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Nitrate Utilization by the Diatom *Skeletonema costatum*

I. KINETICS OF NITRATE UPTAKE[1]

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ABSTRACT

Nitrate uptake has been studied in nitrogen-deficient cells of the marine diatom *Skeletonema costatum*. When these cells are incubated in the presence of nitrate, this ion is quickly taken up from the medium, and nitrite is excreted by the cells. Nitrite is excreted following classical saturation kinetics, its rate being independent of nitrate concentration in the incubation medium for nitrate concentration values higher than 3 micromolar. Nitrate uptake shows mixed-transfer kinetics, which can be attributed to the simultaneous contributions of mediated and diffusion transfer. Cycloheximide and *p*-hydroxymercuribenzoate inhibit the carrier-mediated contribution to nitrate uptake, without affecting the diffusion component. When cells are preincubated with nitrate, the net nitrogen uptake is increased.

Nitrogen appears to be the major nutrient limiting primary production in the oceans of the world (18), as well as in certain freshwater systems. Nitrogen is found in seawater as dissolved N_2 (which cannot be fixed by most marine algae) and as inorganic ions: nitrate, nitrite, and ammonium. These ions are present in concentrations ranging from 0.01 to 50 μM for nitrate, from 0.01 to 5 μM for nitrite, and from 0.1 to 5 μM for ammonium. The concentration of organic forms is usually below 10 μM (15). When all three inorganic ions are present, ammonium is preferentially utilized (4). Nitrate and nitrite have to be reduced to ammonium by means of an energy-dependent enzyme system (nitrate reductase and nitrite reductase) prior to their assimilation by the cell (15).

As nitrate is the most abundant form of N in seawater, marine phytoplankton, mostly composed of diatoms and dinoflagellates, utilizes nitrate as the main N source. Its uptake and reduction constitute major functions in these organisms, since N has been identified as the limiting factor controlling their growth. The relevance of the study of these processes arises from the fact that phytoplankton organisms are the major primary producers of the sea.

The purpose of this paper is to characterize the kinetics of nitrate uptake in a phytoplankton organism of particular ecological significance because of its wide distribution, such as the diatom *Skeletonema costatum*.

MATERIALS AND METHODS

Culture Media and Conditions. Culture media were prepared according to Guillard and Ryther (8), with the modifications proposed by the Woods Hole Oceanographic Institution (25). Cells were maintained in the medium h/2-1. N-deficient cells were obtained by growth on the medium f/2-1 but with 25 μM nitrite as the sole N source. For the antibiotic treatments, the strain was placed in the medium h/2-1A, to which the corresponding amounts of penicillin G and streptomycin were added (24). All media were prepared with synthetic seawater, containing 307.7 mM NaCl, 20.3 mM $MgSO_4$, 8.0 mM KCl, 2.7 mM $CaCl_2$, and 0.48 mM H_3BO_3 in distilled H_2O. The media were adjusted to pH 7.8 with Tris-HCl buffer and autoclaved at 121 C for 30 min.

Batch cultures were used in order to minimize bacterial contamination. The cells were grown autotrophically with continuous artificial light, about 3,500 lux, produced by fluorescent tubes (Sylvania cool-white, F 65 T 12/CW), on a horizontal shaker (140 rpm) at 20 ± 2 C.

Strain. The diatom used in this study was a strain of *S. costatum* (Grev.) Cleve, generously provided by the Instituto de Investigaciones Pesqueras, C.S.I.C., Laboratorio de Castellón, Castellón, Spain. For maintenance purposes, the strain was grown in 100-ml Erlenmeyer flasks containing 50 ml of culture medium h/2-1. The cultures were grown under the conditions described above. Fresh medium was inoculated every week with 2 ml of the preceding culture. The purity of the strain was controlled by means of periodic observations under the phase contrast microscope.

Kinetics and Assessment of Growth. For the study of growth kinetics, cells were grown in 2-liter Erlenmeyer flasks, containing 800 ml of medium f/2-1, with varying amounts of different inorganic salts as the only N source. The cultures were inoculated with 20 ml of a culture grown for 5 days on ammonium containing (h/2-1) medium. Aliquots were taken, under sterile conditions, at regular intervals. Growth was measured by a variety of techniques, turbidimetrically (at 750 nm), hemocytometrically, and by dry weight, with consistent results. Cell density was also measured as Chl *a*, after acetone extraction of the cell pigments (19). Absorption spectra of the acetone extracts were also used to determine the Margalef index (11) A_{430}/A_{465}, which is the ratio between the absorbancy of the whole of the pigments and the absorbancy of Chl *a*. This index varies with the different populations and environmental conditions, and has been considered to reflect the physiological state of the cell.

Axenic Cultures. Axenic cultures were achieved as proposed by the Woods Hole Oceanographic Institution (24) by repeated inoculation of antibiotic-containing media (h/2-1A). After three or four steps of antibiotic treatment, the axenic character of the strain was checked by inoculating a medium containing synthetic seawater (1 liter), peptone (10.0 g), and yeast extract (1.0 g) (pH 7.4) with 1 ml of the antibiotic-treated culture. This medium was incubated for at least 48 hr in the dark, and bacterial growth was investigated turbidimetrically, and by microscopic observation. Antibiotic treatments were performed when the cultures became contaminated.

Nitrogen-deficient Cells. Cells were grown in 2-liter Erlenmeyer flasks containing 800 ml of medium f/2-1, 25 μM in nitrite, under the conditions described above. The inoculum consisted of 20 ml

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of a 48-hr-old culture, grown on the same medium, except that the initial concentration of nitrite was 50 μM. The culture was incubated for 120 hr, although no nitrite could be detected in the medium after 62 hr. At the end of the culture, the biomass corresponded to 40 ± 5 μg Chl *a*/l. These cells were considered N-deficient and used for nitrate uptake assays.

Nitrate Uptake Assay. Nitrate uptake was assayed by a modification of the method of Eppley and Coatsworth (3). N-deficient cultures were divided into 100-ml aliquots (3.5–4.5 μg Chl *a*) in 250-ml Erlenmeyer flasks, to which different amounts of $NaNO_3$ (0–15 μM) were added. The cells were incubated under the conditions described above. The incubation times were chosen in such a way that at low nitrate concentrations, less than 50% of the added nitrate was taken up, and, at high (> 10 μM) nitrate concentrations, the excess nitrate was more than 2 μM. After incubation, the cells were harvested by filtration under reduced pressure on glass-fiber GF/C Whatman filters. The filtrates were stabilized with few drops of chloroform and stored at 4 C for nitrate and nitrite analysis.[4] v_i NO_3^- was calculated as the ratio:

$$\frac{\text{Initial nitrate} - \text{final nitrate} + \text{final nitrite}}{\text{Incubation time} \times \text{amount of Chl } a}$$

and v_o NO_2^- was calculated as the ratio:

$$\frac{\text{Final nitrite}}{\text{Incubation time} \times \text{amount Chl } a}$$

where initial nitrate is the concentration of nitrate in the incubation medium at the beginning of the uptake assay and final nitrate and nitrite the concentrations of these ions in solution at the end of the uptake period, respectively.

Both cases were expressed as nmol/hr × μg Chl *a*.

Nitrate and Nitrite Determination. Nitrite was determined by diazotization with sulfanilamide and N-(1,naphthyl)-ethylenediamine as described by Nicholas and Nason (13).

Nitrate was determined as nitrite after reduction by means of a copper-coated cadmium column, as described by Wood *et al.* (23) except that ammonium chloride was used instead of EDTA as a reduction activator; 15- × 1-cm columns were used with a flow rate of 10 ml/min. Column efficiency was frequently tested by running standards 2, 5, and 10 μM in nitrate. At the beginning of the operation, the system was activated by running a solution 40 μM in nitrate. Samples containing both nitrate and nitrite were assayed for nitrite before and after the reduction step.

RESULTS

Growth of *S. costatum* with Various Nitrogen Sources. The cells were cultured as stated previously for growth experiments, with 25 μM $NaNO_3$, $NaNO_2$, or NH_4Cl as the only N source. Figure 1 shows the kinetics of growth on these three salts. Similar patterns were observed in all cases. Cells growing on ammonium had higher growth rates during the first days than those growing on nitrate or nitrite, because the nitrate and nitrite reductases are adaptive enzymes (20).

The utilization of nitrite for the growth of *S. costatum* was studied at 25 and 50 μM nitrite (Fig. 2). When the initial nitrite concentration was 50 μM, the rate of cell growth and substrate consumption was smaller than in the case of initial concentration of 25 μM. This inhibiting effect was more apparent at higher nitrite concentrations, and has been attributed to some toxic effect of nitrite at high concentrations (6). In the case of 50 μM nitrite, this nutrient disappeared from the medium by the 5th day, and the culture reached the stationary phase after 10 days. When the medium was 25 μM in nitrite, this ion was exhausted after 62 hr,

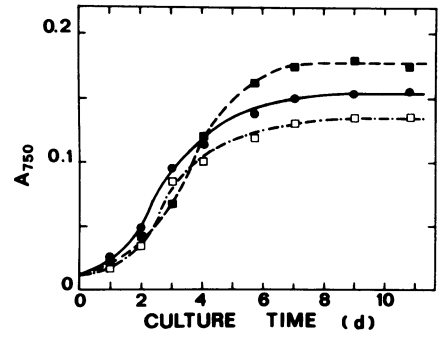

FIG. 1. Growth curves of *S. costatum* with different inorganic N sources: nitrate (■), nitrite (□), ammonium (●).

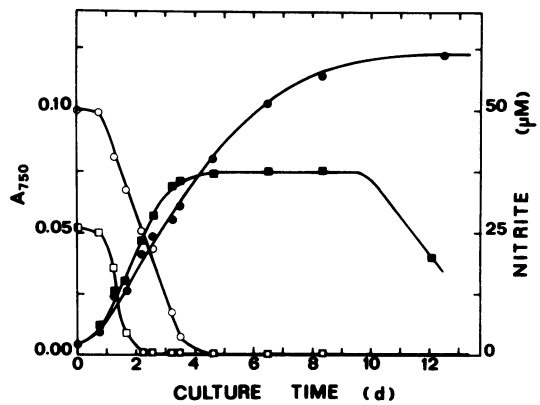

FIG. 2. Nitrite utilization and *S. costatum* growth curves for different initial nitrite concentrations. Fifty μM nitrite: nitrite utilization (○), cell growth (●); 25 μM nitrite: nitrite utilization (□), cell growth (■).

and the stationary phase was reached by the 4th day. The death phase began toward the 10th day. Growth is controlled by the amount of available nitrite and stops shortly after the exhaustion of this nutrient.

The cells grown at an initial 25 μM concentration of nitrite were used for the nitrate uptake experiments when they had reached the stationary phase of growth. The Margalef index (11) was routinely measured in these cells, and similar values, around 3.3, were found in all cases, indicating that the physiological state of the cells was in all cases similar.

Nitrate Uptake and Nitrite Release as a Function of Cell Density. Nitrate uptake from and nitrite release to the incubation medium were simultaneously studied in N-deficient cells at various cell densities. Algal cultures (4.0 μg Chl *a*/100 ml) were diluted with different amounts of sterile N-free f/2-1 medium. $NaNO_3$ was added to 100-ml portions of the different dilutions up to a final concentration of 5 μM. The diluted cultures were incubated for 1 hr as indicated above. At the end of the incubation time the cells were harvested and nitrite and nitrate were determined in the filtrate (Fig. 3A). It can be seen that v_i NO_3^- and v_o NO_2^- were linearly dependent on cell density.

The same experiments were carried out but preincubating the cells with 1 μM nitrate for 4 hr prior to the dilution and 1-hr incubation with 5 μM nitrate. v_i NO_3^- and v_o NO_2^- were again linearly dependent on cell density, but v_i NO_3^- was higher and v_o NO_2^- was lower than in the case of nonpreincubated cells (Fig. 3A).

Figure 3B shows the net N uptake rate (v_i NO_3^- − v_o NO_2^-) as a function of cell density. Cells preincubated with nitrate were able to take up more N than those not preincubated at any of the cell densities under study.

Nitrate Uptake and Nitrite release as a Function of Time.

[4] Abbreviations used: v_i NO_3^-: nitrate uptake rate; v_o NO_2^-: nitrite release rate; CHI: cycloheximide; *p*-HMB: *p*-hydroxymercuribenzoate.

Cultures of N-deficient cells were divided into 100-ml aliquots. $NaNO_3$ was added to all of them, up to a concentration of 10 μM nitrate, and the flasks were incubated for varying lengths of time (15–135 min). As in the previous case, some experiments were performed with cells preincubated with 1 μM $NaNO_3$ for 4 hr. At the end of the incubation time the cells were harvested and the concentrations of nitrite and nitrate determined in the cell-free filtrate (Fig. 4). Nitrate uptake and nitrite release were linearly dependent on time for all of the period under consideration. The positive effect of nitrate preincubation on the net N uptake was also observed here.

Nitrate Uptake and Nitrite Release as a Function of Nitrate Concentration. Effect of Preincubation with Nitrate, CHI and *p*-HMB. The kinetic behavior of nitrate uptake and nitrite release was studied by the standard procedure at the nitrate concentrations naturally found by diatoms in their habitat. In order to investigate the effect of nitrate, CHI, and *p*-HMB on the nitrate uptake and nitrite release, the cells were preincubated for 4 hr with 1 μM nitrate or for 30 min in the presence of CHI (at 5 μg/ml) or *p*-HMB (at 1 mM), respectively.

Figure 5 shows that nitrite release followed a classical saturation kinetics represented by a hyperbolic curve, whether the cells were preincubated with nitrate or not. However, nitrate uptake kinetics followed the hyperbolic pattern at low nitrate concentrations only; for concentrations higher than 6 μM the system did not show a saturation kinetics, but rather a linear dependency on the nitrate concentration outside the cell.

Previous exposure of cells to CHI or *p*-HMB led to a significant decrease of the nitrate uptake (Fig. 6) at low nitrate concentrations,

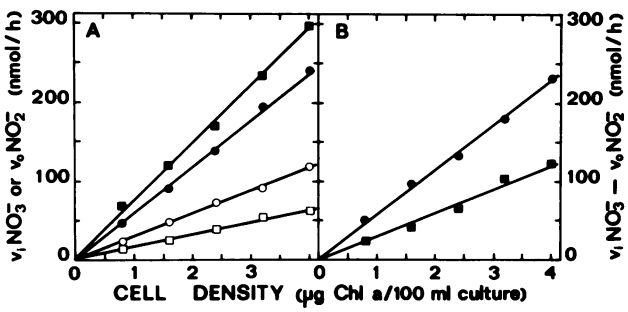

FIG. 3. A: nitrate uptake and nitrite release by *S. costatum* as a function of cell density. Cells preincubated with 1 μM nitrate: v_i NO_3^- (■), v_o NO_2^- (□). Nonpreincubated cells: v_i NO_3^- (●), v_o NO_2^- (○). B: net N uptake as a function of cell density. Cells preincubated with 1 μM nitrate (●); nonpreincubated cells (■). Initial nitrate concentration was 5 μM.

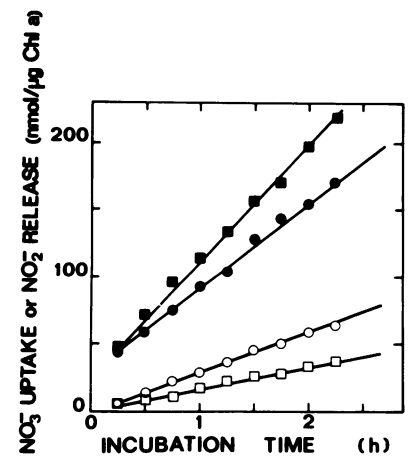

FIG. 4. Nitrate uptake and nitrite release by *S. costatum* as a function of incubation time. Cells preincubated with 1 μM nitrate: nitrate uptake (■), nitrite release (□); nonpreincubated cells: nitrate uptake (●), nitrite release (○). Initial nitrate concentration was 13 μM.

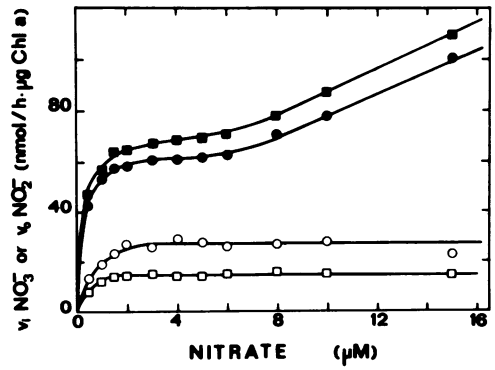

FIG. 5. Nitrate uptake and nitrite release by *S. costatum* as a function of nitrate concentration. Cells preincubated with 1 μM nitrate: v_i NO_3^- (■), v_o NO_2^- (□). Nonpreincubated cells: v_i NO_3^- (●), v_o NO_2^- (○).

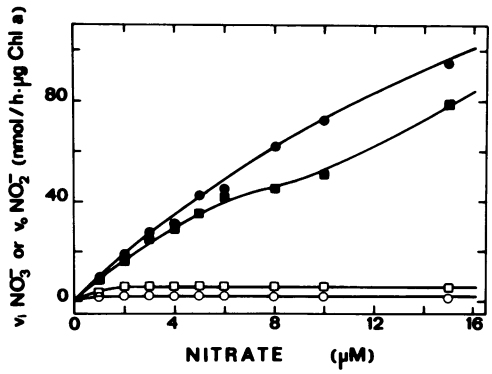

FIG. 6. Effect of CHI and *p*-HMB on nitrate uptake and nitrite release by *S. costatum* as a function of nitrate concentration. Cells preincubated with CHI (5 μg/ml): v_i NO_3^- (■), v_o NO_2^- (□). Cells preincubated with *p*-HMB (1 mM): v_i NO_3^- (●), v_o NO_2^- (○).

and the rate of nitrite release was lowered and was practically negligible.

DISCUSSION

N-deficient algal cultures, which assimilate nitrate rapidly, can be readily prepared by subjecting them to N starvation. The use of such cultures has greatly aided the study of N assimilation (22). N-deficient cells show a series of unusual properties which make them suitable for the study of nitrate assimilation. When they are incubated with nitrate, they are able to take up this ion, to reduce it to nitrite, and to release some of the nitrite to the incubation medium.

In the present work, we have obtained cultures of N-deficient *S. costatum* cells by using nitrite as the limiting N source in the medium. At a 25 μM nitrite concentration, the cultures grew normally (Figs. 1 and 2) without showing any of the toxic effects which have been attributed to nitrite at higher concentrations (6). In addition, this nitrite concentration gives a final cell density suitable for the study of nitrate uptake at physiological concentrations, these nitrite levels being easy to determine by means of the diazotization reaction.

When N-deficient cells were preincubated for 4 hr with 1 μM $NaNO_3$ an increase in nitrate uptake and decrease in nitrite release as compared to control rates were observed. This resulted in a net increase in N assimilation (Fig. 3B). At the end of the 4-hr preincubation, neither nitrate nor nitrite could be detected in the medium.

Uptake rates of nitrate or ammonium by marine phytoplankton yield hyperbolae when plotted against the external concentration of nitrate or ammonium (4, 10). Half-saturation constants (Ks,

concentration supporting half-maximal uptake rate) can be calculated from the hyperbolae using the Michaelis-Menten equation: $v = V S/(Ks + S)$.

The kinetics of nitrate uptake in N-deficient cells of *S. costatum* does not follow the classical saturation kinetics mentioned by other authors (5). When nitrate concentrations become higher than 6 μM, v_i NO_3^- is higher than expected from Michaelian kinetics (Fig. 5). This behavior can be explained by the additive, simultaneous contribution of a linear simple (non-carrier-mediated) diffusion and a hyperbolic, saturation transfer, with a carrier for nitrate. The contribution of single diffusion would become apparent at nitrate concentrations higher than 6 μM.

Classically (21), three types of mixed transfer have been described. In addition to the "simple diffusion *plus* carrier-mediated transport," which has just been mentioned, the existence of two different carriers, with different kinetics constants or of a single, allosteric carrier have been described. Rao and Rains (17) have published the kinetics of nitrate uptake in barley (*Hordeum vulgare*) which appears to be identical to our case. Other similar data have been interpreted either postulating the existence of two carriers or invoking a multiphasic mechanism (14). If there were two different carriers, both proteins should be similarly affected by CHI and the thiol reagents. The hypothetical high concentration carriers, or high concentration forms of the allosteric carriers, seem surprisingly resistant to the action of these rather unspecific reagents. The kinetics of nitrite release is of hyperbolic nature, and should be carrier-associated. A common permease exists for nitrate uptake and nitrite release, provided that their kinetic parameters were different for nitrate and nitrite, as well as for both sides of the membrane. Butz and Jackson (2) have recently proposed that nitrate reductase could be the carrier and catalyst protein which would incorporate and reduce nitrate. Similarly, this enzyme could be the carrier for nitrite excretion.

The increase in nitrate uptake and decrease in nitrite release after nitrate preincubation could be explained by assuming that the permease, and/or the nitrate and nitrite reductases, is nitrate-inducible (7, 12). In cells preincubated with 1 μM nitrate, the cellular levels of these proteins, at the beginning of the nitrate uptake assay, are higher than in the case of the nonpreincubated cells. This leads to the observed increase in N assimilation (Fig. 3B).

p-HMB, like all mercurials, blocks the protein —SH groups. It is a rather unspecific reagent, since it does not discriminate between different sorts of —SH groups. Its blocking action on membrane —SH groups is well known, in such a way that it inhibits many carrier-mediated ion translocation processes. The kinetics of nitrate uptake after *p*-HMB treatment looks similar to a simple diffusion kinetics (Fig. 6). The apparent "enhancement" of diffusion by *p*-HMB and other mercurial reagents has been pointed out for the case of mitochondrial membranes (1).

CHI, which is a well known inhibitor of protein synthesis in eukariotes, affects nitrate transport in a way analogous to the thiol reagents (9) (Fig. 6). In cells preincubated with CHI the nitrate uptake kinetics shows a very small contribution of carrier-mediated transport, probably due to a residual permease level and/or because CHI can increase the intracellular concentration of amino acids. CHI can also alter nitrate uptake by blocking energy transfer and oxidative phosphorylation (16). Its possible simultaneous effect at these different levels would explain its rapid and effective action.

Nitrite release is also affected by the *p*-HMB or CHI pretreatment. These results are understandable since the nitrate reductase activity of *S. costatum*, as well as the enzyme from other sources, is sensitive *in vitro* to thiol reagents and its synthesis can be repressed with CHI (20). In addition, *p*-HMB could affect the nitrite release, as would be expected in the case of carrier-mediated transport.

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