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Nitrate and Ammonium Induced Photosynthetic Suppression in N-Limited *Selenastrum minutum*[1]

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ABSTRACT

Nitrate-limited chemostat cultures of *Selenastrum minutum* Naeg. Collins (Chlorophyta) were used to determine the effects of nitrogen addition on photosynthesis, dark respiration, and dark carbon fixation. Addition of NO_3^- or NH_4^+ induced a transient suppression of photosynthetic carbon fixation (70 and 40% respectively). Intracellular ribulose bisphosphate levels decreased during suppression and recovered in parallel with photosynthesis. Photosynthetic oxygen evolution was decreased by N-pulsing under saturating light (650 microeinsteins per square meter per second). Under subsaturating light intensities (<165 microeinsteins per square meter per second) NH_4^+ addition resulted in O_2 consumption in the light which was alleviated by the presence of the tricarboxylic acid cycle inhibitor fluoroacetate. Addition of NO_3^- or NH_4^+ resulted in a large stimulation of dark respiration (67 and 129%, respectively) and dark carbon fixation (360 and 2080%, respectively). The duration of N-induced perturbations was dependent on the concentration of added N. Inhibition of glutamine 2-oxoglutarate aminotransferase by azaserine alleviated all these effects. It is proposed that suppression of photosynthetic carbon fixation in response to N pulsing was the result of a competition for metabolites between the Calvin cycle and nitrogen assimilation. Carbon skeletons required for nitrogen assimilation would be derived from tricarboxylic acid cycle intermediates. To maintain tricarboxylic acid cycle activity triose phosphates would be exported from the chloroplast. This would decrease the rate of ribulose bisphosphate regeneration and consequently decrease net photosynthetic carbon accumulation. Stoichiometric calculations indicate that the Calvin cycle is one source of triose phosphates for N assimilation; however, during transient N resupply the major demand for triose phosphates must be met by starch or sucrose breakdown. The effects of N-pulsing on O_2 evolution, dark respiration, and dark C-fixation are shown to be consistent with this model.

Nitrogen is an important regulator of photosynthetic carbon flow in both higher plants and algae (2, 12, 14, 15, 22, 30). In higher plants, NH_4^+ enrichment increases the flow of newly fixed carbon into TCA[2] cycle intermediates and amino acids while decreasing the flux into starch and sucrose (2, 14). Phosphoenolpyruvate carboxylase and pyruvate kinase have been identified as key enzymes in regulating this metabolic shift (14, 15, 22).

The significance of N in regulating algal carbon flow increases under N-limitation (7, 20, 27). Responses of N-limited microalgae to N enrichment are light and time dependent. In the dark, N enrichment stimulates both O_2 consumption and CO_2 uptake (19, 24). In the light, N uptake is very rapid (5, 11) and ultimately results in an increase in photosynthesis (13, 27). In many cases, however, N enrichment to N-limited microalgae or natural phytoplankton assemblages results in a temporary suppression of photosynthetic carbon fixation (4, 7, 9, 10, 13, 16, 17, 20, 25–27).

Although there are several reports of N-induced photosynthetic suppression, the phenomenon has yet to be studied in any detail. In this study chemostat cultures were used to produce steady state NO_3^--limited cells of *Selenastrum minutum* (Chlorophyta). The effects of nitrogen resupply on photosynthetic carbon fixation, O_2 evolution, dark carbon fixation, dark respiration, and RuBP concentration are reported. The observed changes enable development of a hypothesis which explains the processes of N-induced photosynthetic suppression in N-limited microalgae.

MATERIALS AND METHODS

Chemostat Culture. *Selenastrum minutum* Naeg. Collins was isolated from Lake Ontario and grown axenically in chemostat culture under NO_3^--limitation at a growth rate of 0.3 d^{-1}. Complete culture conditions were as previously described (8) with the exception that cultures were buffered at pH 8.0 with 50 mM Hepes and grown at a photon flux density of 165 $\mu E \cdot m^{-2} \cdot s^{-1}$. These conditions resulted in steady state cell densities of 0.90 μg Chl $\cdot ml^{-1}$. This concentration was used in all experiments with the exception of those associated with RuBP measurement. Under steady state conditions, the cellular growth rate in a chemostat depends upon the constant flow of a nutrient limited media (in this study, NO_3^--limited) into the growth vessel. Upon addition, this media is mixed rapidly and homogeneously throughout the culture by magnetic stirring and continuous aeration. As each drop enters the culture an equal volume is forced out of the reactor. Consequently, the culture volume remains constant and at steady state the growth rate of the cells must equal the dilution rate of the culture (28). Consequently, the lower the growth rate, the greater the degree of N-limitation. In this study cells were extremely N-limited, growing at 18% of the maximum growth rate. Nitrogen sufficient cells were obtained by growing cells at maximum growth rate (1.68 d^{-1}) in chemostat culture.

Photosynthetic Carbon Fixation. Culture samples (0.90 μg Chl $\cdot ml^{-1}$, 10 mM DIC) were incubated in the presence of $H^{14}CO_3$ (Atomic Energy Commission of Canada, 50 $\mu Ci \cdot ml^{-1}$) with or without nitrogen enrichment. Samples were withdrawn at discrete time intervals and placed in 5.0 ml scintillation vials containing 0.5 ml of stop solution (80% aqueous ethanol, 5% HCOOH), evaporated to dryness and ^{14}C determined as previ-

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[2] Abbreviations: TCA cycle, tricarboxylic acid cycle; RuBP, ribulose bisphosphate; GOGAT, glutamine 2-oxoglutarate aminotransferase (EC. 2.6.1.53); GS, glutamate synthase (EC. 6.3.1.2); DIC, dissolved inorganic carbon; μ, growth rate; Rubisco, ribulose bisphosphate carboxylase; PEP, phosphoenolpyruvate; TP, triose phosphate.

ously described (7). Incubations took place at a light intensity of 165 $\mu E \cdot m^{-2} \cdot s^{-1}$ unless otherwise indicated.

Oxygen Exchange. Photosynthetic O_2 evolution was monitored with a Clarke-type O_2 electrode (Hansatech Ltd., King's Lynn, England) at 165 $\mu E \cdot m^{-2} \cdot s^{-1}$ unless otherwise stated. Culture aliquots (1.5 ml, 0.90 μg Chl $\cdot ml^{-1}$) were transferred to the electrode cuvette and bubbled with N_2 to depress O_2 concentration. The cuvette was sealed and the rate of O_2 evolution was observed. Dark respiration was measured as O_2 consumption in the dark.

Dark Carbon Uptake. Culture aliquots (10 ml) were preincubated in capped, blackened 20 ml scintillation vials at 20°C for 5 min. $H^{14}CO_3$ and the appropriate N concentration were added and the samples incubated for 15 min. Incubations were terminated by gentle filtration (<100 mm Hg) and acid-stable ^{14}C assessed as previously described (27).

Inhibitors and Analogs. Azaserine, an inhibitor of GOGAT, was added to a concentration of 1 mM. Following preincubation for 30 min, experiments were initiated. Methylamine, ethylamine, tungstate, and chlorate were used at a concentration of 50 μM. To partially inhibit TCA cycle activity, cells were preincubated for 60 min in the dark with 10 mM fluoroacetate.

Inorganic Nitrogen Analysis. Samples were filtered through Whatman 934-AH glass fiber filters. NO_3^- content of filtrates was determined using the method of Strickland and Parsons (23) modified for flow-through sample injection. NH_4^+ was measured with an Orion 9512 NH_3 electrode.

Rubisco Extraction. Rubisco was extracted from commercially obtained spinach. Deribbed leaves (0.25 kg) were homogenized in 200 ml of sucrose isolation medium (0.35 M sucrose, 25 mM Hepes NaOH [pH 7.6], 2 mM EDTA, 2% w/v PVP) for approximately 30 s with an Ultra-Turrax. The homogenate was filtered through six layers of cheesecloth and the filtrate spun at 4,000g for 2.5 min at 4°C. The chloroplast pellet was suspended in lysis buffer (25 mM Tris HCl [pH 8.0], 10 mM $MgSO_4 \cdot 7H_2O$, 2% w/v PVPP) for 15 min and the combined lysed fractions from 1.5 kg of leaves were spun at 15,000g for 15 min at 4°C. The supernatant, termed the stromal fraction, was loaded onto a 50 ml DEAE Sephacel ion-exchange column and washed with elution buffer (10 mM Tris HCl [pH 8.0], 0.5 mM EDTA, 10 mM β-mercaptoethanol). Protein was eluted using a linear gradient from 0 to 0.5 M KCl in elution buffer. The Rubisco-enriched fractions were pooled and brought to 70% saturation with $(NH_4)_2SO_4$ at 4°C. The pellet was redissolved in 10 ml elution buffer and further purified by passage through a Sephacryl S-300 gel filtration column (2.5 by 90 cm). Fractions containing Rubisco were pooled and brought to 50% saturation with $(NH_4)_2SO_4$ at 4°C. The precipitate was frozen at −20°C until use. Purity was confirmed by PAGE.

RuBP Assay. Cells were harvested and concentrated to approximately 35 μg Chl $\cdot ml^{-1}$ by gentle centrifugation and placed in a water-jacketed cuvette (20°C) illuminated with two Kodak 600H projectors. DIC was added to a final concentration of 60 mM and cells were preadapted under these conditions for 20 min. Following preincubation, 750 μl samples were removed at regular intervals over a 70 min period, placed directly into ice-cold $HClO_4$ (10% final concentration), and immediately frozen in liquid N_2. Twenty-five min after the initiation of sampling, either 4 mM $NaNO_3$ or NH_4Cl was added. Thawed samples were neutralized with 5 M KOH and the resulting precipitate removed by centrifugation at 8000g for 5 min. Aliquots (100 μl) of the supernatant were added to serum-stoppered vials containing 405 μl assay buffer (125 mM Bicine, 25 mM $MgCl_2$, 0.25 mM EDTA, 1 mM DTT) and 20 μl of 200 mM $H^{14}CO_3$ (60 $\mu Ci \cdot nmol^{-1}$). Incubations were initiated by addition of 20 μl (8 mg $\cdot ml^{-1}$) of activated Rubisco (21) and were terminated after one h by the addition of 500 μl of kill solution (80% aqueous ethanol, 5% HCOOH). Samples were evaporated to dryness and acid-stable ^{14}C assessed by liquid scintillation. Parallel experiments were run in which the rate of photosynthetic carbon fixation and the concentrations of NO_3^- or NH_4^+ were determined.

Other Measurements. DIC, Chl, and cell numbers were determined as previously described (8).

RESULTS

N-Sufficient Cells. Nitrate sufficient cells showed no short-term photosynthetic response to NO_3^- or NH_4^+ enrichment. Furthermore, there was no effect on dark carbon fixation, dark respiration or intracellular RuBP concentration (data not shown).

N-Limited Cells. *Photosynthetic Carbon Fixation.* Under NO_3^--limitation addition of NO_3^- or NH_4^+ resulted in short-term suppression of photosynthetic carbon fixation (70 and 40%, respectively). The duration of the suppression was dependent on the concentration of added N but the magnitude of the suppression was constant (Fig. 1). Following suppression, photosynthetic rates returned to the control rate (Fig. 1).

Light intensity affected the degree of NO_3^--induced suppression. At high light intensities (>100 $\mu E \cdot m^{-2} \cdot s^{-1}$) the magnitude of suppression was between 50 and 70% but under light-limiting intensities (31 $\mu E \cdot m^{-2} \cdot s^{-1}$) carbon fixation was suppressed only 34% (Table I). In the presence of azaserine, photosynthetic carbon fixation was not affected by NO_3^- (Fig. 2) or NH_4^+ pulsing (data not shown). Nitrogen analogs (methylamine, ethylamine, tungstate, chlorate) had no effect on photosynthetic carbon fixation in N-limited cells.

Photosynthetic O_2 Evolution. Addition of NO_3^- to NO_3^--limited cultures of *S. minutum* resulted in a slight (24%) reduction of O_2 evolution (Fig. 3A; Table II). Inhibition of N assimilation by azaserine alleviated this effect (Table II). Partial inhibition of

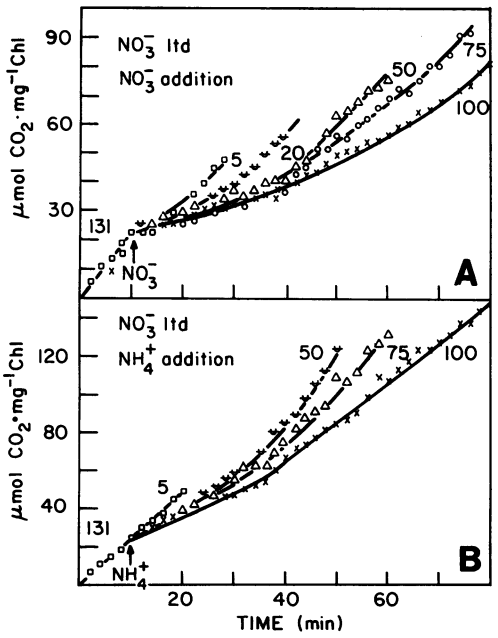

FIG. 1. Effect of N addition on short-term photosynthetic carbon fixation in NO_3^--limited cultures of *S. minutum* (μ=0.3 d^{-1}). A, NO_3^- enrichment to either 5, 20, 50, 75, or 100 μM took place at min 10. The average initial rate of photosynthesis was 131 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$. The average postsuppression rate of photosynthesis was 133 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$. B, NH_4^+ enrichment to either 5, 50, 75, or 100 μM took place at min 10. The average initial rate of photosynthesis was 131 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$. The average postsuppression rate of photosynthesis was 132 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$.

Table I. *Effects of Light Intensity on Photosynthetic Carbon Fixation for a 10 Minute Period Following NO_3^- Addition*

Light Intensity	Control Rate	Suppression + 50 μM NO_3^-
$\mu E \cdot m^{-2} \cdot s^{-1}$	$\mu mol\ CO_2 \cdot mg^{-1}\ Chl \cdot h^{-1}$	%
31	54.5	33.6
100	151.2	63.9
310	187.2	60.6
1200	187.2	49.8

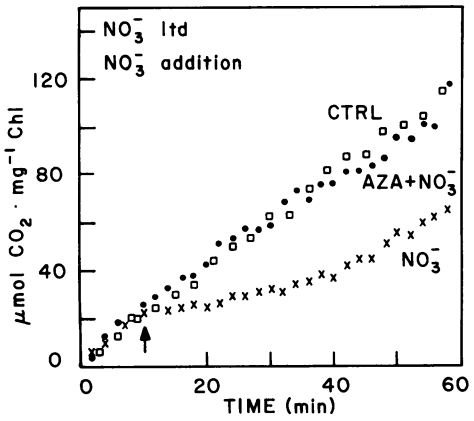

FIG. 2. Effect of NO_3^- addition (50 μM) on photosynthetic carbon accumulation in NO_3^--limited *S. minutum* in the presence or absence of azaserine. For treatments in which GOGAT was inhibited, cells were preincubated with 1 mM azaserine for 0.5 h. The control rate of photosynthesis was 134 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$. Control unenriched cells, (□); cells pulsed with NO_3^- in the presence of azaserine, (●); cells pulsed with NO_3^- in the absence of azaserine, (×).

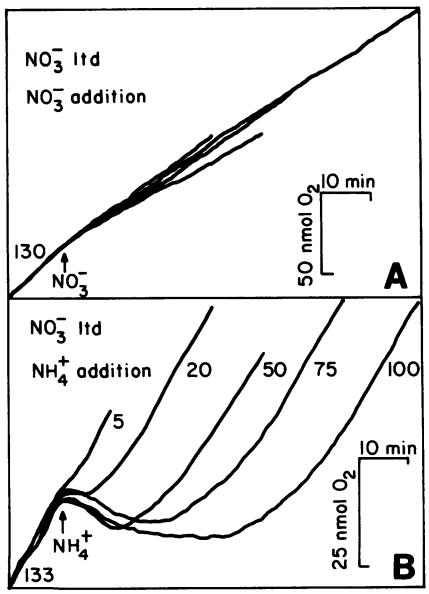

FIG. 3. Short-term responses of photosynthetic O_2 evolution to several concentrations of N in NO_3^--limited *S. minutum* (μ=0.3 d^{-1}). A, NO_3^- enrichment at 5, 20, 50, 75, and 100 μM. The average initial rate of O_2 evolution was 130 μmol $O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$. The average postsuppression rate of O_2 evolution was 122 μmol $O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$. B, NH_4^+ enrichment at 5, 20, 50, 75, and 100 μM. The average initial rate of O_2 evolution was 133 μmol $O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$. The average postsuppression rate of O_2 evolution was 124 μmol $O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$.

Table II. *Effects of Azaserine (AZA) and Fluoroacetate (FA) on Photosynthetic Oxygen Evolution for a 10 Minute Period following NO_3^- or NH_4^+ Addition (50 μM)*

Treatment	Rate	Change
	$\mu mol\ O_2 \cdot mg^{-1}\ Chl \cdot h^{-1}$	%
Control	132.3	
Control + FA (45%)[a]	121.5	
Control + AZA	144.8	
+NO_3	100.1	−24.4
+NO_3 + FA (45%)[a]	190.4	+56.7
+NO_3 + AZA	111.2	−23.2
+NH_4	−46.9	−135.5
+NH_4 + FA (46%)[a]	13.6	−88.8
+NH_4 + AZA	102.7	−29.1

[a] The percent inhibition of the TCA cycle by fluoroacetate, as measured by the change in dark respiration, is given in parentheses.

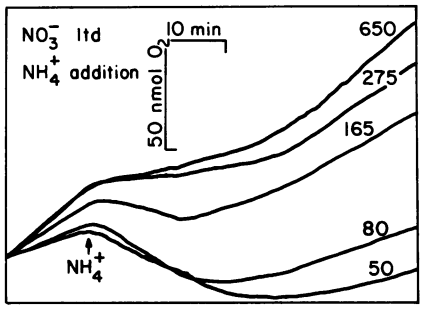

FIG. 4. Effect of light intensity on O_2 evolution in response to 50 μM NH_4^+ pulsing. The initial rates of O_2 evolution were 60, 82, 131, 168, and 169 μmol $O_2 \cdot mg^{-1}$ Ch $\cdot h^{-1}$ for 50, 80, 165, 275, and 650 $\mu E \cdot m^{-2} \cdot s^{-1}$, respectively. The postsuppression rates of O_2 evolution were 47, 69, 120, 160, and 171 μmol $O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$ for 50, 80, 165, 275, and 650 $\mu E \cdot m^{-2} \cdot s^{-1}$, respectively.

TCA cycle activity with fluoroacetate resulted in a 57% increase in the rate of O_2 evolution following NO_3^- pulsing (Table II).

Addition of NH_4^+ to NO_3^--limited *S. minutum* resulted in the consumption of O_2 in the light (Fig. 3B; Table II). The duration of this response was dependent on the concentration of added NH_4^+ (Fig. 3B). Following the period of O_2 consumption, O_2 evolution resumed at rates similar to the controls. The presence of azaserine alleviated this effect (Table II). Partial inhibition of the TCA cycle with fluoroacetate eliminated the period of O_2 consumption but still resulted in a decrease in O_2 evolution following NH_4^+ pulsing (Table II). The response of O_2 evolution to NH_4^+ pulsing was light dependent. Increasing light intensity above 165 $\mu E \cdot m^{-2} \cdot s^{-1}$ resulted in the alleviation of O_2 consumption following NH_4^+ pulsing (Fig. 4). Decreases in light intensity (<165 $\mu E \cdot m^{-2} \cdot s^{-1}$) resulted in higher rates of O_2 consumption following NH_4^+ addition. Nitrogen analogs (methylammonium, ethylammonium, tungstate, chlorate) had no effect on O_2 evolution.

Dark Respiration. Addition of 50 μM NO_3^- or 50 μM NH_4^+ resulted in a 67 and 129% stimultion in dark O_2 consumption, respectively (control rate of 91 μmol $O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$). This stimulation was rapid and constant during the period of photosynthetic suppression.

Dark Carbon Fixation. Addition of NO_3^- caused a 4.6-fold stimulation of dark carbon fixation while NH_4^+ caused a 22-fold stimulation. In the presence of azaserine the control rate of dark carbon fixation was four times greater in unenriched samples but there was no effect of pulsing with NO_3^- or NH_4^+ (Table III).

RuBP Measurements. Pulsing with NO_3^- resulted in a large decrease in the rate of carbon fixation (Fig. 5A) which coincided with a large decrease in cellular RuBP (Fig. 5B). The return of

Table III. *Effects of NO_3^- or NH_4^+ Addition (50 μM) on Dark Carbon Fixation in the Presence or Absence of Azaserine (AZA)*

Treatment	Dark CO_2 Fixation	Stimulation
	μmol mg⁻¹ Chl·h⁻¹ ± SE	%
Control	2.01 ± 0.18	
$+NO_3^-$	9.18 ± 0.43	+357
$+NH_4^+$	43.8 ± 3.32	+2079
Control + AZA	8.90 ± 0.70	
$+NO_3^-$ + AZA	8.06 ± 0.16	−9
$+NH_4^+$ + AZA	7.71 ± 0.49	−13

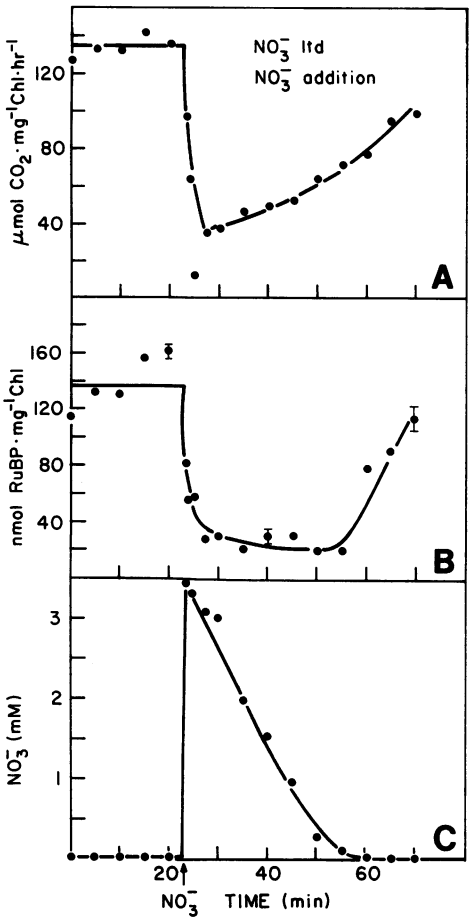

FIG. 5. Changes in photosynthetic carbon fixation, intracellular RuBP level, and exogenous NO_3^- concentration in response to a NO_3^- pulse. The average initial rate of photosynthesis was 134 μmol CO_2·mg⁻¹ Chl·h⁻¹. N assimilation in chemostat cells was 8.1 μmol N·mg⁻¹ Chl·h⁻¹. The average initial RuBP concentration was 139 nmol·mg⁻¹ Chl. During the period of suppression, the average rates of photosynthesis and N assimilation were 39.3 μmol CO_2·mg⁻¹ Chl·h⁻¹ and 60.4 μmol N·mg⁻¹ Chl·h⁻¹, respectively and the average RuBP concentration was 20 nmol·mg⁻¹ Chl. Representative standard errors for RuBP concentrations are given.

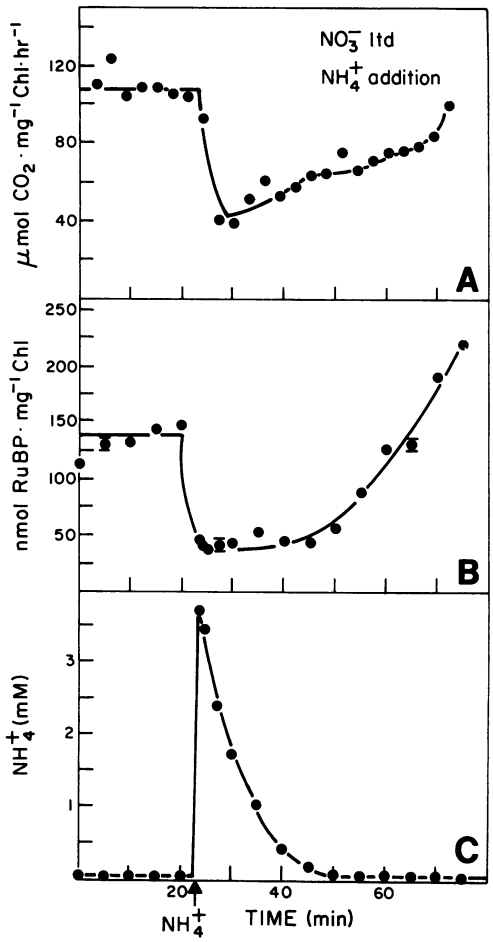

FIG. 6. Changes in photosynthetic carbon fixation, intracellular RuBP level and exogenous NH_4^+ concentration in response to an NH_4^+ pulse. The average initial rate of photosynthesis was 107 μmol CO_2·mg⁻¹ Chl·h⁻¹. N assimilation in chemostat cells was 8.1 μmol N·mg⁻¹ Chl·h⁻¹. The average initial RuBP concentration was 133 nmol·mg⁻¹ Chl. During the period of suppression, the average rates of photosynthesis and N assimilation were 78.7 μmol CO_2·mg⁻¹ Chl·h⁻¹ and 66.4 μmol N·mg⁻¹ Chl·h⁻¹, respectively and the average RuBP concentration was 37 nmol·mg⁻¹ Chl. Representative standard errors for RuBP concentrations are given.

Table IV. *Effect of NO_3^- or NH_4^+ Addition (50 μM) on Intracellular RuBP Levels in the Presence or Absence of Azaserine (AZA)*

Treatment	RuBP Content	Change
	nmol·mg⁻¹ Chl ± SE	%
Control	141.8 ± 4.4	
$+NO_3^-$	25.5 ± 1.9	−83
$+NH_4^+$	43.0 ± 2.6	−70
Control + AZA	144.8 ± 7.0	
NO_3^- + AZA	143.8 ± 2.6	0
NH_4^+ + AZA	130.3 ± 3.0	−10

photosynthesis to the control rate coincided with the recovery of RuBP concentration and the disappearance of NO_3^- from the medium (Fig. 5A, B, C). Similar results were obtained for NH_4^+ pulsing (Fig. 6), except that RuBP levels and photosynthesis decreased to a lesser extent (Fig. 6A, B). The recovery of photosynthesis and intracellular RuBP was concomitant with the disappearance of NH_4^+ from the medium (Fig. 6C). In the presence of azaserine, pulsing with NO_3^- or NH_4^+ had no effect on the levels of RuBP (Table IV). The relationship between photosynthetic carbon fixation and RuBP concentration during these experiments were indicative of saturation kinetics (Fig. 7).

DISCUSSION

N pulsing of NO_3^--limited cells resulted in different effects on photosynthetic O_2 evolution and carbon fixation. NO_3^- enrichment resulted in a 70% suppression of carbon fixation (Fig. 1A) but only a 24% suppression of O_2 evolution (Fig. 3A). In contrast,

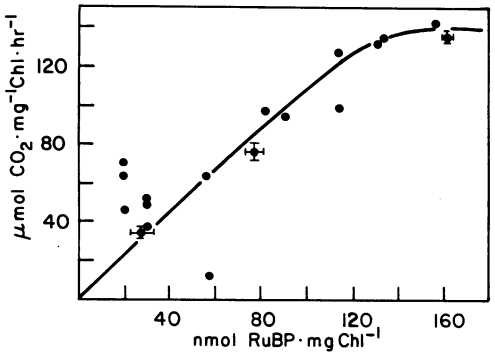

FIG. 7. Relationship between intracellular RuBP concentration and photosynthetic carbon fixation as determined from the data in Figure 5. Representative standard errors are given (Figs. 5, 6).

NH_4^+ enrichment resulted in a 40% suppression of carbon fixation (Fig. 1B) and the consumption of O_2 in the light (Fig. 3B). In interpreting these data it is important to realize that the processes of photosynthetic O_2 evolution and $^{14}CO_2$ fixation are distinct. Net O_2 evolution is the net result of O_2 production from water photolysis and O_2 consumption by mitochondrial respiration and other reactions. The observed rate of photosynthetic $^{14}CO_2$ fixation represents the net accumulation of acid stable counts due to Rubisco and PEP carboxylase activities after photo- and dark respiratory $^{14}CO_2$ release in the light. It has been shown that CO_2 release in the light is much lower in algae than higher plants (3) and consequently the rates of net and gross carbon fixation may be very similar.

Based on these considerations, several hypotheses are proposed to explain the mechanism of N-induced photosynthetic suppression. One explanation is that this phenomenon is the result of a transient uncoupling event caused by excessive levels of intracellular NH_3 resulting from NH_4^+ addition (1). Similarly, since NO_3^- and NO_2^- reduction are rapid in comparison to NH_4^+ assimilation (6, 29), intracellular NH_3 pools could also result from NO_3^- pulsing. Several lines of evidence indicate that uncoupling is not the cause of photosynthetic suppression. First, cells growing under N-sufficient conditions did not exhibit suppression in response to NO_3^- or NH_4^+ pulsing. Second, the concentration of NH_4^+ employed was well below that required for uncoupling (1). Finally, inhibition of GOGAT by azaserine protected photosynthetic CO_2 fixation, O_2 evolution, and RuBP concentration from NO_3^- or NH_4^+-induced suppression (Fig. 2; Tables II and IV). We can therefore conclude that photosynthetic suppression is dependent on N assimilation (Tables II–IV; Fig. 2) and is not an uncoupling phenomenon.

A subsequent hypothesis is that the suppression of photosynthetic carbon fixation, in cells acclimated to low N supply, may be a result of competition for metabolites between N assimilation and CO_2 fixation (7, 9, 27). The transient supply of new N could result in the diversion of photogenerated reductant and ATP from the Calvin cycle to N assimilation. This would reduce RuBP regeneration and consequently cause a suppression of carbon fixation. Furthermore, the increased carbon skeleton demand for NH_4^+ assimilation by GS/GOGAT would most likely be met through increased TCA cycle activity. The source of carbon for increased TCA cycle activity would be triose phosphates from the Calvin cycle. A sudden drain on Calvin cycle intermediates would cause a decrease in RuBP regeneration and result in a decrease in carbon fixation. A schematic representation of the biochemical pathways associated with this hypothesis is presented in Figure 8.

The observation that RuBP levels decrease following NO_3^- or NH_4^+ addition and that this decrease coincides with the period of N assimilation is consistent with this hypothesis (Figs. 5, 6). The relationship between RuBP concentration and photosynthesis is indicative of saturation kinetics. This correlation between RuBP concentration and photosynthesis suggests that during the period of photosynthetic suppression. RuBP regeneration was limiting carbon afixation (Fig. 7). Carbon skeleton requirements for the assimilation of equimolar amounts of NO_3^- and NH_4^+ would be similar and result in a comparable drain from the Calvin cycle. Because NO_3^- reduction has an additional reductant requirement (18) one would predict a larger decrease in RuBP and a greater suppression of C-fixation following NO_3^- addition. Our results are consistent with these predictions. RuBP levels decreased to 20 nmol·mg^{-1} Chl in response to NO_3^- pulsing but to only 37 nmol·mg^{-1} Chl following NH_4^+ addition (Figs. 5, 6). Figures 1 and 6 show that the greatest suppression in carbon fixation followed NO_3^- addition. It would also be expected that the duration of suppression would be dependent on the time required to assimilate the N pulse and consequently on the concentration of added N. This is consistent with the data presented (Fig. 1).

Rapid removal of α-ketoglutarate from the TCA cycle to provide carbon skeletons for N assimilation requires anapleurotic reactions to replenish TCA cycle intermediates. These reactions would be expected to occur via the production of oxaloacetate from the carboxylation of PEP by PEP carboxylase and increased pyruvate kinase activity. Previous work on higher plants has shown that NH_4^+ increased the flow of recently fixed carbon into TCA cycle intermediates through an increase in PEP carboxylase and pyruvate kinase activities (2, 14). This resulted in an increased photosynthetic carbon flux into amino acid biosynthesis (15, 22). Our hypothesis predicts that N pulsing should increase dark C-fixation. In *S. minutum*, NH_4^+ pulsing resulting in a 22-fold increase in dark C-fixation, whereas NO_3^- addition resulted in a 4.6-fold stimulation (Table III). The lower stimulation resulting from NO_3^- addition is consistent with the decreased rate of NO_3^- assimilation in the dark (18, 24). The observation that N addition at low light intensities resulted in decreased suppression of photosynthetic carbon fixation provides indirect evidence that dark C-fixation was also stimulated in the light (Table I). To maintain TCA cycle integrity, removal of one molecule of α-ketoglutarate would require the input of one oxaloacetate derived via PEP carboxylase. Consequently, the rate of dark carbon fixation should be roughly equivalent to the rate of NH_4^+ assimilation. The measured rate of NH_4^+ assimilation (66.4 μmol NH_4^+·mg^{-1} Chl·h^{-1}, Fig. 6) was similar in magnitude to the enhancement in dark carbon fixation (43.8 μmol CO_2·mg^{-1} Chl·h^{-1}, Table III).

This hypothesis is also consistent with the observed changes in net O_2 exchange in response to N addition. If photosynthetic carbon fixation were the only sink for photogenerated reductant, N addition should result in an equivalent decrease in O_2 evolution. In fact, NH_4^+ pulsing in *S. minutum* resulted in O_2 consumption in the light (Fig. 3). We propose that this may be the result of increased mitochondrial O_2 consumption associated with the increased TCA cycle activity previously proposed (Fig. 8). Consistent with this are three observations. First, NH_4^+ addition resulted in a large stimulation of dark respiration. Second, increased light intensity relieved the effect of NH_4^+ addition on O_2 exchange (Fig. 4). Finally, partial inhibition of the TCA cycle with fluoroacetate decreased the consumption of O_2 following NH_4^+ addition (Table II). NO_3^- reduction, unlike NH_4^+, requires a significant amount of photogenerated reductant and consequently supports O_2 evolution independent of CO_2 fixation (18). Consequently, the decrease in O_2 evolution associated with both decreased CO_2 fixation and increased TCA cycle activity would be partially offset by the increase in O_2 evolution associated with NO_3^- reduction. This explains why NO_3^- addition resulted in only a slight decrease in O_2 evolution (Fig. 3). As expected,

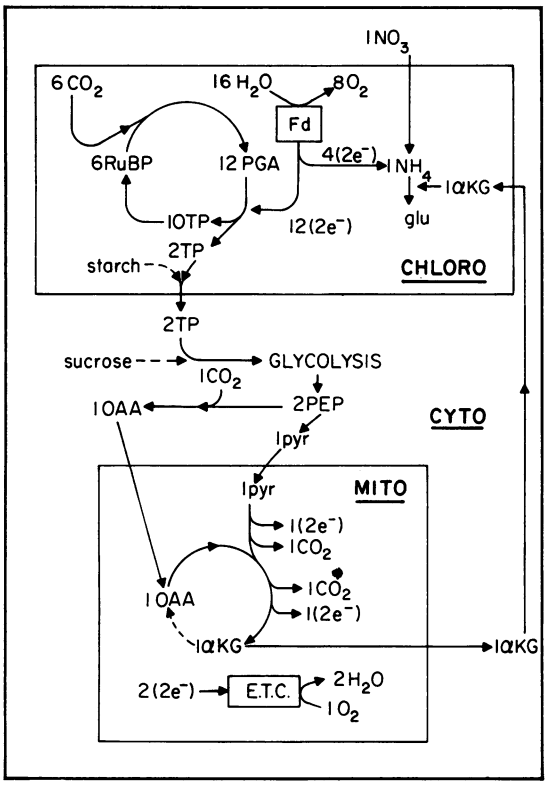

FIG. 8. Proposed interactions between the Calvin cycle and N assimilation during photosynthetic suppression in response to N pulsing in N-limited *S. minutum*. The theoretical carbon stoichiometry required for N assimilation through the proposed pathway is given. This stoichiometry assumes that the Calvin cycle provides all the carbon for N assimilation and that the TCA cycle operates only to provide α-ketoglutarate for N assimilation (via GS/GOGAT). Calculations based on this stoichiometry indicate that the minimum rate of CO_2 fixation required (362 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$) to support the measured rate of N assimilation (60.4 μmol $N \cdot mg^{-1}$ Chl $\cdot h^{-1}$) during N resupply is far in excess of that actually observed (39.3 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$). This implies that although one source of TP for N assimilation is the Calvin cycle a major portion of TP demand is met by starch or sucrose breakdown. Abbreviations: α-KG, α-ketoglutarate; E.T.C., electron transport chain; Fd, ferredoxin; glu, glutamate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; pyr, pyruvate; RuBP, ribulose bisphosphate; TP, triose phosphate.

partial inhibition of TCA cycle activity with fluoroacetate resulted in an increase in O_2 evolution following NO_3^- addition relative to the controls (Table II).

In conclusion, the data presented in this study can be explained on the basis of competition for carbon skeletons and photogenerated reductant between the processes of photosynthetic CO_2 fixation and N assimilation (Fig. 8). We suggest that sudden N resupply to NO_3^--limited cultures of *S. minutum* results in increased TCA cycle activity associated with the supply of carbon skeletons for NH_4^+ assimilation. Increased demands on TCA cycle intermediates requires anapleurotic reactions to maintain cycle activity. One source of carbon for these reactions is suggested to be Calvin cycle triose phosphates, the depletion of which decreases RuBP regeneration thereby limiting carbon fixation. However, based on stoichiometric calculations, the major source of TP for N assimilation during N resupply must be from starch or sucrose breakdown (Fig. 8). The consumption of O_2 in the light which follows NH_4^+ addition is a result of the decreased reductant required for carbon fixation and an increase in O_2 consumption associated with increased TCA cycle activity. The requirement of photogenerated reductant for NO_3^- reduction results in higher rates of O_2 evolution following NO_3^- addition when compared to NH_4^+ addition. This hypothesis allows us to establish a number of predictions, the testing of which should provide further insight into the relationship between C and N metabolism in N-limited microalgae.

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