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 colorimetric 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 NH₄Cl at a final concentration of 75 μM. 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 H₂SO₄ trap to remove trace ammonia. Cell densities were maintained at 3 to 7 × 10⁵ and 0.4 to 2 × 10⁶ cells ml⁻¹ for *Dunaliella tertiolecta* and *Thalassiosira pseudonana* respectively.

¹³N was produced on the 60 inch (152.4 cm) cyclotron at Brookhaven National Laboratory using the ¹⁰O (p, α) ¹³N 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. ¹³N-nitrate was purified by ion-exchange HPLC (Chasko & Thayer 1981) and ¹³N-ammonium produced from reduction of ¹³N-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 ¹³NH₄⁺ solutions were measured by the phenol-hypochlorite method (Solorzano 1969) and ¹³NO₃⁻ by HPLC (Chasko & Thayer 1981). The purified ¹³N-labeled substrates were added to cell suspensions (maintained under constant illumination and at constant temperature) to give final concentrations of 300 nM and 30 to 40 nM 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 submerging 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

¹³N-nitrate and -ammonium were rapidly taken up and incorporated into protein by NH₄⁺-limited *Thalassiosira pseudonana* (Fig. 1a). ¹³N-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 ¹³N 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 ¹³NH₄⁺ 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 ¹³N incorporation into protein (Zehr et al. 1988) are comparable to long-term uptake experiments, where the rate limiting step of ¹³N uptake and cycling is the uptake of ¹³NO₃⁻.
uptake becomes incorporation (Dugdale & Wilkerson 1986). The rapid incorporation of \( \text{NO}_3^- \)-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).

\( ^{15}N \) was immediately detected as \( ^{15}N \)-nitrite in ammonium-limited cells of \textit{Thalassiosira pseudonana} and \textit{Dunaliella tertiolecta} (Fig. 3, data for \textit{D. tertiolecta}

![Fig. 3. Intracellular reduction of \( ^{15}\text{NO}_3^- \) to \( ^{15}\text{NO}_2^- \) by ammonium-limited \textit{Thalassiosira pseudonana} (3H). The cell suspension was incubated with \( ^{15}\text{NO}_3^- \) for 5 s (○) and 5 min (●) in the light. (See text for details of extraction of \( ^{15}\text{NO}_3^- \) and \( ^{15}\text{NO}_2^- \)).](image)

not shown). The appearance of \( ^{15}N \) 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 \( ^{15}N \). 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).

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 (\textit{Anabaena}) grown on \( \text{NH}_4^+ \) assimilated \( ^{15}\text{NO}_3^- \). These results suggest that some NR is synthesized and remains active in most, if not all, algae during growth on \( \text{NH}_4^+ \). However, \( ^{15}N \)-nitrate accumulated more rapidly than \( ^{14}N \)-nitrite, indicating that nitrate reduction was the rate limiting step in N-incorporation (Fig. 3).

The incorporation of a saturating pulse of \( ^{15}N \) 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 \( ^{15}N \) 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 \textit{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 \( ^{15}N \) 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 \( ^{15}\text{NH}_4^+ \) 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 \( ^{15}\text{NO}_3^- \) 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 nM is 16% of the rate measured at 300 nM. Therefore, the \( ^{15}\text{NH}_4^+ \)-incorporation rate at 40 nM will be ca. 16% of the \( ^{15}\text{NH}_4^+ \)-incorporation at 40 nM 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 nM.

The results show that ammonium-limited phytoplankton are capable of transporting, reducing, and incorporating \( \text{NO}_3^- \) 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
Goldman, J. C. (1985) Seasonal oxygen cycling, use of the Brookhaven cyclotron facility. Fowler and D. Schlyer for their help and cooperation in the Health and Environmental Research. We thank Joanna activity and stability of nitrate reductase. Plant Physiol. 85: 1593-1627. Additional support was provided by the US Dept of Energy. Office of Health and Environmental Research. We thank Joanna Fowler and D. Schlyer for their help and cooperation in the use of the Brookhaven cyclotron facility.

LITERATURE CITED


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