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Short Communication

Regulation of Nitrate Reductase in *Chlorella vulgaris*[1]

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F. W. SMITH[2] AND JOHN F. THOMPSON
Department of Agronomy, Cornell University, and United States Plant, Soil and Nutrition Laboratory, Soil and Water Conservation Research Division, Agricultural Research Service, United States Department of Agriculture, Ithaca, New York 14850

Studies of Syrett and Morris on the utilization of nitrate and ammonium by *Chlorella* have shown that ammonium is assimilated preferentially (14). These investigators also demonstrate that the level of nitrate reductase is increased by adding nitrate and decreased by adding ammonium to *Chlorella* cultures (7). However, since these studies suggested that a product of ammonium assimilation rather than ammonium *per se* was responsible for decreasing the nitrate reductase increase, a number of amino acids (as products of ammonium metabolism) were tested as possible inhibitors.

MATERIALS AND METHODS

Growth and Handling of *Chlorella*. *Chlorella vulgaris* Beijerinck var. viridis (Chodat), obtained initially from the University of Indiana algal collection, was used as experimental material. Stock cultures were maintained on dextrose agar slants (10).

Cultures were grown on nutrient medium containing ammonium chloride as the sole nitrogen source. This medium had the following composition: glucose, 1.0%; NH_4Cl, 25.0 mM; $MgSO_4$, 5.0 mM; KH_2PO_4, 53.5 mM; K_2HPO_4, 13.4 mM; FeNaEDTA, 0.1 mM; Mo, 20 ng/g; Mn, 40 ng/g; Cu, 3 ng/g; Zn, 70 ng/g. The initial pH of the medium was 6.15. Cultures were grown in continuous light (approximately 500 ft-c) on a reciprocal shaker (30 cycles per min) at 21 C.

Cells for inoculum were grown in 20 ml of nutrient medium in 125-ml Erlenmeyer flasks for 2 days. Inoculum cultures were maintained by transferring approximately 1 ml of 2-day-old inoculum culture to 20 ml of fresh medium.

Experimental material was grown in 1 liter of nutrient medium in 4-liter Erlenmeyer flasks. These flasks were inoculated with 1 ml of suspension (0.014 ml of cells) from a 2-day-old inoculum culture. Growth was accompanied by a rapid drop in the pH of the medium which limited further growth 6 to 7 days after inoculation (Fig. 1). Cells were harvested after 6 days with the exception of those used with aspartic and glutamic acids.

Preparation of *Chlorella* Cells for Nitrate Reductase Assay. Extracts of *Chlorella* cells were obtained by breaking the cells in a "Nossal" type (9) mechanical disintegrator (Lourdes Instrument Corporation, Old Bethpage, N. Y.)[3]. Cells were transferred from centrifuge tubes into ice-cold 25-ml stainless steel disintegrator cups with 15 ml of an extraction medium containing 0.1 M tris-HCl buffer, pH 7.5; 0.3 mM Na_2EDTA; and 0.1 mM mercaptoethanol. Seven milliliters of ice-cold acid-washed glass beads (350–450 μ) were added to each cup. The neck of the cup was thoroughly coated with stopcock grease, and all air was removed and replaced with nitrogen prior to capping. The cells were then disintegrated by shaking in the Nossal shaker for 40 sec while being kept cold by circulating ice water around the disintegrator cup. No intact cells remained after disintegration. The homogenate was decanted into ice-cold centrifuge tubes, and particulate material was removed by centrifugation at 25,000*g* at 0 to 2 C for 20 min. The supernatant from this centrifugation was the crude extract used for assays.

Nitrate Reductase Assay. One-tenth milliliter of crude enzyme extract was used to determine nitrate reductase activity by the previously described method (13). Reaction mixtures were incubated at 30 C for 15 min. Nitrate reductase activity was directly proportional to the amount of extract and time of incubation up to 30 min.

Protein Assay. The protein content of extracts was determined by the method of Lowry *et al.* (5) using bovine serum albumin as a standard.

Preparation of *Chlorella* Cells for Nitrate Reductase Induction Studies. After a small aliquot of culture was removed for determination of packed cell volume (8), cells were harvested by centrifuging at 2000*g* for 5 min and decanting the supernatant. The cells were washed once in distilled water and three times in nitrogen-free medium (nutrient medium containing all components except ammonium chloride). The cells were then resuspended in sufficient nitrogen-free medium to give a cell suspension containing 1 ml of packed cells in 20 ml of cell suspension. This suspension was shaken in the light for 1 hr to insure utilization of any accumulated ammonium before performing induction studies.

Induction Studies. A 10-ml aliquot for the final cell suspension obtained by the above procedure was added to 50-ml Erlenmeyer flasks. Each of these flasks contained a compound to be tested as an inhibitor of nitrate reductase synthesis or of protein synthesis (see Tables I and II) dissolved in 0.5 ml of water. The flasks were placed on a reciprocal shaker (50 cycles per min) in a water bath at 21 to 23 C. After the cultures had been preincubated in the dark for 30 min, 0.5 ml of 0.1 M KNO_3 was added to each flask, and the cells were incubated in the dark for 2.5 hr. At the end of the incubation period, the cells were transferred to 15-ml centrifuge tubes and centrifuged at 4000*g* for 2 min. The supernatant was removed, and the cells were washed twice by resuspending in deionized

[1] Department of Agronomy Paper No. 910.

[2] Present address: Division of Tropical Pastures, C.S.I.R.O., The Cunningham Laboratory, St. Lucia, Queensland, 4067, Australia.

[3] Trade names and company names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product listed by the U. S. Department of Agriculture or the New York State College of Agriculture.

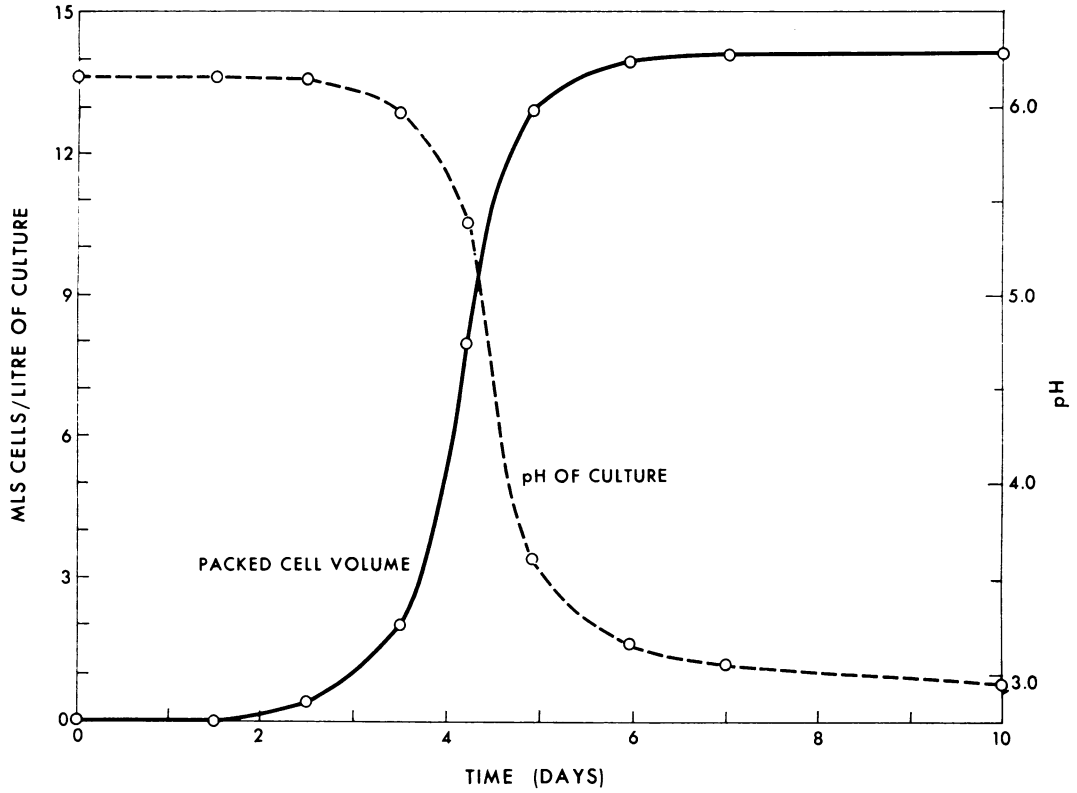

FIG. 1. Effect of growth period on packed cell volume and pH of culture medium.

distilled water and centrifuging. The cells were broken in a Nossal disintegrator and assayed for nitrate reductase.

All treatments were run in duplicate and there was good agreement between duplicate results. Controls that were assayed for nitrate reductase activity at the time of adding KNO_3 to other flasks (zero induction time controls) were run with each experiment.

Measurement of Rate of Growth of *Chlorella* Cultures in Media Used in Induction Studies. Experiments were performed in 18- × 150-mm culture tubes. Compounds to be tested (Figs. 3 and 4) and KNO_3 were dissolved in nitrogen-free culture medium and pipetted into the tubes. *Chlorella* cells that had been harvested and washed, as previously described, were suspended in nitrogen-free culture medium. An aliquot of this cell suspension sufficient to give an initial concentration of 1.0 to 2.0 ml of cells per liter of culture was pipetted into each culture tube. The final volume of culture in each tube was 10 ml, and the concentration of KNO_3 and the compounds tested were 5.0 mM. These cultures were incubated at 25 C by shaking in a water bath. Cultures were also mixed on a Vortex shaker every 30 min. All treatments were run in triplicate. Growth rate was measured by following the change in turbidity at 600 nm.

RESULTS AND DISCUSSION

Using the methods detailed above, it was found that nitrate reductase is increased in *Chlorella* by nitrate with maximum induction at concentrations above 3 mM (Fig. 2). Since the increase in nitrate reductase that resulted from adding nitrate was inhibited by actinomycin D, cycloheximide, and puromycin (Table I) as in radish cotyledons (3) and barley roots (13), it is probable that *de novo* synthesis of messenger RNA and protein is required.

Tests of a number of nitrogenous compounds (protein amino acids, ornithine, citrulline, urea, and ammonium) revealed that ammonium chloride, urea, aspartic acid, glutamic acid,

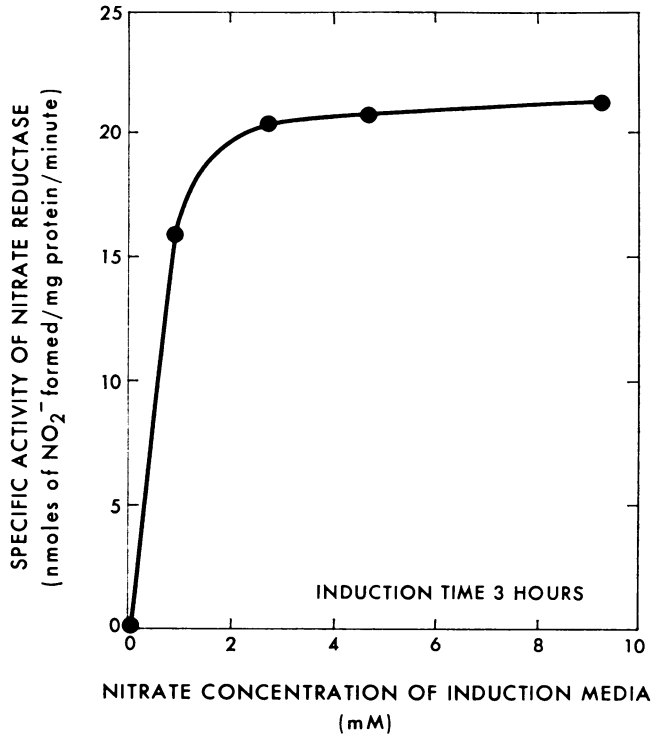

FIG. 2. Effect of KNO_3 concentration on the level of nitrate reductase in *Chlorella* cells grown on NH_4Cl as a nitrogen source. There was no detectable nitrate reductase prior to addition of potassium nitrate.

leucine, histidine, arginine, citrulline, ornithine, and lysine inhibited the nitrate reductase increase induced by nitrate *in vivo* (Table II). None of the latter compounds (10^{-2} and 10^{-3} M) affected nitrate reductase activity *in vitro*, indicating that the effect *in vivo* was not a result of inhibition of nitrate reductase. It was also important to establish that the inhibition was due to a specific effect of these compounds on nitrate reductase and not a general effect of protein synthesis. Using growth as a test of generalized inhibition of protein synthesis, only leucine and histidine (of the compounds inhibiting nitrate reductase increase) inhibited growth (Figs. 3 and 4). Hence, the inhibitory effects of leucine and histidine may be due to effects on growth.

Table I. *Effect of Inhibitors of Protein Synthesis on the Induction of Nitrate Reductase Synthesis in Chlorella*

Chlorella cells were harvested by centrifugation, resuspended in a nitrogen-free medium, and shaken 1 hr. An aliquot of the suspension was shaken with various inhibitors for 30 min prior to the addition of KNO_3 to give a concentration of 5 mM. After the suspensions were shaken 2.5 hr longer at 21 to 23 C, the cells were harvested, broken, and assayed for nitrate reductase by methods detailed in the text.

Inhibitor	Concentration	Specific Activity[1]
	μg/ml	*% of control*
Actinomycin D	40	53
Cycloheximide	1	14
Puromycin	500	60

[1] Nitrate reductase activity was 21.5 nmoles NO_2^- produced/mg protein·min. Standard error = ±5%.

The inability to demonstrate effects of glutamic acid and aspartic acid on 6-day-old cultures (Table II) are possibly due to their inability to absorb these two amino acids. Lynch and Gillmor (6) have shown that, probably as a result of its negative charge, glutamic acid is not taken up by *Chlorella pyrenoidosa* readily.

Inhibition of the development of nitrate reductase activity by ammonium (Table II) confirms the findings of Morris and Syrett (7, 14) who also observed that ammonium did not inhibit nitrate reduction when a carbon source was lacking. The latter observation implies that a product of ammonium assimilation is responsible for its inhibitory effect on nitrate reductase synthesis. This implication is supported by the rapid metabolism of ammonium by *Chlorella* (1).

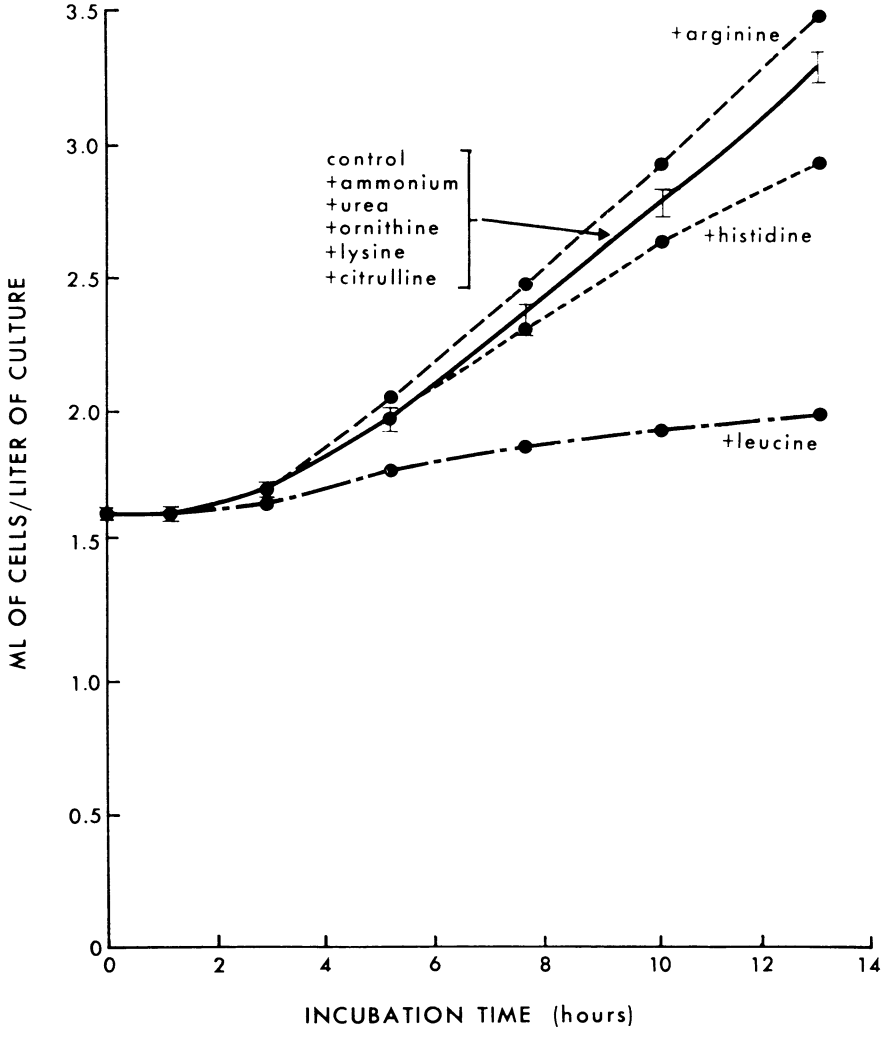

FIG. 3. Effect of some nitrogenous compounds on the rate of growth of *Chlorella* cultures in medium containing KNO_3.

Table II. *Effect of Some Nitrogenous Compounds on the Induction of Nitrate Reductase in Chlorella Cells*

Chlorella cells were treated as in Table I except that nitrogenous compounds were used in place of inhibitors.

Compound[1]	Specific Activity[2]
	% of control
Ammonium chloride	7
Urea	32
L-Aspartic acid[3]	46
L-Glutamic acid[3]	41
L-Leucine	31
L-Histidine	72
L-Arginine	8
L-Citrulline	79
L-Ornithine	13
L-Lysine	69
Other compounds[4]	100

[1] The concentration of test compounds in the induction medium was 5 mM.

[2] Six batches of cells were used in testing all amino acids. The nitrate reductase activity of the controls ranged from 19.1 to 26.7 nmoles NO_2^- produced/mg protein·min.

[3] Ten-day-old cultures were used for tests with aspartic acid and glutamic acid. Six-day-old cultures were used for other compounds.

[4] L-Asparagine, L-glutamine, L-alanine, glycine, L-isoleucine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-valine, γ-amino-butyric acid. Standard error = ±8%.

The inhibition of nitrate reductase formation by urea, arginine, and citrulline could be explained by conversion to ammonium. *Chlorella* has an ATP-dependent urease (4, 11, 15) and arginine desimidase (12). Indirect evidence (2) suggests that *Chlorella* also contains a citrullinase.

Since ammonium, aspartic acid, ornithine, and lysine could cause an increase in glutamic acid by reductive amination and transamination, it is conceivable that glutamic acid is the only inhibitor of nitrate reductase synthesis.

It is possible that the nitrogen compounds inhibit nitrate reductase synthesis by interfering with the uptake of nitrate. This is considered unlikely because ammonium assimilation does not affect nitrate uptake (14). However, it would be difficult to provide conclusive evidence against this possibility because *Chlorella* accumulates nitrate to a very low level. This low level of accumulation implies that nitrate level in *Chlorella* is closely controlled at a low concentration so that it would be difficult to detect any inhibition of nitrate uptake.

These experiments show that eight nitrogenous compounds inhibit the increase in nitrate reductase in *Chlorella* in the presence of nitrate but exert no effect on nitrate reductase activity. Since the increase in nitrate reductase is reduced by inhibitors of RNA polymerase and protein synthesis, it is likely that nitrate reduction in *Chlorella* is controlled by repression of enzyme synthesis and not by feedback inhibition.

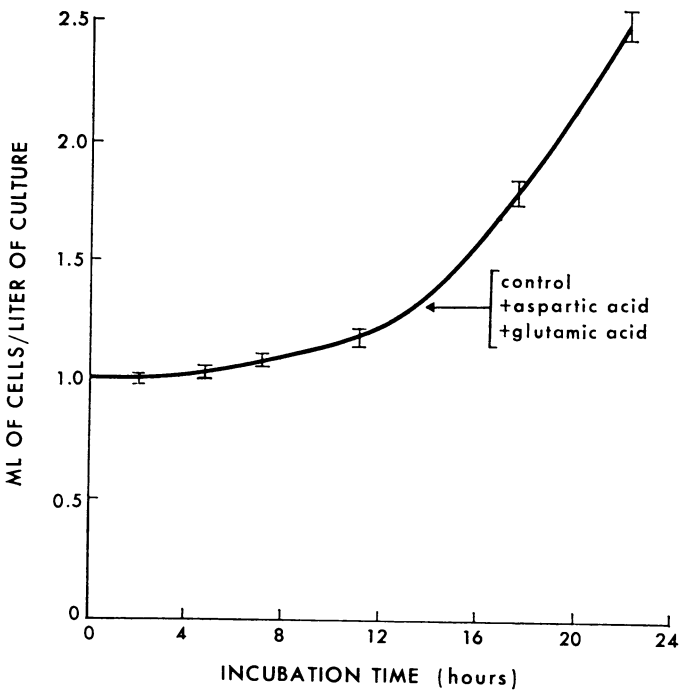

FIG. 4. Effect of aspartic and glutamic acids on the rate of growth of *Chlorella* cultures in media containing KNO_3.

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