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Rapid incorporation of $^{13}NO_3$ by NH_4-limited phytoplankton

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ABSTRACT: Nitrate reductase, the enzyme which catalyzes the reduction of nitrate to nitrite, is repressed by ammonium and induced by the presence of nitrate. In oligotrophic oceans, inorganic nitrogen concentrations are low and it is believed that phytoplankton primarily use regenerated ammonium. Under these conditions, nitrate reductase activity should be reduced. We investigated the metabolism of nitrate in ammonium-limited chemostats of marine phytoplankton using the short-lived radiotracer ^{13}N. We found that nitrate is rapidly taken up, reduced and incorporated into protein by ammonium-limited phytoplankton due to the constitutive activity of nitrate reductase. These results provide a mechanism for the utilization of episodic pulses of nitrate, which have been suggested to be responsible for a significant fraction of nitrate-based production in the open ocean.

INTRODUCTION

Primary production in the oceans is supported by 2 major forms of nitrogen: 'regenerated' nitrogen (primarily ammonium) which is reduced and is provided by the recent decomposition of organic matter in the water column, and 'new' nitrogen (nitrate) which is oxidized and diffuses or is advected into the euphotic zone from deep water (Dugdale & Goering 1967). It is generally believed that ammonium is a major source of nitrogen for phytoplankton in oligotrophic oceans (Dugdale & Goering 1967, Eppley et al. 1973, 1979). However, recent studies have suggested that new production may be more important than previously thought (Jenkins & Goldman 1985, Platt & Harrison 1985) and supported by episodic mixing events (Platt & Harrison 1985) or mixing which is not reflected in the vertical profiles of nitrate concentrations (Hayward 1987). These mechanisms require that phytoplankton, which may have been growing primarily on ammonium, become rapidly capable of transporting, reducing and incorporating nitrate.

Ammonium and nitrate can be used simultaneously when both forms are supplied (Eppley & Renger 1974, Bienfang 1975), but ammonium is usually used in preference to nitrate (McCarthy et al. 1977, Dortch & Conway 1984). Whereas NH_4^+-N can be directly assimilated into amino acids, NO_3^--N must be reduced to NH_4^+ before the nitrogen can be assimilated (Falkowski 1983). The reduction is catalyzed by an NAD(P)H-dependent enzyme, nitrate reductase (NR), the regulation of which is not well understood. While nitrate transport is constitutive (Falkowski 1975, Balch 1987), NR is inducible (Morris & Syrett 1965, Eppley & Renger 1974). NR activity can be quickly repressed by ammonium and the enzyme activity can be high when cells are starved of nitrogen (Morris & Syrett 1965). The activity of the enzyme is usually determined by colorometric assay of nitrite formation in cell-free extracts (Morris & Syrett 1965, Eppley et al. 1969); the sensitivity of this assay is low unless incubations are relatively long (tens of minutes). NR activity is labile in vitro, and susceptible to proteolysis unless precautions are taken (Ingemarsson 1987). Consequently, low levels of enzyme activity are difficult to detect and preserve.

We used the radionuclide ^{13}N ($t_{1/2}$ = 9.96 min) to trace nitrate uptake, reduction and incorporation in ammonium-limited cultures of marine phytoplankton to investigate the effect of growth on ammonium on in vivo NR activity. This technique made it possible to trace nitrogen in vivo during short time intervals, and avoid artifacts due to extraction, substrate enrichment and length of incubation.

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METHODS

Continuous cultures of *Thalassiosira pseudonana* (Woods Hole clone 3H) and *Dunaliella tertiolecta* (Woods Hole clone DUN) were maintained in ammonium-limited chemostats using an artificial seawater medium (Goldman & McCarthy 1978) as described by Zehr et al. (1988). Nitrogen was supplied as NH_4Cl at a final concentration of 75 μ*M*. The chemostats (0.8 to 3 l) were grown under continuous illumination (100 μEinst m^{-2} s^{-1}) at 18 °C, and were bubbled with air which had been passed through a 0.1 *N* H_2SO_4 trap to remove trace ammonia. Cell densities were maintained at 3 to 7×10^5 and 0.4 to 2×10^6 cells ml^{-1} for *Dunaliella tertiolecta* and *Thalassiosira pseudonana* respectively.

^{13}N was produced on the 60 inch (152.4 cm) cyclotron at Brookhaven National Laboratory using the ^{16}O (p, α) ^{13}N reaction and a small volume (0.5 to 1.0 ml) water target. The water target was bombarded with 18 MeV protons for 10 min at a current of 15 μA. ^{13}N-nitrate was purified by ion-exchange HPLC (Chasko & Thayer 1981) and ^{13}N-ammonium produced from reduction of ^{13}N-nitrate with DeVarda's alloy (Cooper et al. 1987), distillation and purification with ion-exchange resin (Bio-Rad AG 1X8) (Zehr et al. 1988). Final concentrations of $^{13}NH_4^+$ solutions were measured by the phenol-hypochlorite method (Solorzano 1969) and $^{13}NO_3^-$ by HPLC (Chasko & Thayer 1981). The purified ^{13}N-labeled substrates were added to cell suspensions (maintained under constant illumination and at constant temperature) to give final concentrations of 300 n*M* and 30 to 40 n*M* for ammonium and nitrate, respectively. At each time point cells were filtered onto 1 μm pore size Nuclepore filters (uptake), or filtered and proteins precipitated with cold 5 % trichloroacetic acid (incorporation) as described by Zehr et al. (1988). Filters were counted in a Packard gamma counter and counts per minute (cpm) were decay-corrected to a common time.

Internal nitrate and nitrite pools were extracted by submersing filtered cells in 1 ml boiling distilled water for 1 min. The 1 ml sample was centrifuged in a microcentrifuge. Nitrate and nitrite in the extract were separated by ion-exchange HPLC (Chasko & Thayer 1978) and 0.3 ml (0.3 min) fractions collected and counted in a Packard gamma counter.

RESULTS AND DISCUSSION

^{13}N-nitrate and -ammonium were rapidly taken up and incorporated into protein by NH_4^+-limited *Thalassiosira pseudonana* (Fig. 1a). ^{13}N-nitrate was also rapidly incorporated by ammonium-limited *Dunaliella tertiolecta* growing at high (0.8 d^{-1}) and low (0.3 d^{-1}) growth rates (Fig. 1b). Incorporation of ^{13}N was 95 % inhibited by the addition of cycloheximide, an inhibitor of cytoplasmic mRNA translation, confirming that the TCA-precipitable material was protein (Fig. 2). We have previously shown that the short-term (5 to 15 min) rate of incorporation of $^{13}NH_4^+$ into TCA-precipitable material is directly related to steady state growth rate, and does not reflect transient uptake phenomena (Zehr et al. 1988). Short-term measurements of ^{13}N incorporation into protein (Zehr et al. 1988) are comparable to long-term uptake experiments, where the rate limiting step of

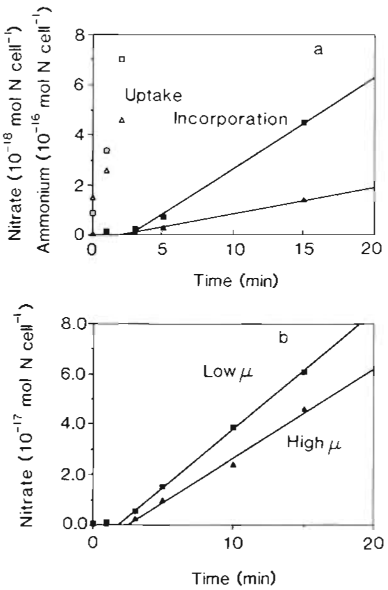

Fig. 1. (a) Uptake and incorporation of ^{13}N-ammonium (□, ■) and ^{13}N-nitrate (△, ▲) by *Thalassiosira pseudonana* (3H) grown in ammonium-limited chemostats. (b) Incorporation of ^{13}N-nitrate by *Dunaliella tertiolecta* growing at 0.8 d^{-1} (▲) and 0.3 d^{-1} (■) in ammonium-limited chemostats. (See text for details of methods)

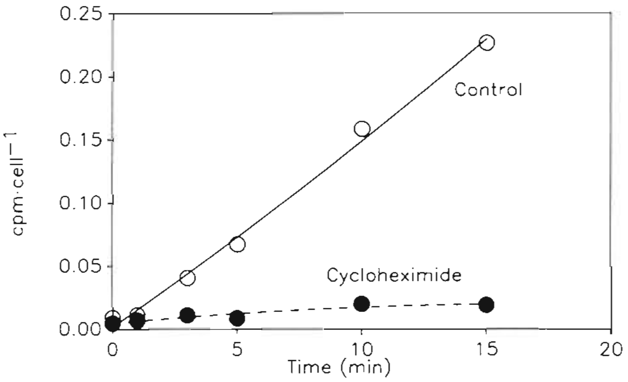

Fig. 2. Inhibition of incorporation of ^{13}N-nitrate into TCA-insoluble material by cycloheximide. *Dunaliella tertiolecta* was grown in an ammonium-limited chemostat at 0.3 d^{-1} and 100 μg ml^{-1} of cycloheximide added 1 h before addition of the radioisotope. The control and inhibitor-treated samples were exposed to $^{13}NO_3^-$ of identical specific activity

uptake becomes incorporation (Dugdale & Wilkerson 1986). The rapid incorporation of NO_3^--nitrogen into protein indicates that nitrate was immediately used as an alternate nitrogen source to support growth in ammonium-grown cells, regardless of the degree of N-deficiency (growth rate).

^{13}N was immediately detected as ^{13}N-nitrite in ammonium-limited cells of *Thalassiosira pseudonana* and *Dunaliella tertiolecta* (Fig. 3, data for *D. tertiolecta* not shown). The appearance of ^{13}N in the nitrite fraction is probably not a result of a significant increase in the nitrite pool, but rather to the rapid turnover of a small pool which is heavily labeled with ^{13}N. The rapid reduction of nitrate to nitrite indicates that NR was present and active, even though nitrate had not been a major nitrogen source for these cultures. Although nitrate was present from contamination of the inorganic salts, it was less than 0.3 % of total inorganic nitrogen (several hundred nanomolar).

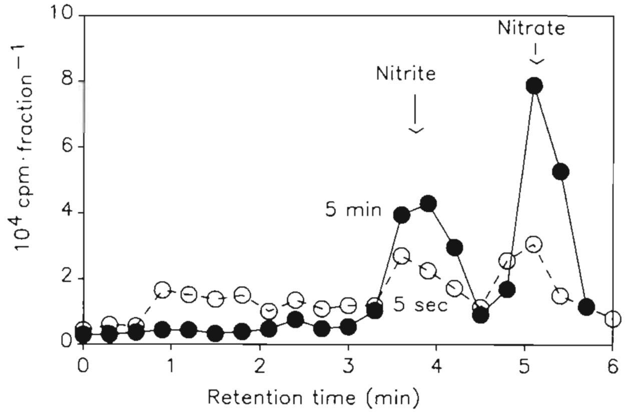

Fig. 3. Intracellular reduction of $^{13}NO_3$ to $^{13}NO_2$ by ammonium-limited *Thalassiosira pseudonana* (3H). The cell suspension was incubated with $^{13}NO_3$ for 5 s (○) and 5 min (●) in the light. (See text for details of extraction of $^{13}NO_3^-$ and $^{13}NO_2^-$)

The immediate reduction of nitrate (within seconds) by eukaryotic algae growing on ammonium has not previously been demonstrated. Meeks et al. (1983) found that N-fixing cyanobacteria *(Anabaena)* grown on NH_4^+ assimilated $^{13}NO_3^-$. These results suggest that some NR is synthesized and remains active in most, if not all, algae during growth on NH_4^+. However, ^{13}N-nitrate accumulated more rapidly than ^{13}N-nitrite, indicating that nitrate reduction was the rate limiting step in N-incorporation (Fig. 3).

The incorporation of a saturating pulse of ^{13}N into protein underestimates the growth rate due to the isotope dilution by unlabeled nitrogen in the internal free amino acid pool (Zehr et al. 1988). In the experiments described here, the added concentration of nitrogen was kept low to avoid inhibition of uptake of nitrate by ammonium and to maintain a nitrogen-deficient environment. The effect of isotope dilution is even more pronounced in these experiments than with saturating pulses, since the internal pool does not become saturated with tracer as rapidly due to the lower transport rates. This is clear from the data which show a lower ^{13}N incorporation rate in high growth rate cultures than in low growth rate cultures (Fig. 1b). During uptake of a saturating pulse of nitrogen, the specific activity of internal nitrogen pools become dominated by the rapid influx of nitrogen and approaches the specific activity of the external nitrogen pool. Since transport rates are lower at low substrate concentrations, the difference between uptake rate and incorporation rate is reduced. The ratio of uptake to incorporation in *Thalassiosira pseudonana* was 8 and 14 for nitrate and ammonium, respectively, for low growth rate (0.3 d^{-1}) cultures in this study, but was 40 to 50 during rapid uptake of a saturating pulse of ammonium (Zehr et al. 1988). Therefore, the measured rate of ^{13}N incorporation underestimates protein synthesis rates as a function of the external N concentration, as well as the size of the internal free amino acid pool. The kinetics of $^{13}NH_4^+$ ammonium uptake have previously been determined (J. Zehr unpubl.).

We calculated the absolute contribution of nitrate uptake to ascertain whether the radioactive flux was significant. Calculation of the absolute nitrogen uptake and incorporation rates from measurements of radioactivity requires some assumptions. Because $^{13}NO_3$ is the primary radiolabeled product produced in the cyclotron reaction, its specific activity is significantly higher than that of ammonium, and because of the short half-life of the isotope it is virtually impossible to determine the specific activity in real time. We therefore added equal amounts of radioactive ammonium or nitrate to the cultures and determined the specific activity as soon as possible after the exposure (within an hour). We corrected for the difference in substrate concentrations in nitrate and ammonium uptake measurements based on the specific activity and measurements of uptake kinetics (J. Zehr unpubl.). The calculated ammonium incorporation rate at an external concentration of 40 n*M* is 16 % of the rate measured at 300 n*M*. Therefore, the $^{13}NH_4^+$-incorporation rate at 40 n*M* will be ca 16 % of the $^{13}NH_4^+$-incorporation at 40 n*M* due to the decreased specific activity of the amino acid pool. Given these assumptions, nitrate incorporation was ca 18 % of the calculated total nitrogen (ammonium + nitrate) uptake at 40 n*M*.

The results show that ammonium-limited phytoplankton are capable of transporting, reducing, and incorporating NO_3^-, even when nitrate is a trivial source of nitrogen for growth in situ. Although it has been shown that phytoplankton simultaneously take up nitrate and ammonium when both are present (Eppley

& Renger 1974, Bienfang 1975), it was thought that nitrate would not be incorporated to a great extent unless the cells had been exposed to nitrate in order to induce NR activity (Eppley et al. 1973, Eppley & Renger 1987). Our findings show that some NR activity is constitutive in cells growing under ammonium-limited conditions regardless of growth rate, in addition to during nitrogen-starvation (Morris & Syrett 1965).

Previous work has shown that NR activity reflects the 'history' of the cells and may remain at high levels for some time following removal of the nitrate source (Blasco et al. 1984). In this study, the cells had been grown solely on ammonium. Potential NR activity may be even higher in natural populations of phytoplankton which have had prior exposure to nitrate.

The measured uptake rate from experiments where the ^{15}N-ammonium or ^{15}N-nitrate additions increase the ambient concentration can approach the growth rate regardless of the nitrogen compound which was used prior to the experiment (e.g. Harrison et al. 1983). This could lead to an overestimate of total N uptake or growth rate by a factor of 2, since both tracers ($^{15}NO_3^-$ and $^{15}NH_4^+$) will be measuring ammonium assimilation and incorporation if NR activity is not limiting. However, the nitrate incorporation rate will be limited by NR activity, which will be some fraction of the growth rate. In this study, NR activity was only 18 % of total nitrogen incorporation in cells which had been growing solely on ammonium. If potential NR activity were higher, due to prior exposure of the cells to nitrate (Blasco et al. 1984), the measured nitrate incorporation rate could lead to errors in calculations of the percentage of total production supported by nitrate or the rate of growth on nitrate. Total nitrogen uptake rates or growth rates calculated from summing nitrogen uptake from individual nitrogen sources (Kokkinakis & Wheeler 1987) will be particularly sensitive to perturbations of nitrate or ammonium concentrations by the 'tracer' additions. This phenomenon may partially explain the unrealistically high growth rates calculated by summing $^{15}NO_3^-$ and $^{15}NH_4^+$ derived growth rate estimates (Kokkinakis & Wheeler 1987).

The results of this study strongly suggest that phytoplankton contain a fraction of constitutive nitrate reductase activity which can be used to rapidly reduce nitrate and allow its utilization in protein synthesis in the event of an episodic injection of nitrate into the euphotic zone.

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