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Effect of Triacontanol on *Chlamydomonas*[1]

I. STIMULATION OF GROWTH AND PHOTOSYNTHETIC CO_2 ASSIMILATION

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ABSTRACT

Treatment of *Chlamydomonas reinhardtii* cells, cultured at 5% CO_2, with 1 to 1000 micrograms triacontanol (TRIA) per liter resulted in 21 to 35% increases in cell density, 7 to 31% increases in total chlorophyll, and 20 to 100% increases in photosynthetic CO_2 assimilation. The increase in CO_2 fixation 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 one order of magnitude above the optimum concentration established for higher plants. The necessity for larger concentrations of TRIA may be due to destabilizing effects of Ca^{2+} and K^+ present in the *Chlamydomonas* growth medium. These ions caused flocculation of the colloidally dispersed TRIA in apparent competition with binding of [^{14}C]TRIA to *Chlamydomonas* cells. 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 after treatment up to 3 days. *Chlamydomonas* cells that had been grown at low-CO_2 (air) did not respond to TRIA, and transfer of high-CO_2 (5%) grown 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 CO_2 is probably the species of inorganic carbon utilized by control and TRIA-treated *Chlamydomonas* cells.

Triacontanol is a 30-carbon, straight-chain primary alcohol, possessing plant growth regulator properties discovered in 1977 (18). The effects of TRIA[3] on plant growth, development, and metabolism have recently been summarized (16). 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, 16, 19). Proper procedures for formulation and application of TRIA are now available (14, 19).

The most profound effect of TRIA on plants is an increase in dry weight (10, 13, 14, 17). 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 ribulose-P_2 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). The maximum per cent increase in the dry weight of plants caused by TRIA treatment is also approximately 15 to 30% (16, and references therein). Therefore, it was postulated that TRIA may alleviate O_2 inhibition of photosynthetic CO_2 assimilation. However, an increase in photosynthetic CO_2 assimilation in tomato or *Chlamydomonas* in response to TRIA treatment was not observed (7, 11). Our investigations with *Chlamydomonas* showed that TRIA stimulated photosynthetic CO_2 assimilation, and increased cell growth. Increased cell growth in response to TRIA treatment, 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 medium (20) or in 0.25 L Erlenmeyer flasks with 0.1 L of growth medium. The cultures were continuously mixed with an Eberbach 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 μmol/s·m^2, and the temperature was maintained between 21° and 23°C with fans to move room air over the flasks. Cell density was determined with a hemacytometer after suspending the cells in 5% glycerol. Starting cell density after inoculation was typically 100 to 500 cells/μl and the density reached approximately 1×10^4 cells/μl after 3 d of growth. Algae growth with these conditions was sigmoidal. After a 6-h lag, cell density increased logarithmically for about 48 to 60 h and then slowed to a stationary phase of growth. Chl was determined by the method of Arnon (1). TRIA was applied as a sterile aqueous colloidal dispersion (800 to 1000 μg/ml stock concentration) containing TAS as the dispersive agent, present at 1% the level of TRIA (14). TAS alone or distilled H_2O 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 1000*g*, washed once with an equal volume of 50 mM Hepes-KOH buffer (pH 7.5), suspended in the same buffer to give a Chl concentration of 10 to 50 μg/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 × 5 cm flat-bottom glass

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[3] Abbreviations: TRIA, triacontanol; TAS, sodium tallow alkyl sulfate; C_i, inorganic carbon (HCO_3^- and CO_2).

vials; these 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 $\mu mol/s \cdot m^2$ of PAR at the top of the cell suspension.

After addition of 1 to 10 mM $KH^{14}CO_3$ (0.14–0.54 $\mu Ci/umol$) to the cell suspensions, photosynthetic CO_2 assimilation was initiated with light. Aliquots (0.1–0.5 ml) of the cell suspensions were removed at various intervals and mixed with an equal volume of 2 N HCl. These samples were evaporated to dryness at 85°C, and after cooling, 0.5 ml H_2O and 4.5 ml of scintillation cocktail (2 L toluene, 1 L Triton X-100, 12 g 1,4-bis[2-(5-phenyloxazolyl)]benzene, 0.15 g 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene) 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 with 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-μl plastic microfuge tubes in the light (100 $\mu mol/s \cdot m^2$) at 25°C. The tubes contained from bottom to top 20 μl of 1 M glycine, 65 μl silicone oil (1:1, v/v, Wacker AR 20 and AR 200), and 250 μl 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, 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 Millipore AP 25 filter with an 8-μm pore size. Aliquots of $CaCl_2$ or KCl solutions (0.1–1.0 M) were added to colloidally dispersed [^{14}C]TRIA (450 μl), and after incubation at 25°C for 4 min, the dispersion was filtered and samples (150 μl) 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) removed with time 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) resuspended in 200 μl 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 (20).

Chemicals. Octacosanol ($CH_3[CH_2]_{26}CH_2OH$), TRIA, TAS, and [15,16-^{14}C]TRIA (23.2 $\mu Ci/\mu mol$) were from the Proctor and Gamble Co. (Miami Valley Laboratories, Cincinnati, OH). The octacosanol and TRIA were provided as aqueous colloidal dispersions (800–1000 μg/ml) with approximately 3×10^{11} particles/ml. $NaH^{14}CO_3$ (40–60 $\mu Ci/\mu mol$) 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 valiance, was tested in each investigation with an F-ratio. When appropriate, an F-ratio was also determined for treatment variance with trend analysis or nonorthogonal comparisons. In some tests, treatment means were compared with an LSD value.

RESULTS

TRIA Stimulation. *Chlamydomonas* cells tested with TRIA showed increases in Chl (7%), and photosynthetic CO_2 assimilation (41%) during the first 3 d of treatment (Table I). Aliquots of these same cells when transferred and cultured for an additional 3 d, without additional treatment, showed 31% more Chl and a 38% increase in photosynthetic CO_2 assimilation compared

Table I. *Effect of TRIA on Cell Density, Chl, and Photosynthetic CO_2 Assimilation by High-CO_2 Grown Chlamydomonas Cells.*

For the first cultures the total growth media for control and TRIA (100 μg/L) treated cultures were inoculated with *Chlamydomonas* cells at approximately 575 cells/μl and 1.0- to 0.1-L aliquots of inoculated media were added to 3-L Fernbach or 0.25-L Erlenmeyer flasks, respectively. The results are the average of seven experiments in which TAS (10 μg/L) or distilled H_2O were used as controls. 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 $KH^{14}CO_3$. The second cultures were started from aliquots of the 3-d-old control (0.5 ml) and TRIA (0.4 ml) treated cultures. These were equal in total Chl and were transferred to fresh growth medium (100 ml) without TRIA and grown for 3 additional d. Each observation is the mean of three experiments with duplicate determinations.

Treatment	Cell Density	Chl		Photosynthetic CO_2 Assimilation
	cells/μl × 10^4	*μg/ml*	*pg/cell*	*$\mu mol/h \cdot mg$ Chl*
First culture (1–3 d)				
Control	1.13	10.0	0.88	51.5
TRIA	1.37	10.7*	0.78	72.8**
Second culture (3–6 d)				
Control	1.20	10.5	0.88	40.5
TRIA	1.62*	13.8*	0.85	55.8*

*, F-ratio for comparison of treatments is significant at 5% level. **, F-ratio for comparison of treatments is significant at 1% level.

to untreated cells. An increase in cell density was not apparent during the first culture, but was evident during the second culture (Table I). The absence of an increase in cell density with TRIA treatment during the first culture was probably due to the presence of *Chlamydomonas* cells in different states of division (diad, tetrad, octad), thus the total cell number was not always accurately determined using a hemacytometer. This resulted in a large error variance for the cell density data (coefficient of variation, 37%). The increase in cell density during the second culture, with TRIA-treated cells, might be expected since *Chlamydomonas* cells began to enter the stationary phase of growth after about 48 to 60 h, when nutrients and other factors limit the potential for further growth due to increased photosynthetic CO_2 assimilation.

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 Chl basis (Fig. 1). In newly inoculated cultures, there was a rapid increase in the rate of photosynthetic CO_2 assimilation. However, this increase was larger in TRIA-treated cells than in the control. In the majority of studies this increase 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

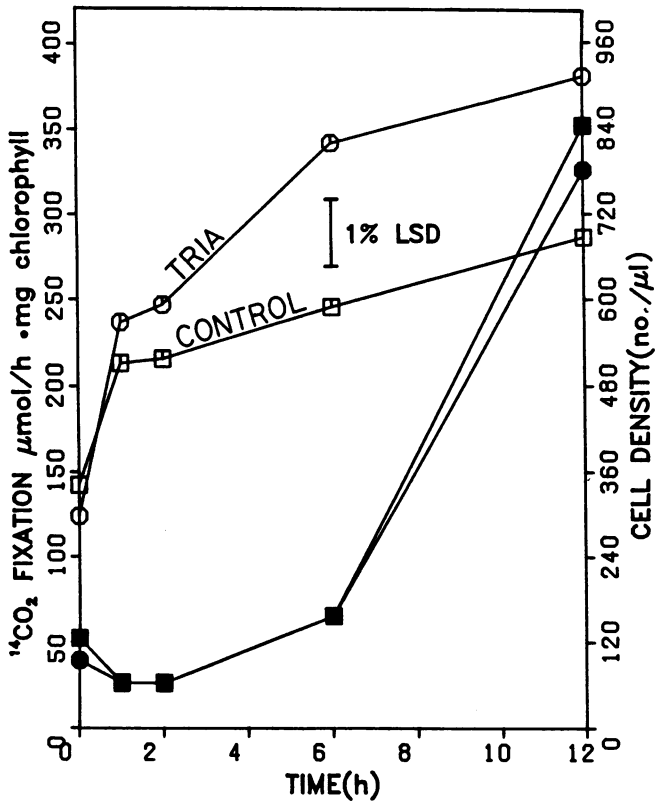

FIG. 1. Effect of TRIA on cell density and photosynthetic CO_2 assimilation in *Chlamydomonas* with time. After treatment, 100 ml samples were removed for determination of cell density (■, ●), and photosynthetic CO_2 assimilation with 10 mM $KH^{14}CO_3$ (□, ○) with time in control (TAS, 1 μ/L) (□, ■) and TRIA (100 μg/L) (○, ●) treated 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.

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

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

Treatment		Photosynthetic CO_2 Assimilation
TRIA	TAS	
μg/L		μmol/h·mg Chl
0	10	31.9[a]
1	0.01	46.0
10	0.10	48.8
100	1	48.5
1000	10	52.4
5% LSD		14.8
1% LSD		20.3

[a] The F-ratio for comparison of the control with all treatments was significant at the 1% level, and there was no statistical difference between concentrations.

Table III. *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 two experiments with duplicate determinations. There were no significant differences between treatments.

Treatment		Photosynthetic CO_2 Assimilation
TRIA	TAS	
μg/L		μmol/h·mg Chl
0	10	61.6
1	0.01	59.8
10	0.10	61.6
100	1	64.3
1000	10	53.0

leveled off at 10 μg/L without further significant increases in photosynthetic CO_2 assimilation (Table II). The analysis of variance showed that 92% of the total variance was due to the single degree of freedom for the nonorthogonal comparison of control *versus* all TRIA treatments, demonstrating that *Chlamydomonas* cells became saturated with low levels of TRIA (10 μl/L) with respect to stimulation of photosynthetic CO_2 assimilation. The lowest concentration of TRIA that resulted in a significant increase in photosynthetic CO_2 assimilation (10 μ/L), was two orders of magnitude above the optimum dose of TRIA established for higher plants (14). The necessity for this relatively large concentration of TRIA led to investigations into 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 lowered two orders of magnitude when the particle size of the TRIA formulation was decreased. The colloidal dispersion of TRIA used in our 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 0.1–0.8 μm) 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

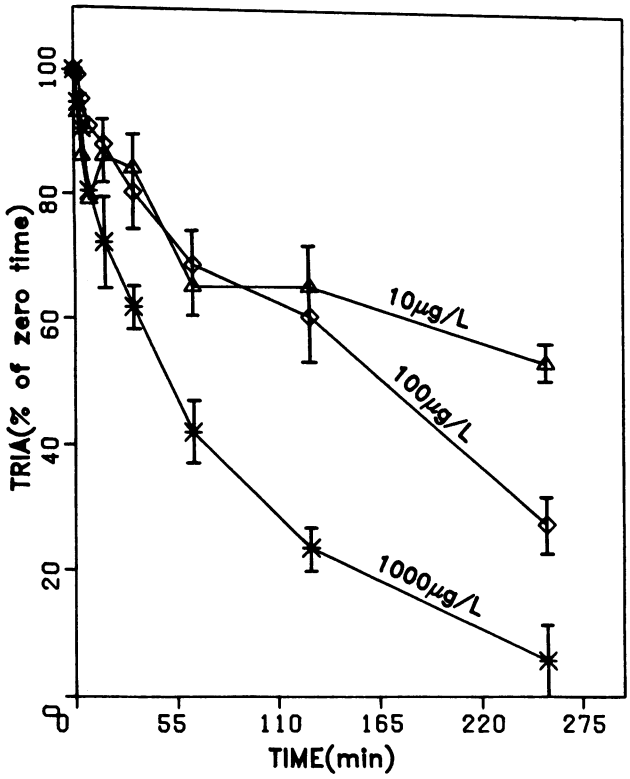

FIG. 2. Effect of *Chlamydomonas* growth medium on the particle size of colloidally dispersed [^{14}C]TRIA. [^{14}C]TRIA was added to 20 ml of *Chlamydomonas* growth medium and samples (1.2 ml) removed after different times for estimation of flocculated TRIA by failure to pass through an 8-μm filter. Each observation is the mean of duplicate determinations ±SE.

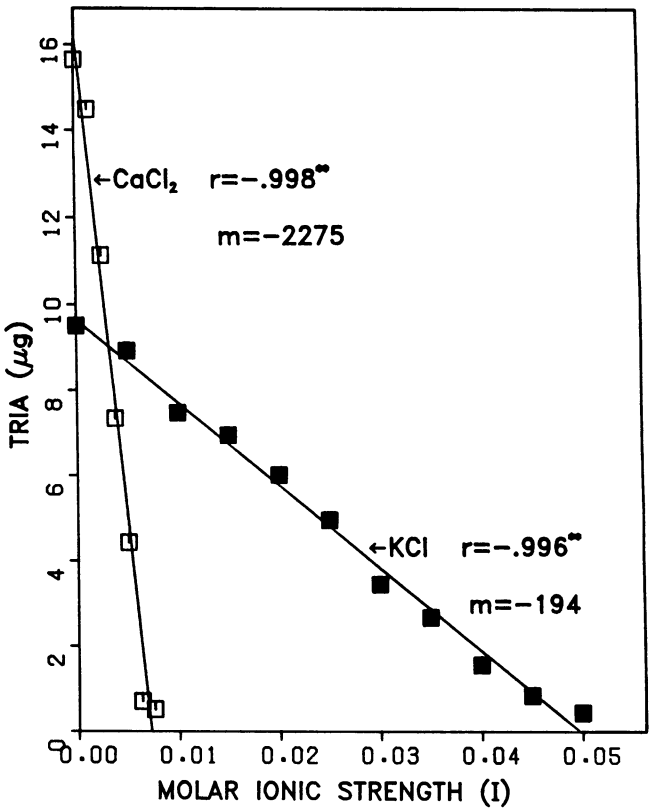

FIG. 3. Ca^{2+} and K^+ induced flocculation of colloidally dispersed [^{14}C]TRIA. Aliquots (0.5 ml) of the TRIA dispersion (210 μ/ml for Ca^{2+} and 180 μg/ml for K^+) were incubated with $CaCl_2$ (□) or KCl (■) at 25°C for 4 min, filtered, and the [^{14}C]TRIA remaining in the filtrate determined. Each observation is the mean of three determinations. The range in the SE measurements was 0.001 to 0.140. The r-values from linear regression analysis for the loss of TRIA in the filtrate with increasing ionic strength are significant at the 1% level. The slopes (m, μg TRIA/L) of the two lines are shown for comparison of the effectiveness of the two cations.

when treated with flocculated TRIA (Table III). Therefore, it was postulated that the necessity for large concentrations of TRIA, and the ineffectiveness of the concentrations of TRIA greater than 10 μg/L at eliciting further significant increases in photosynthetic CO_2 assimilation, was 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-μm filter, decreased with time in a logarithmic manner after addition of 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 μm in diameter after 165 and 50 min with 100 and 1000 μg/L TRIA, respectively. With the lower concentration of TRIA (10 μg/L), the percentage of TRIA present as particles larger than 8 μm 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 III). Since particle size was not quantitated in these experiments, it is possible that the TRIA remaining as particles less than 8 μm in diameter (Fig. 2) were still considerably larger than the initial particle size and are inactive. K^+ and Ca^{2+} 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^{2+}, there was a rapid increase in the particle size (Fig. 3). The slopes of the two regression lines indicated that Ca^{2+} ions were approximately 12 times more effective at initiating this flocculation than K^+ ions. The level of these two cations in the growth medium (0.2 mM Ca^{2+} and 29.4 mM K^+) are within the range of Ca^{2+} 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 collodially dispersed TRIA, and flocculated TRIA failed 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 were treated with [^{14}C]TRIA under conditions favorable for flocculation (*i.e.* when in the growth medium) TRIA particles became bound to the cells (Fig. 4). The binding of TRIA particles to *Chlamydomonas* cells reached saturation in about 20 min 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}] 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 Figure 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),

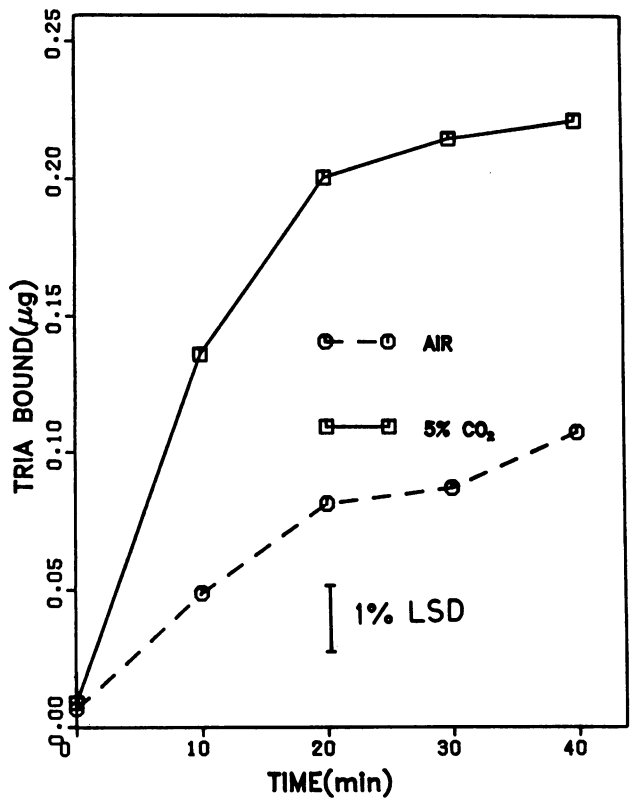

FIG. 4. Binding of [^{14}C]TRIA to *Chlamydomonas* cells. [^{14}C]TRIA (66.7 μg/ml) was added to cells suspended in growth medium (2.9 × 10^4 cells/μl) and incubated at 25°C for various periods of time. The incubations were terminated by centrifugation through a silicone oil layer, and the bound [^{14}C]TRIA measured. 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 duplicate determinations ±SE.

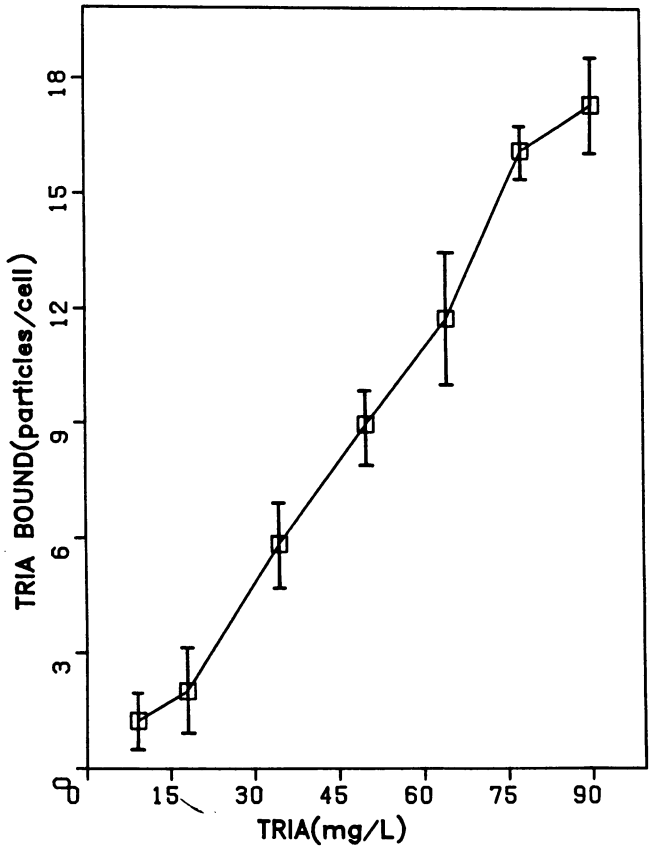

FIG. 5. Effect of TRIA concentration on the number of TRIA particles bound to *Chlamydomonas* cells. Cell suspensions (3.0 × 10^4 cells/μl) were incubated with [^{14}C]TRIA for 30 min and then centrifuged through silicone oil to measure bound [^{14}C]TRIA. The number of TRIA particles bound per cell was calculated from the total cell number and the particle density of the [^{14}C]TRIA dispersion. Blanks consisted of [^{14}C] TRIA added to growth medium that did not contain *Chlamydomonas* cells. Each observation is the mean of duplicate determinations ±SE.

since flocculated TRIA did not migrate through the silicone oil layer. This technique was adapted for small volumes of cell suspensions, but because the specific activity of the [^{14}C]TRIA was low, higher concentrations of TRIA had to be used than 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 used was not a solution.

Effect of Culture Age on Photosynthesis 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 μg/L) for 1 d, photosynthetic CO_2 assimilation increased 13%; by 3 d it had increased to approximately 100%. The decrease in CO_2 assimilation is probably due to limited nutrients and light availability in older algal cultures.

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 TRIA-treated *Chlamydomonas* cells (Table IV). Octacosanol alone had no effect on photosynthetic CO_2 assimilation; therefore, the effect of TRIA on this process appears to be 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 is presented in Table V. *Chlamydomonas* cells grown on high-CO_2 excrete a percentage of photosynthetically fixed $^{14}CO_2$ as glycolate under nonsaturating levels of CO_2 (20). Since TRIA has been shown to effect plasma membrane function and integrity in isolated barley root vesicles (A. P. Lesniak, S. K. Ries 1984 An increase in plasma membrane associated ATPase activity in barley after triacontanol treatment. J. Am Soc Hort Sci 19: 580), it was postulated that TRIA alters 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. TRIA-treated *Chlamydomonas* cells had increased ^{14}C in all of these fractions up to 1 h after addition of $KH^{14}CO_3$ due to the increased rate of photosynthetic CO_2 fixation. ^{14}C distribution among the soluble products was also examined by 2-dimensional paper chromatography and autoradiography, but there was no apparent 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 C_i within the cells. This mechanism is induced when cells are transferred from high-CO_2 to low-CO_2 (5). Only

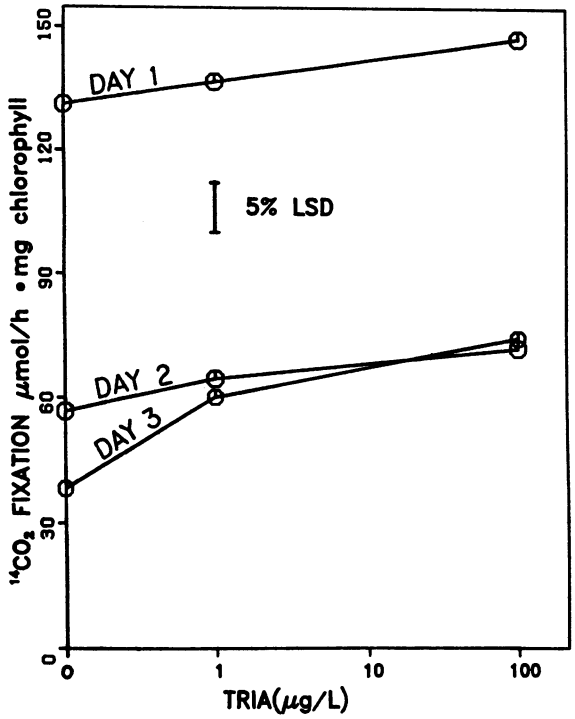

FIG. 6. Effect of TRIA (1 of 100 μ/L) on photosynthetic CO_2 assimilation by *Chlamydomonas* as affected by culture age. After inoculation and treatment, 100-ml samples were removed after 1, 2, and 3 d of growth for determination of photosynthetic CO_2 assimilation (10 mM $KH^{14}CO_3$). Photosynthetic 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 is significant at the 5% level. The LSD shown is for comparison of any two data points.

Table IV. *Effect of Colloidally Dispersed Octacosanol (100 μg/L) on the Increase in Photosynthetic CO_2 Assimilation by TRIA (100 μg/L) Treated Chlamydomonas Cells*

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

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

Chlamydomonas cells that had been cultured with high-CO_2 showed an increase in photosynthetic CO_2 assimilation in response to treatment with TRIA (Table VI). *Chlamydomonas* cultures grown with low-CO_2 (air) had a higher rate of CO_2 fixation with low-CO_2 (1 mM HCO_3^-) because of the C_i pump, but this rate of photosynthetic CO_2 assimilation was not stimulated by TRIA. 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 about 6 h (Fig. 7). The time course of this loss is similar to that for induction of the C_i accumulation system (5). The reason that there was no TRIA stimulation of CO_2 fixation by air-grown *Chlamydomonas* is unknown. Decreased binding of TRIA to cells cultured with air has already been mentioned. Because of the higher rate of CO_2 fixation by the algae with the C_i pump, other factors limiting CO_2 fixation (perhaps stimulated by TRIA) might not be apparent. The data also indicate that TRIA treatment may bear some relationship to the mechanism for accumulating C_i normally associated with cells grown at low-CO_2.

CO_2 is considered to be the species of C_i that diffuses across the plasma membrane of *Chlamydomonas* cells grown at low- or high-CO_2 (15). 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 near pH 8.0 (3, 15). 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 inhibited in both control and TRIA-treated *Chlamydomonas* cells. Though not statistically significant at the higher pH, because of low CO_2 fixation rates, the per cent stimulation due to TRIA was nearly the same.

DISCUSSION

Treatment of *Chlamydomonas* cultures with 1 to 1000 μg TRIA/L resulted in significant increases in cell density, total Chl, and photosynthetic CO_2 assimilation. The increase in Chl was dependent upon increases in cell density since there was no change in the amount of Chl 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 Chl or cell density. TRIA stimulation of photosynthetic CO_2 assimilation was most pronounced in *Chlamydomonas* cells that were entering the stationary phase of growth. The inhibition of TRIA activity by equal concentrations of octacosanol indicates that the increase in photosynthetic CO_2 assimilation was specific for TRIA. 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 a decrease in the binding affinity or absorption of TRIA. 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 which is similar to the time period for development of the C_i pump and carbonic anhydrase. The effect of pH on photosynthetic CO_2 assimilation by control and TRIA-treated *Chlamydomonas* cells was consistent with claims that CO_2 is the C_i species crossing the plasmalemma of *Chlamydomonas* cells (15).

The colloidally dispersed formulations of TRIA, which are considered the best formulations of TRIA available for experimental purposes (14, 16), 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. An increase in particle size and/or flocculation of TRIA formulations is antagonistic to the response of both higher plants and algae to TRIA and therefore, the presence of contaminating cations in the water used for preparing and diluting

Table V. *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 cultured for 2 d as indicated in Table I. Tests were conducted with additions of 1 mM $KH^{14}CO_3$ (final concentration) added at 0, 20, 40 min to maintain approximately 1 mM $KH^{14}CO_3$. Each observation is the mean of two experiments with duplicate determinations.

Time	Total Fixation[a]		^{14}C Distribution[b]					
			Excreted		Soluble		Insoluble	
	Control	TRIA	Control	TRIA	Control	TRIA	Control	TRIA
min	*μmol/mg Chl*				*% of total counts*			
10	1.9	2.2	1.3	1.4	41	40	58	58
20	3.8	4.4	3.4	4.1	34	35	63	61
30	6.5	7.8	3.9	4.6	34	34	63	62
40	9.1	10.7	4.8	5.8	31	31	64	63
50	12.3	14.6	4.7	5.9	30	30	66	64
60	14.3	18.2	5.2	5.4	30	29	65	65

[a] The rate of photosynthetic CO_2 assimilation with 10 mM $KH^{14}CO_3$ in TRIA-treated cells (133 μmol/h·mg Chl) was significantly higher (5% level) than the control rate (109 μmol/h·mg Chl). [b] The F-ratios for the comparison of controls with TRIA for the excreted, soluble, and insoluble fractions were not significant.

Table VI. *Effect of TRIA on Photosynthetic CO_2 Assimilation by Chlamydomonas Cells Grown at High- or Low-CO_2.*

Cells were treated and cultured for 3 d as in Table I. 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 two experiments with duplicate determinations.

Treatment	Photosynthetic CO_2 Assimilation[a]	
	High-CO_2 Culture	Low-CO_2 Culture
	μmol/h·mg Chl	
TAS (1 μg/L)	20.3	79.4
TRIA (100 μg/L)	30.2	78.2

[a] The F-ratio for the difference in response of the algae to TRIA applied under the different culture atmospheres was significant at the 1% level.

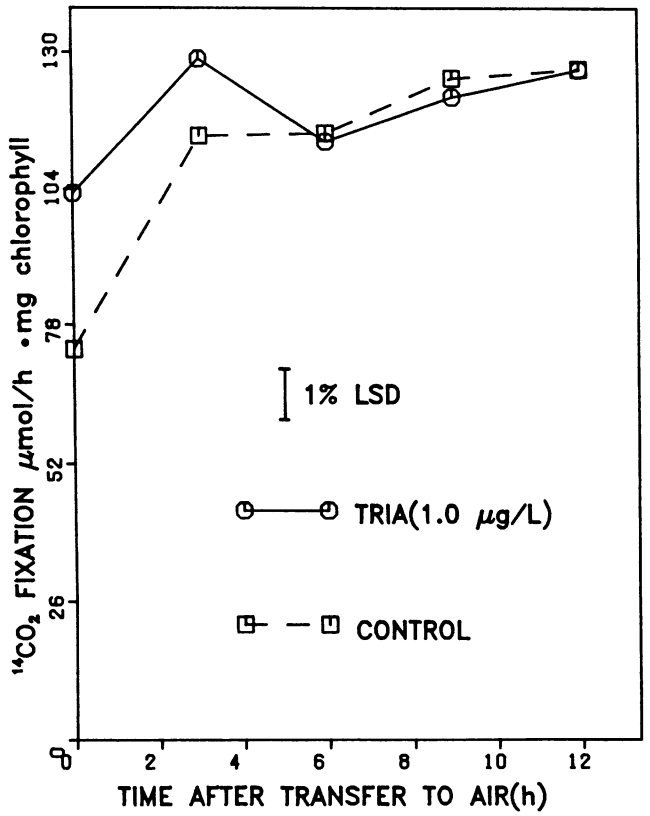

FIG. 7. Photosynthetic CO_2 assimilation in control (TAS) (0.01 μg/L) and TRIA (1.0 μg/L) treated *Chlamydomonas* cells before and after transfer to low-CO_2 (air). Cultures were grown for 3 d with 5% CO_2 as in Figure 1. The cells were harvested, resuspended in fresh media, and aerated with air in the light similar to culturing on low-CO_2. Samples were removed at zero time and at 3-h intervals for determination of photosynthetic CO_2 assimilation with 1 mM $KH^{14}CO_3$. Each observation is the mean of two experiments with duplicate determinations. The F-ratio for the interaction of TRIA with linear time is significant at the 1% level. The LSD shown is for comparison of any two data points.

colloidally dispersed TRIA may be a crucial factor in achieving consistent and reproducible results. Though the TRIA dispersions were not stable in *Chlamydomonas* growth medium, *Chlamydomonas* cells apparently bind 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. Our evidence suggests 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 by the binding of TRIA to the cells. Since these two processes occur simultaneously during treatment of *Chlamydomonas* cultures with colloidally dispersed TRIA, there may be a 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 aggregated 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 their response to TRIA.

TRIA treatment may effect 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. Under conditions of high-CO_2, ribulose-P_2 levels would be expected to play an important role in determining the maximum rate of photosynthetic

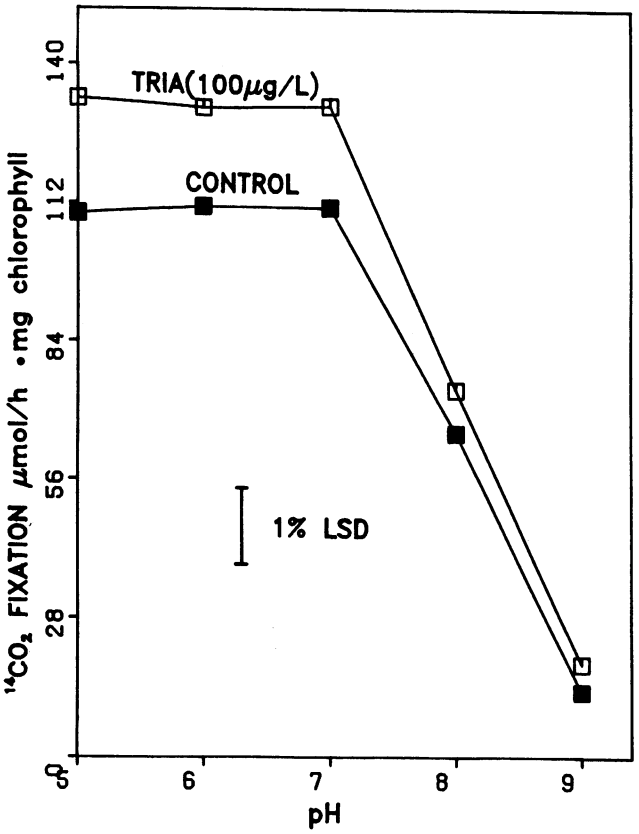

FIG. 8. Photosynthetic CO_2 assimilation in control (TAS, 1 μg/L) and TRIA (100 μg/L) treated *Chlamydomonas* cells. Cultures were inoculated and treated, and after 2-d growth with 5% CO_2, samples (500 ml) were removed for determination of photosynthetic CO_2 assimilation with 1 mM $KH^{14}CO_3$ at 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 levels is significant at the 1% level. The LSD shown is for comparison of any two data points.

CO_2 assimilation in algae and higher plants. Ribulose-P_2 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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