, a period of time similar to that for induction of the inorganic carbon accumulation mechanism (14). This suggests that there may be other changes in Chlamydomonas cells during the development of the inorganic carbon accumulation mechanism, in addition to their aquiring the ability to accumulate inorganic carbon. TRIA may have affected one of these processes.

The presence of the C_i accumulation mechanism alone does not necessarily correlate with increased photosynthetic CO_2 assimilation (2). The activity of RuBP carboxylase assayed with activiting and non activating conditions was higher in extracts from TRIA-treated Chlamydomonas cells. This increase was not a result of increased enzyme levels, but was due to an increase in the specific activity of the enzyme. Variations in the specific activity of RuBP carboxylase in plant species has been postulated to be associated with the presence or absence of a CO_2 concentrating mechanism (5, 24). The value reported for Chlamydomonas cells (24) cultured at low CO_2 (air) (6.7 $\pm$ 0.18 umol CO_2/min·mg enzyme) is substantially higher than the values determined here for Chlamydomonas cells cultured at 5% CO_2 (Table V). These results suggest that during the induction of the inorganic carbon

accumulation mechanism in Chlamydomonas cells there may be changes in the specific activity of RuBP carboxylase.

The loss in the TRIA stimulation of photosynthetic CO_2 stimulation with high CO_2 cultured Chlamydomonas cells upon transfer to low CO_2 (14) could be due to an increase in the specific activity of RuBP carboxylase in control cells. The increased specific activity of RuBP carboxylase in TRIA-treated Chlamydomonas cells may be due to differences in inactivation of the enzyme during extraction and assay. Although proteolytic digestion of RuBP carboxylase decreases catalytic activity, it does not necessarily decrease the binding of CABP and hence determinations of the level of active RuBP carboxylase (22). Substantial variations in the specific activity of RuBP carboxylase could be a result of differing proteolytic activity in crude extracts of plants. If TRIA treatment reduces proteolytic activity in Chlamydomonas cells this could account for the increased specific activity of RuBP carboxylase observed in these cells. However, TRIA has not been reported to affect the activity of proteolytic enzymes.

Changes in the levels of chloroplast metabolites, many of which can affect RuBP carboxylase activity (17), may also influence the specific activity of the enzyme from Chlamydomonas by maintaining the enzyme in the active ternary complex during extraction. The extract from TRIA-treated Chlamydomonas cells did not influence the RuBP carboxylase activity in extracts from control cells in an anomalous manner. Thus, there does not appear to be a positive effector of RuBP carboxylase activity in the extract from TRIA-treated Chlamydomonas cells. RuBP will also stabilize the RuBP carboxylase enzyme in an active state under conditions of low CO_2. Since the level of RuBP in Chlamydomonas cells was increased with TRIA, regardless of the CO_2

concentration, perhaps these increased levels of RuBP resulted in a higher specific activity of extractable RuBP carboxylase.

Based on a current model of photosynthesis (7, 8) the limiting steps in photosynthesis may vary between either RuBP carboxylase activity or electron-transport capacity (hence RuBP levels), and the level of RuBP should reflect the rate-limiting step. For Chlamydomonas cells cultured at 5% CO_2 the in situ rate of photosynthetic CO_2 assimilation should be limited by the RuBP regeneration rate. TRIA-treated Chlamydomonas cells assayed for photosynthetic CO_2 assimilation at saturating levels of CO_2 show 40% to 60% higher levels of RuBP. Therefore, the increased growth of Chlamydomonas cells cultured at 5% CO_2 (14) may be due to increased levels of RuBP. At less than saturating levels of CO_2, photosynthetic CO_2 assimilation is limited by the activity of RuBP carboxylase and not RuBP. Under these conditions, TRIA-treated Chlamydomonas cells would still show increased photosynthetic CO_2 assimilation, since the specific activity of RuBP carboxylase is higher than that in control cells. However, RuBP levels were also significantly increased in TRIA-treated Chlamydomonas cells at less than saturating levels of CO_2 where the level of RuBP was in considerable excess of the active site concentration of RuBP-carboxylase. This percent increase in RuBP levels was equal or similar to the percent increase in photosynthetic CO_2 assimilation with TRIA-treatment. Rather than discount this observation as coincidence, a search for similar observations by other researchers showed that RuBP levels may influence the rate of photosynthetic CO_2 assimilation even when in excess of the RuBP carboxylase active site concentration (3). Observations on the levels of RuBP and photosynthetic CO_2 assimilation

in bean (Phaseolus vulgaris L.) leaves, suggested that RuBP limitation of photosynthetic CO_2 assimilation can occur at RuBP levels that are in considerable excess of RuBP carboxylase active site concentrations.

The increase in the specific activity of RuBP carboxylase and level of RuBP in TRIA-treated Chlamydomonas cells can explain the increase in photosynthetic CO_2 assimilation of intact cells over a large range of CO_2 levels. TRIA treatment may affect photosynthetic electron transport in Chlamydomonas cells, since the rate of RuBP synthesis is dependent on this process. However, the increase in RuBP levels in TRIA-treated Chlamydomonas cells in the absence of CO_2 also suggests that the level of chloroplast metabolites available for RuBP synthesis may also be increased in these cells. TRIA-treated Chlamydomonas cells may have an increased ability to mobilize carbon reserves within the chloroplast for synthesis of RuBP. In rice (Oryza sativa L.) and soybean (Glycine max L.) leaves TRIA increased the activity of starch phosphorylase and decreased levels of starch (13).

If TRIA causes changes in higher plants similar to those described here for Chlamydomonas, these observations may explain the increase in dry weight associated with TRIA treatment. However, photosynthetic CO_2 assimilation by higher plants is controlled by some mechanisms not present in algae, such as stomatal conductance. Therefore, changes in RuBP levels and specific activity of RuBP carboxylase similar to those observed in Chlamydomonas cells, may not lead to increased photosynthetic CO_2 assimilation in higher plants treated with TRIA. Furthermore, the increase in dry weight by higher plants treated with TRIA is not entirely light dependent (23). Our results with Chlamydomonas cells treated with TRIA may not be the same as the effects of TRIA on higher plants, but Chlamydomonas cells proved

to be a valuable tool for studying the biological effects of TRIA on plants.

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SUMMARY

Growth and photosynthetic CO_2 assimilation by Chlamydomonas reinhardtii cells is a suitable bioassay for TRIA activity. The rapidity of the increase in CO_2 assimilation makes this parameter a better indicator for the TRIA response than increases in cell density. The report by other researchers that TRIA affected the sensitivity of photosynthetic CO_2 assimilation to O_2 in Chlamydomonas cells (14), was not supported in these studies. Apparently, the enhancement of photosynthetic CO_2 assimilation by TRIA-treated Chlamydomonas cells is due to increased RuBP concentrations and an increase in the specific activity of RuBP-carboxylase.

The type of TRIA formulation is an important consideration when conducting research with TRIA (18, 22). The most recent and effective formulation of TRIA (18) is not stable in the presence of K^+ and Ca^{++}. It is possible that this instability may limit the effectiveness of TRIA in situations where contaminating cations are present in the water used for preparation of TRIA. The cation-induced flocculation of colloidally dispersed TRIA by K^+ and Ca^{++} is similar to the flocculation of clay particles by cations of the Hofmeister series. Both involve neutralization of negative charges on colloidal surfaces. In addition to K^+ and Ca^{++}, pH may also be important in determining the stability of colloidally dispersed TRIA, since H^+ is the second most

effective cation for flocculating clay colloids. Some experimental sprayers used for plant research utilize compressed CO_2 as a propellent. Under these conditions flocculation of colloidally dispersed TRIA could be facilitated by the decrease in pH caused by dissolved CO_2.

Although the research with TRIA is still young, there are several observations that lead to a reasonable hypothesis regarding the metabolic changes that take place in plants treated with TRIA. Ries and Wert (22) reported that the effect of TRIA on dry weight gain in rice plants was greater with low light intensities (measured as % increase over controls). The results obtained with TRIA-treated Chlamydomonas cells offers an explanation for this observation. The synthesis of RuBP is dependent on electron transport and subsequent ATP synthesis, both of which are reduced at low light intensities. When RuBP levels are low, photosynthetic CO_2 assimilation is RuBP-limited and this forms the basis of the light saturation curve for plant photosynthetic CO_2 assimilation (3, 8, 9). Therefore, increases in RuBP levels by TRIA treatment would be expected to have a larger effect on photosynthetic CO_2 assimilation at low light intensities than at high intensities where RuBP-levels can be saturating. Increased RuBP levels in Chlamydomonas cells treated with TRIA may also be related to previous observations obtained with whole plants on P_i uptake and carbohydrate levels. Apparently, TRIA treatment can result in increased P_i uptake by roots (16, 20, 21, 26) and reduced levels of starch (17) in the leaves of several higher plant species. An increase in the activity of starch phosphorylase with TRIA treatment has also been shown (16). Starch synthesis and degradation in chloroplasts is

controlled by the ratio of [PGA]/[P_i] which in turn is dependent upon the activity of the phosphate translocator present on the inner membrane (27). Manipulation of cytoplasmic P_i levels by mannose feeding has shown that the concentration of P_i in the cytoplasm may be as important in controlling the rate of photosynthetic CO_2 assimilation, as P_i is at determining the proportion of carbon which enters starch (2, 15). Increases in cytoplasmic P_i levels in response to TRIA treatment, could lead to increased photosynthetic CO_2 assimilation, decreased starch synthesis, and increased RuBP levels. Recently, it was demonstrated that TRIA can alter both the physical and biochemical properties of vesicles prepared from barley (Hordeum vulgare L.) root plasma membranes (19). Perhaps TRIA stimulates the activity of the mechanism responsible for P_i uptake at the plasma membrane. The exchange of triose phosphates from the chloroplast stroma for cytoplasmic P_i is increased with increasing levels of P_i in the cytoplasm (27). Since sucrose synthesis is dependent on triose phosphates from the chloroplast perhaps the level of sucrose is also increased in plants treated with TRIA. Increased levels of sucrose may stimulate the growth of sinks such as newly expanding leaves. The increase in dry weight gain by rice plants treated with TRIA occurs primarily through increased growth of the newly expanding leaves (1, 23, 24, 25). Inorganic phosphate plays a regulatory role in the response of plants to TRIA both in the laboratory and field (unpublished observations). The primary effect of TRIA on plant metabolism may take place through changes in the availability or transport of P_i at a cellular level.

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