Plant Physiol. (1974) 54, 629–637

Purification and Characterization of the Nitrate Reductase from the Diatom *Thalassiosira pseudonana*[1]

Received for publication February 8, 1974 and in revised form June 3, 1974

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ABSTRACT

The assimilatory nitrate reductase (NADH: nitrate oxidoreductase, E.C. 1.6.6.2.) from the marine diatom *Thalassiosira pseudonana*, Hasle and Heimdal, has been purified 200-fold and characterized. The regulation of nitrate reductase in response to various conditions of nitrogen nutrition has been investigated.

Nitrate reductase activity is repressed by the presence of ammonium *in vivo*, and its synthesis is derepressed when ammonium is absent. The derepression process is sensitive to cycloheximide and apparently requires protein synthesis. Repression of enzyme activity by ammonium is neither inhibited nor delayed by the presence of cycloheximide. *In vitro*, ammonium does not inhibit enzyme activity.

NADH is the physiological electron donor for the enzyme in a flavin-dependent reaction. Spectral studies have indicated the presence of a *b*-type cytochrome associated with the enzyme. It is possible to observe enzymatic oxidation-reduction reactions which represent partial functions of the over-all electron transport capacity of this enzyme. Nitrate reductase will accept electrons from artificial electron donors such as reduced methyl viologen in a flavin-independent reaction. Further, dithionite-reduced flavin adenine dinucleotide can donate electrons to the enzyme to reduce nitrate to nitrite. Finally, the nitrate reductase will exhibit a diaphorase activity and reduce the artificial electron acceptor mammalian cytochrome *c* in flavin-adenine-dinucleotide-dependent reaction.

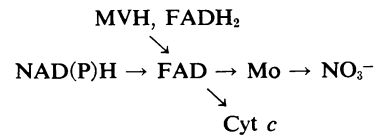
Inhibition studies with potassium cyanide, sodium azide, and *o*-phenanthroline have yielded indirect evidence for metal component(s) of the enzyme.

The inhibition of the NADH-requiring enzyme activities by *p*-hydroxymercuribenzoate has shown that an essential sulfhydryl group is involved in the initial portion of the electron transport. Heat treatment exerts an effect similar to the *p*-hydroxymercuribenzoate inhibition; namely, the NADH-requiring activities are rapidly inactivated, whereas the terminal nitrate-reducing activities are relatively stable to heat.

The *T. pseudonana* nitrate reductase molecule has the hydrodynamic properties of an ellipsoid with a frictional coefficient of 1.69 and a molecular weight of 330,000.

The ability to utilize nitrate as a nitrogen source occurs in many bacteria, fungi, algae, and higher plants. Nitrate reductase, the first enzyme in the pathway of nitrate assimilation, is generally found to be a molybdoflavoprotein that utilizes NADH or NADPH as the physiological electron donor (4, 11, 19).

The transfer of electrons from NAD(P)H to nitrate can be envisioned as two sequential enzymatic activities: an FAD-dependent NAD(P)H diaphorase, and a terminal nitrate reductase. The electron transport pathway can be represented as follows.[2]



A Cyt *b*-557 which appears to function in the electron transport pathway between FAD and molybdenum has been identified in *Neurospora* (6) and *Chlorella vulgaris* (25).

An NAD(P)H diaphorase activity can be measured independently of nitrate reduction by substituting mammalian Cyt *c* as an electron acceptor from NAD(P)H in an FAD-dependent reaction. The terminal nitrate reductase activity can be measured by utilizing artificial electron donors such as MVH or $FADH_2$ for nitrate reduction. Either the diaphorase or terminal nitrate reductase can be selectively inhibited, but the two have not been physically separated with retention of activity. Thus, the NAD(P)H nitrate reductase activity probably represents the physiological function of the enzyme, and the associated activities: NAD(P)H-Cyt *c* reductase, MVH-nitrate reductase, and $FADH_2$-nitrate reductase represent partial functions of the over-all electron transport capacity of this enzyme.

In spite of the fact that nitrate reductase has been intensively studied (4), very little research has been undertaken concerning the nitrate reductase of bacterial or algal species populating oceans and estuaries. Nitrate reduction is a particularly vital function of marine algae because nitrogen has been identified as the rate-limiting nutrient and controlling factor for the growth of these organisms (22). The purpose of this research was to investigate the regulation and enzymology of nitrate assimilation in a marine alga and to gain further insight into this process through a comparison of this algal nitrate reductase with the other nitrate reductases which have been characterized.

The marine alga chosen for investigation, *Thalassiosira pseudonana*, is a small centric diatom. Clones of this species inhabit a range of environments from the nutrient-rich brackish

[1] This research was supported by the National Institutes of Health Grant GM 18955 and a P. F. duPont Fellowship.

[2] Abbreviations: NADH → NO_3^-: NADH-nitrate reductase; $FADH_2$ → NO_3^-: reduced flavin adenine dinucleotide nitrate-reductase; MVH → NO_3^-: reduced methyl viologen-nitrate reductase; NADH → Cyt *c*: NADH-Cyt *c* reductase; pHMB: *p*-hydroxymercuribenzoate.

estuaries to the nutrient-poor mid-ocean regions (9). This relatively cosmopolitan species can be considered as a representative marine organism and is interesting because of its adaptability to a wide range of conditions. This investigation utilized clone 3H of *T. pseudonana,* which was isolated from an estuary and is able to survive in a wide range of salinities and temperatures and is capable of utilizing a variety of nitrogen sources.

A preliminary account of this research has been presented (2).[3]

MATERIALS AND METHODS

Culture Conditions. Bacteria-free cells of *Thalassiosira pseudonana,* Hasle and Heimdal (10), clone 3H, were a gift of Dr. Robert Guillard. The cells were routinely maintained on sterile media f/2 prepared according to Guillard and Ryther (9), employing artificial sea water (Instant Ocean) made up to a concentration which was 80% that of average sea water. The diatoms were maintained and subcultured in 100 ml of the above media in 250-ml flasks. Cultures were transferred to 1.5 liters of media in 3-liter flasks, and bubbled with a steady stream of air. After approximately 5 days of growth, the contents of a 3-liter flask were used to inoculate 15 liters of f/2 media in a 5-gallon glass carboy. Growth was continued for 5 more days with continuous illumination from two banks of Gro-Lux fluorescent lamps, and with aeration by a 5% CO_2-air mixture. The ambient temperature was approximately 23 C. Cells from eight carboys were harvested by a Sorvall continuous flow centrifugation apparatus at a speed of 12,000*g,* and a flow rate of approximately 400 ml/min. The cells were washed with cold artificial sea water, and then stored at −15 C for a period ranging from 12 hr to 2 months. Forty to sixty grams of packed cells were routinely obtained from 120 liters of media.

Assays. NADH-nitrate reductase activity was determined by assaying for the enzymatic production of nitrite, as modified from Garrett and Nason (7). The concentration of nitrite was determined colorimetrically by a modification of the diazo coupling procedure described by Nicholas and Nason (20). Alternatively, activity was measured by determining the rate of oxidation of NADH by following the decrease in absorbance at 340 nm in the above reaction mixture doubled to give a final volume of 1 ml. Enzyme was added in sufficient amounts to give a decrease in absorbance of 0.04 to 0.2/min as measured by the Beckman DU spectrophotometer or by the Cary 14 recording spectrophotometer.

$FADH_2$-nitrate reductase activity, reduced methyl viologen-nitrate reductase activity, and NADH-Cyt *c* reductase activity were assayed according to Garrett and Nason (7).

Protein concentration was measured either colorimetrically by a modification of the Folin method described by Lowry (17) with crystalline BSA as the standard, or by the ratio of absorbance at 280 nm and 260 nm as described by Layne (13). The activity units for NADH-nitrate reductase, $FADH_2$-nitrate reductase, and MVH-nitrate reductase are defined as the formation of 1 nanomole of nitrite/min. The NADH-Cyt *c* reductase activity units are defined as the reduction of one nanomole of Cyt *c*/min. Specific activity is activity units/mg protein.

The double reciprocal Lineweaver-Burk plots for the determination of the K*m* values were drawn according to coordinates obtained from analysis of the data by the least squares method using a Wang Series 370 electronic calculator.

Purification of NADH-Nitrate Reductase and Associated Activities. All purification procedures were carried out at 0 to 4 C. Buffers contained 1 mM mercaptoethanol and 1 mM EDTA and were adjusted to pH 7.9. In a typical purification preparation, 180 g of frozen *T. pseudonana* cells were homogenized in a 1-liter Waring Blendor operated at low speed and containing 540 ml of 0.2 M phosphate buffer (3 ml/g of cells) and 18 g of insoluble PVP. Polyvinyl polypyrrolidone was added to absorb and precipitate plant phenols and tannins which interfere with enzyme extraction (12). Homogenization was performed in three equal sized batches for 30 sec each, then the crude homogenate was centrifuged for 30 min at 16,000*g* to yield a dark green supernatant solution, designated the crude extract (fraction 1).

Solid ammonium sulfate was added slowly with constant stirring to give a final concentration of 45% saturation. Stirring was continued for an additional 45 min, then the enzyme extract was centrifuged for 15 min at 16,000*g*. The pink supernatant was decanted and the pellet was redissolved in 0.2 M phosphate buffer, yielding the 0 to 45% ammonium sulfate precipitate (fraction 2).

The nucleic acids and much of the Chl and other pigments were precipitated by the addition of 30% (w/w) polyethylene glycol (Carbowax 6000) in the proportion of 1 ml/3 ml of fraction 2. This solution was stirred for 30 min, then centrifuged for 15 min at 20,000*g* to yield a tan colored supernatant solution, which was designated the polyethylene glycol treatment (fraction 3).

Fraction 3 was desalted in two 100-ml size Dow ultrafiltrators (code No. b/HFU-1) while approximately 10 liters of 50 mM phosphate buffer passed through each of the units. There is usually some loss of protein since the ultrafiltrator only retains proteins of 30,000 mol wt or greater.

Dialyzed fraction 3 was then applied to a DEAE-cellulose column (Whatman DE-52, 4.5 × 13 cm, bed volume 206 ml), previously equilibrated with 50 mM phosphate buffer. The column was washed with the same buffer, then eluted with a linear gradient generated from 300 ml each of 50 mM and 450 mM phosphate buffer. The fractions with the highest specific activity eluted at approximately 0.3 M phosphate and were pooled to form the DEAE eluate (fraction 4).

Solid ammonium sulfate was added to fraction 4 with constant stirring to give a final concentration of 30% ammonium sulfate saturation. Stirring was continued for 30 min, then the solution was centrifuged for 15 min at 20,000*g*. To the resulting supernatant solution was added solid ammonium sulfate to give a final concentration of 45% saturation. This ammonium sulfate-treated fraction was allowed to stand at 4 C overnight, then centrifuged for 15 min at 20,000*g*. The resulting pellet was redissolved in 50 mM phosphate buffer and designated the 30 to 45% ammonium sulfate precipitate (fraction 5). The enzyme may be stored frozen at this stage at −20 C in 30% glycerol with little loss of activity.

Fraction 5 was desalted by dialysis for 3 hr against three 2-liter changes of 10 mM phosphate buffer, and subsequently applied to a hydroxylapatite column (1.5 × 6 cm, bed volume 10.5 ml), previously equilibrated with 10 mM phosphate buffer. The column was eluted with a series of phosphate buffers of increasing molarity: 10 mM, 50 mM, 100 mM, 200 mM, and 450 mM. NADH-nitrate reductase eluted at 200 mM phosphate, and the fractions with the highest specific activity were pooled and designated the pooled hydroxylapatite eluate (fraction 6).

Fraction 6 was then precipitated by the addition of solid ammonium sulfate with constant stirring to a final concentration of 50% saturation. Stirring was continued for 30 min, then the extract was centrifuged for 15 minutes at 20,000*g*. The supernatant solution was discarded and the 0 to 50% ammonium sulfate precipitate (fraction 7) was redissolved in 1 ml of 50 mM phosphate buffer.

[3] These results are presented in greater detail in the dissertation submitted by N.K.A. to the University of Virginia in partial fulfillment of the requirements for the Ph.D. degree.

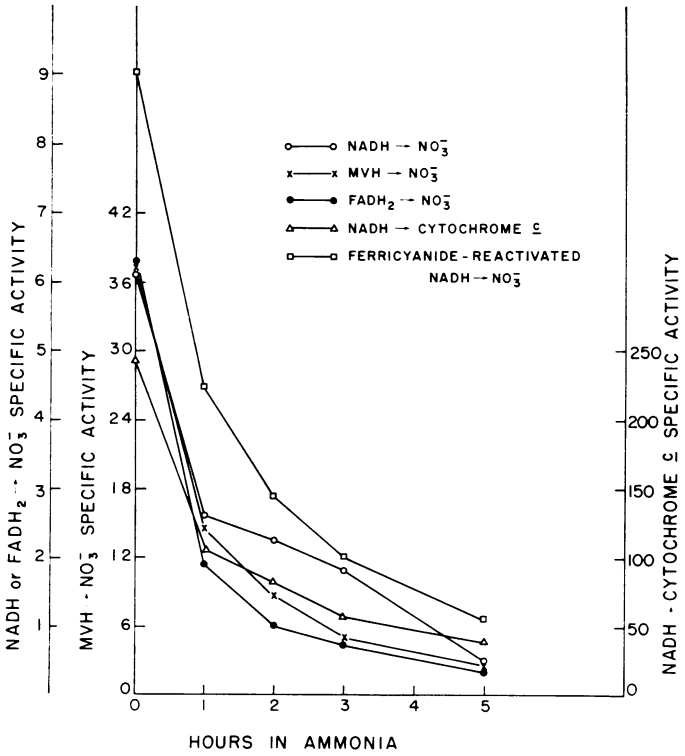


FIG. 1. Inactivation of NADH-nitrate reductase and associated activities by ammonium chloride. *T. pseudonana* cells were grown on media f/2 with 0.3 mM nitrate and possessed nitrate reductase. At the time given as zero, ammonium chloride was added to give a final concentration of 1 mM. At the indicated times, aliquots of cells were collected by centrifugation for 10 min at 16,000*g*, then washed in nitrogen-free sea water and centrifuged. The pellet of cells was resuspended in one ml of cold 0.2 M phosphate buffer, pH 7.9, with 1 mM EDTA and 1 mM mercaptoethanol. One to ten mg of insoluble PVP were added. The cell suspension was sonicated for 20 sec then centrifuged for 10 min at 12,000*g*. The resulting suspernatant solution was assayed by the standard methods. The ferricyanide-reactivated NADH-nitrate reductase was prepared by incubating the enzyme extract with 0.2 mM ferricyanide and phosphate buffer, pH 7.9, for 5 min at 0 C. The enzyme extract was warmed to room temperature, then the other components of the assay mixture were added and the reaction was initiated as usual.

Fraction 7 was applied to a Sephadex G-200 gel filtration column (2.5 × 37.5 cm, bed volume 184 ml), previously equilibrated with 50 mM phosphate buffer, and eluated with the same buffer. The column was developed by upward flow with a pressure head of 12 cm, causing a flow rate of 12 ml/hr. The Sephadex eluates containing the nitrate reductase activity were pooled and concentrated by ultrafiltration, yielding the Sephadex G-200 eluates (fraction 8) and representing a 190-fold purification with 5% recovery of enzymatic activity.

Instant Ocean was purchased from Aquarium Systems, Inc., Eastlake, Ohio; PVP from Calbiochem; DEAE cellulose (Whatman DE-52) from H. Reeve Angel; and hydroxylapatite was purchased from Clarkson Chemical Co. as Hyapatite-C.

RESULTS

***In Vivo* Regulation of Nitrate Reductase.** The time course of inactivation of the NADH-nitrate reductase and its associated activities by ammonium is seen in Figure 1. When nitrate-grown *T. pseudonana* cells possessing nitrate reductase were transferred to f/2 media in which ammonium chloride was the sole nitrogen source, or when ammonium chloride was added to the nitrate f/2 media, there was a rapid decline in the four associated activities. In contrast to the results reported with *Chlorella* (15) and *Chlamydomonas* (16), the diaphorase activity, as measured by the NADH-Cyt *c* reductase activity, decreased with the other activities. The decline in the nitrate reductase activity was not delayed or prevented by the addition of cycloheximide (5 μg/ml) with the ammonium chloride (not shown), indicating that the rapid decline in the enzymatic activity was not mediated by synthesis of new protein.

Cycloheximide was added to cells possessing nitrate reductase growing in nitrate media. The nitrate reductase activity declined slowly with more than 50% of the activity remaining after 5 hr. The simple cessation of protein synthesis does not account for the rapid decline in nitrate reductase activity caused by ammonium.

When enzyme extracts from nitrate-grown *T. pseudonana* cells were preincubated at 0 C for 5 min with ferricyanide, there was some increase in nitrate reductase activity, as indicated in Figure 1. When extracts from cells possessing an ammonium-inactivated nitrate reductase were preincubated with ferricyanide, the activity of the enzyme was never restored to the activity of the initial nitrate-grown state; in fact, the rate of decline of the ferricyanide-treated nitrate reductase activity exactly parallels the rate of decline of the ammonium-inactivated extracts.

Figure 2 illustrates the induction of nitrate reductase activity

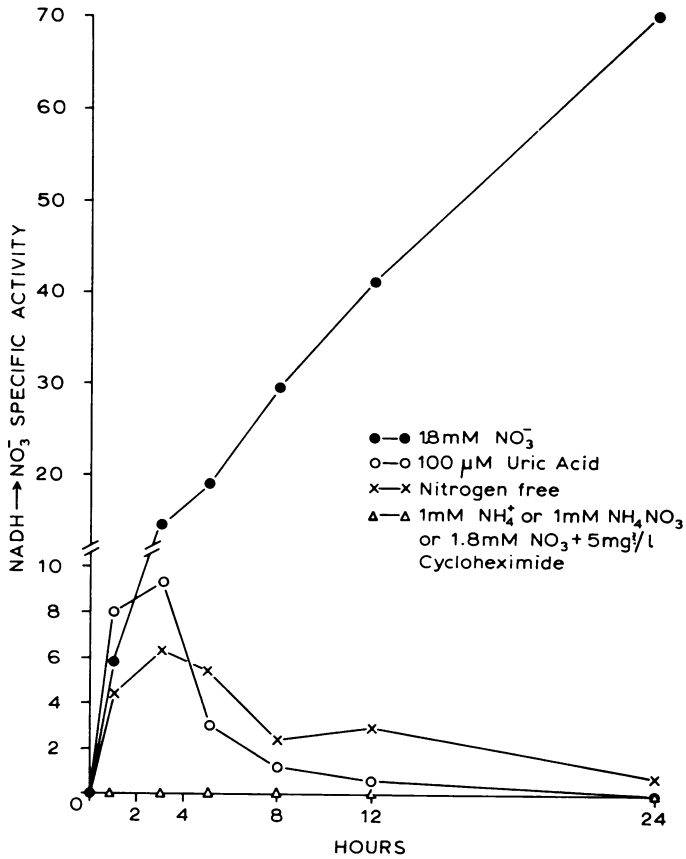


FIG. 2. Effect of various nitrogen sources on the induction of nitrate reductase activity. *T. pseudonana* cells were grown in ammonium f/2 media, washed with nitrogen-free sea water, then resuspended in media f/2 with the different nitrogen sources at time zero. At the indicated intervals, the cells were collected and extracts were prepared as indicated in the legend to Fig. 1. The NADH-nitrate reductase activity was assayed by the standard procedure.

when the *T. pseudonana* cells were transferred to various nitrogen sources. When the diatoms were transferred from ammonium media, in which they express no nitrate reductase activity, to nitrate f/2 media, there was a rapid increase in nitrate reductase activity. The four activities associated with the enzyme increased coordinately (not shown). A small amount of constitutive Cyt *c* reductase activity was present in ammonium-grown cells, but upon transfer to nitrate media, there was a marked increase in the NADH-Cyt *c* reductase activity.

When ammonium-grown cells were transferred to nitrogen-free media, there was a significant increase in nitrate reductase activity, which indicated the derepressible nature of the nitrate reductase synthesis in this organism. The decline in activity at 5 hr is probably due to exhaustion of the available cellular nitrogen, compounded with the normal turnover of the nitrate reductase. In the presence of uric acid, nitrate reductase activity was also derepressed; however, the rapid decline in nitrate reductase activity after 5 hr might be attributed to the metabolism of the uric acid which could produce a pool of intracellular ammonium, which in turn would cause repression of the nitrate reductase.

No nitrate reductase activity was present when the cells were exposed to ammonium nitrate, indicating that the ammonium-repressive effect predominated. Nitrate reductase activity was not induced in the presence of nitrate plus cycloheximide or uric acid plus cycloheximide.

Purity of Enzyme. In eight similar purification preparations, as described under "Materials and Methods," specific activities ranging from 660 to 3,120 units of NADH-nitrate reductase/mg protein were obtained, with an average purification of 211-fold. Typical results from an enzyme purification are shown in Table I.

Polyacrylamide gel electrophoresis at pH 8.3 performed with fraction 8 protein, specific activity 2050, revealed the presence of one major band and two minor bands, as seen in Figure 3.

Identical, unstained gels were frozen and sliced into 2-mm sections with the Mickle gel slicer. Each section was placed in a tube of cold assay buffer and pulverized with a glass rod. The tubes were warmed to room temperature, the components of the assay mixture were added, and the enzymatic activity was assayed. The peak of enzymatic activity of the NADH-nitrate reductase, MVH-nitrate reductase, and $FADH_2$-nitrate reductase corresponded to the position of the major band on the stained gel. The recovery of the NADH-nitrate reductase activity was very poor, possibly due to inhibition by the ammonium persulfate used to polymerize the gels.

PROPERTIES OF NITRATE REDUCTASE

Behavior of Nitrate Reductase on Sephadex G-200 Gel Filtration Columns. Figure 4 shows the elution profile of nitrate reductase from an analytical Sephadex G-200 gel filtration column. The void volume is indicated by the position of the peak of blue dextran, which was excluded from the gel beads and eluted at a volume of 68 ml. All the activities of the nitrate reductase eluted coincidently, indicating that they all are associated with the same protein complex.

The Stokes or molecular radius of the nitrate reductase determined from gel filtration data by employing the methods of Gelotte (8) and Ackers (1) is 77A.

The sedimentation coefficient or $s^{0,725}_{20,w}$ value for nitrate reductase was obtained by sucrose density gradient centrifugation experiments according to the method of Martin and Ames (18). A mixture of partially purified nitrate reductase and the marker enzymes catalase and yeast alcohol dehydrogenase was layered on a linear sucrose gradient solution prepared from 15.5 and 33% (w/v) sucrose solutions in 0.1 M phosphate buffer. The $s_{20,w}$ value for nitrate reductase was calculated from the relationship:

$$\frac{\text{distance migrated by unknown}}{\text{distance migrated by known}} = \frac{s_{20,w} \text{ of unknown}}{s_{20,w} \text{ of known}}$$

Table I. *Summary of Purification of NADH-Nitrate Reductase and Its Associated Activities*

Fraction	Volume	Total Protein	NADH-Nitrate Reductase			NADH-Cyt *c* Reductase			$FADH_2$-Nitrate Reductase			MVH-Nitrate Reductase		
			Total activity	Specific activity	Recovery	Total activity	Specific activity	Recovery	Total activity	Specific activity	Recovery	Total activity	Specific activity	Recovery
	ml	*mg*	*units*	*units/mg*	%	*units*	*units/mg*	%	*units*	*units/mg*	%	*units*	*units/mg*	%
1. Crude extract	480	5760	62,400	10.8	100	984,320	167	100	36,000	6.2	100	432,000	75	100
2. 45% $(NH_4)_2SO_4$ precipitate	140	3500	43,400	12.4	70	1026,200	293	103	37,240	10.6	103	392,000	112	90
3. Polyethylene glycol treatment	137	2192	42,470	19.4	68	824,740	376	84	24,660	11.2	68	290,440	132	67
4. DEAE-cellulose eluates	277	759	12,188	16	20	146,256	193	15	11,080	14.6	30	138,500	182	32
5. 30–45% $(NH_4)_2SO_4$ precipitate	10.5	182	12,480	68.3	20	105,000	574	11	5,355	29	29	94,500	517	21
6. Hydroxylapatite eluates	112	28	9,620	350	15	70,500	2,500	7	5,476	195	15	65,128	2,325	15
7. 50% $(NH_4)_2SO_4$ precipitate	2	13	9,676	756	15	61,000	4,800	6	5,305	414	14	61,632	4,815	14
8. Sephadex G-200 eluates	15	1.5	3,137	2,050	5	14,250	9,700	1	1,650	1,122	4	24,000	16,326	5

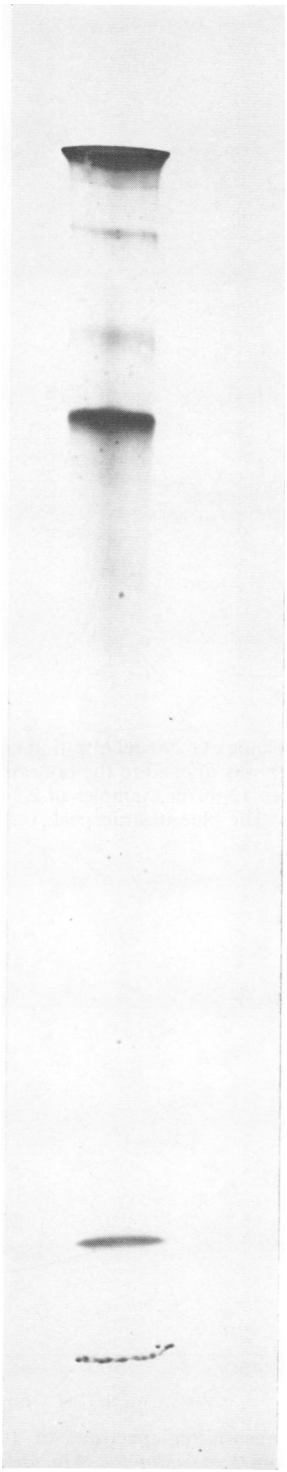


FIG. 3. Polyacrylamide gel electrophoresis of *T. pseudonana* nitrate reductase. Gels of 7.5% polyacrylamide were polymerized with ammonium persulfate and were 80 mm long with a 10-mm stacking gel (5). The stacking gel is not shown. Protein from fraction 8 (54 μg) with 0.05 ml of 0.1% bromphenol blue in 10% (w/v) sucrose was applied in a sample volume of 0.15 ml to the gel. Electrophoresis was performed for approximately 3 hr at 3 ma/tube at 4 C. The gels were stained with 1% buffalo black in 7.5% (v/v) acetic acid for 5 min, then the unreacted dye was removed by washing with 7.5% (v/v) acetic acid. The band at the bottom of the gel is the bromphenol blue tracking dye and the protein at the top of the gel is denatured protein.

The average sedimentation coefficient for nitrate reductase from *T. pseudonana* is 10.5. The data from three experiments are shown in Table II. All the activities which are associated with the NADH-nitrate reductase were assayed and found to sediment together.

The mol wt of nitrate reductase was estimated by the method of Siegel and Monty (24) utilizing both the Stokes radius (77A, as determined by gel filtration) and the sedimentation coefficient (10.5, as determined by sucrose density gradient centrifugation). Based on these values, the molecular weight of nitrate reductase was calculated to be 330,000 daltons. The frictional coefficient, a relative indication of the axial ratio of the molecule, was determined to be 1.69.

Absorption Spectrum. The visible absorption spectrum (Fig. 5) of the purified nitrate reductase (concentrated fraction 8, 0.42 mg/ml) from *T. pseudonana* indicates that the dithionite-reduced enzyme has absorption maxima at 554 and 423 nm. Although the sample was too dilute to reveal a typical β-absorption band in the 525-nm region, the observed absorption bands compare favorably with those of the *b*-type Cyt which has been identified in the *Neurospora* nitrate reductase (6) with absorption maxima at 557, 528, and 423 nm, and also with the *b*-type Cyt associated with the *Chlorella* nitrate reductase (25) with absorption bands at 556, 525, and 420 to 423.

pH Optimum. There is a broad pH optimum for nitrate reductase between pH 7 and 8 for the four associated activities (Fig. 6). The buffers potassium phosphate, citrate-phosphate, tris-phosphate, and tris-HCl at 0.1 M concentration were equally effective with both the MVH-nitrate reductase and the NADH-Cyt *c* reductase activities. Tris-phosphate buffer gave less activity for the NADH-nitrate reductase and $FADH_2$-nitrate reductase activities. Citrate-phosphate buffer appeared to stimulate the $FADH_2$-nitrate reductase activity.

Substrate Affinities. The apparent K*m* calculations for the substrates of the four activities of nitrate reductase are seen in Table III. Typical hyperbolic *v versus s* functions which yielded linear Lineweaver-Burk plots were obtained with all substrates and cofactors. NADPH was tested as an electron donor for the NADH-nitrate reductase and the NADH-Cyt *c* reductase activities, but the maximal velocity attained with this compound was very low (4% of that with NADH).

Effect of Heat Treatment. Figure 7 shows that mild heat treatment has a differential effect on the NADH-nitrate reductase and its associated activities. There is a rapid loss of both the NADH-nitrate reductase and the NADH Cyt *c* reductase activities, in which virtually all the NADH-nitrate reductase and more than 90% of the NADH-Cyt *c* reductase activity is lost after 5 min at 40 C. The $FADH_2$-nitrate reductase and MVH-nitrate reductase activities retain at least 80% of their original activity after 30 min. The latter two activities can be destroyed by treatment at 55 C for 30 min.

EFFECT OF INHIBITORS

***p*-Hydroxymercuribenzoate.** The mercurial, *p*-hydroxymercuribenzoate, which binds to sulfhydryl groups on proteins, is qualitatively similar to the heat treatment in its effect. The inhibition of nitrate reductase activity by increasing concentrations of pHMB is shown in Figure 8. The NADH-dependent activities, namely the NADH-nitrate reductase and NADH-Cyt *c* reductase were 50% inhibited at a concentration of 2 μM pHMB. The MVH-nitrate reductase and $FADH_2$-nitrate reductase were 50% inhibited only at concentrations greater than 100 μM pHMB, a concentration at which the inhibitor may exert nonspecific effects.

Metal-binding Agents. Metal-binding agents showed a contrasting pattern of inhibition. The three activities for which

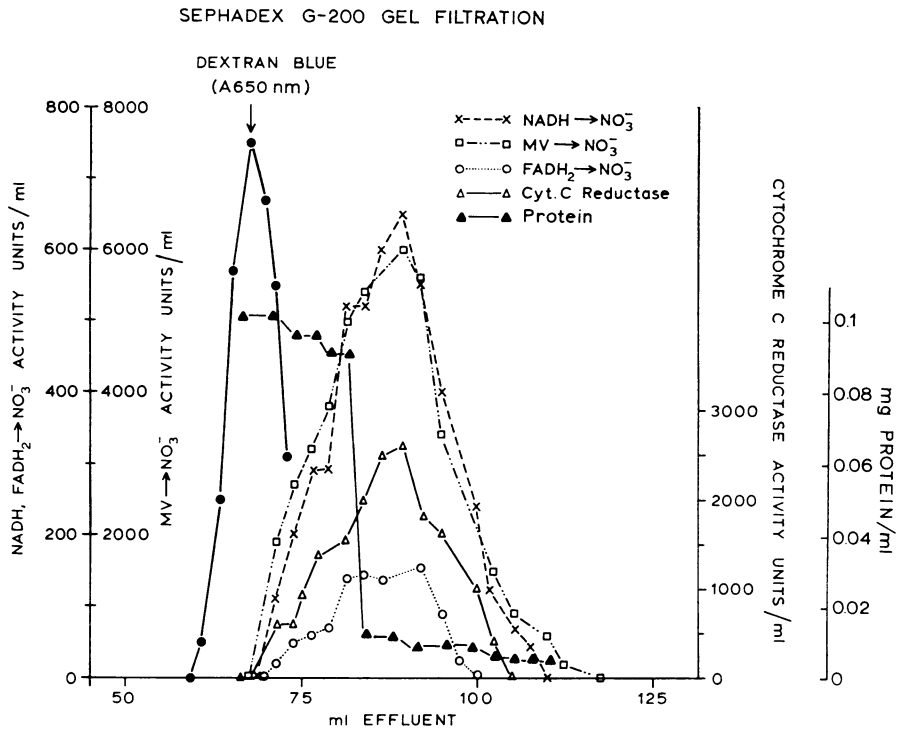


FIG. 4. Typical elution profile of NADH-nitrate reductase and associated activities on a Sephadex G-200 gel filtration column. A 1-ml aliquot of fraction 7 (specific activity 1250) containing 2.8 mg of protein in 50 mM phosphate buffer was applied to the bottom of a Sephadex G-200 column (2.5 × 37.5 cm, bed volume 184 ml) and developed upward with a gravity flow rate of 12 ml/hr. Samples of 2.5 ml were collected and assayed for NADH-nitrate reductase and its associated activities by the standard procedures. The blue dextran peak was determined in a separate experiment because nitrate reductase appears to adsorb to the blue dextran.

Table II. *Summary of Sucrose Density Gradient Centrifugation Data*

Gradient Volume	Catalase		Yeast Alcohol Dehydrogenase		NADH-nitrate Reductase		
	Distance migrated	Known s_w	Distance migrated	Known s_w	Distance migrated	s_w (calculated from catalase)	s_w (calculated from alcohol dehydrogenase)
drops	*drops*		*drops*		*drops*		
253	129	11.3	85	7.4	121	10.6	10.5
237	121	11.3	81	7.4	113	10.3	10.6
272	134	11.3	92	7.4	128	10.3	10.5

nitrate is the electron acceptor, namely, the NADH-nitrate reductase, MVH-nitrate reductase, and the $FADH_2$-nitrate reductase, were all inhibited to approximately the same extent by KCN and NaN_3 as shown in Table IV. The NADH-Cyt *c* reductase activity was not susceptible to this type of inhibition.

There was significant inhibition of the NADH-nitrate reductase and the NADH-Cyt *c* reductase activities with *o*-phenanthroline, but not the MVH-nitrate reductase or $FADH_2$-nitrate reductase activities. Bathophenanthroline, another metal-chelating agent, appears to inhibit all the activities associated with the NADH-nitrate reductase.

Nitrogen Compounds. A variety of nitrogen compounds were tested to determine if nitrate reductase was susceptible to negative feedback inhibition *in vitro*. All the compounds were assayed under conditions in which the concentration of nitrate was rate-limiting in order to test for competition with nitrate. The following compounds at a final concentration of 10 mM were tested: NH_4Cl, L-glutamine, L-phenylalanine, L-alanine, L-valine, glycine, L-glutamic acid, L-aspartic acid, and urea. None had any inhibitory effect.

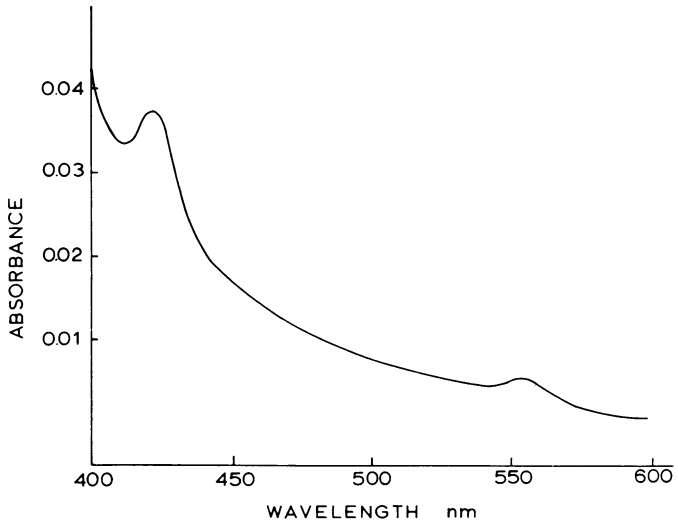


FIG. 5. Visible absorption spectrum of the dithionite-reduced nitrate reductase from *T. pseudonana*. The absorption spectrum was determined with a Cary Model 14 recording spectrophotometer equipped with the 0 to 0.1 slidewire.

The possibility that nitrite would act as an electron acceptor from nitrate reductase was investigated. The rate of NADH oxidation was followed at 340 nm with purified fraction 8 protein in an assay system in which nitrite replaced nitrate in the reaction mixture. No NADH oxidation was observed. When nitrate and nitrite were both present at a concentration of 5 mM, the rate of NADH oxidation was equal to that observed

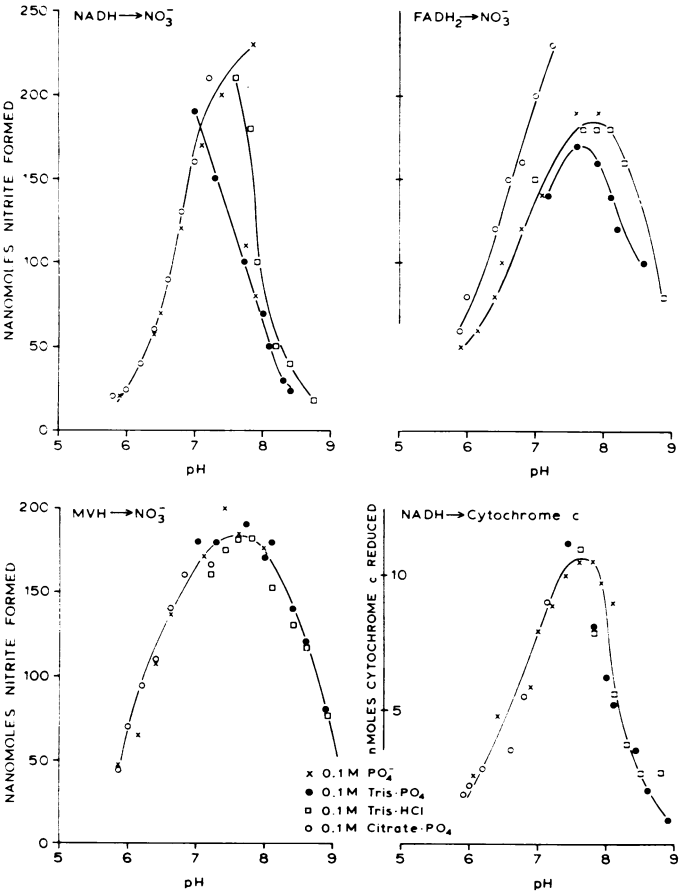


FIG. 6. Effect of pH and various buffers on the NADH-nitrate reductase and its associated activities. The standard assay protocol was followed employing enzyme from fraction 8 except for the substitution of the various buffers at a concentration of 0.1 M.

Table III. *Apparent Km Values of Substrates for the NADH-Nitrate Reductase and Associated Activities*

Substrate	NADH-Nitrate Reductase	NADH-Cyt *c* Reductase	$FADH_2$-Nitrate Reductase	MVH-Nitrate Reductase
		μM		
Electron donor				
NADH	15	13		
FAD			420	
Methyl viologen				6
Electron acceptor				
Nitrate	62		16	330
Cyt *c*		0.6		
Cofactor				
FAD	0.07	0.01		
FMN	10	11		

with nitrate alone. Thus, nitrite is not a substrate for nitrate reductase, nor does it competitively inhibit the interaction between nitrate reductase and its substrate nitrate.

DISCUSSION

The dominant feature in the regulation of the nitrate reductase from *T. pseudonana* is the inactivation and repression of the enzyme by ammonium, which is the end product of the nitrate assimilatory pathway. No nitrate reductase activity is present when the cells are grown in an ammonium media. Further, when ammonium chloride is added to the media in which the diatoms possess nitrate reductase and are utilizing nitrate, there is a rapid loss in the pre-existing nitrate reductase activity, which can be seen in the parallel decline of the four activities associated with this enzyme. This inactivation of the nitrate reductase by ammonium could not be reversed *in vitro* by oxidation of the enzyme with ferricyanide, as was reported to occur with *Chlorella fusca* (15) and *Chlamydomonas* (16).

The rapid decrease in nitrate reductase activity caused by ammonium does not require protein synthesis. However, the effect of ammonium is not simply a cessation of the nitrate reductase synthesis, because pre-existing nitrate reductase activity does not undergo such a rapid decline when *de novo* protein synthesis is inhibited by cycloheximide. Ammonium does not appear to inactivate the enzyme directly since ammonium does not inhibit the reductase in enzyme extracts. The rapid decline of nitrate reductase activity caused by ammonium *in vivo* appears to be due to an inactivation of the enzyme which is somehow indirectly mediated by ammonium. Ammonium exerts end product repression and prevents the appearance of nitrate reductase activity and also rapidly inactivates pre-existing nitrate reductase to stop the synthesis of ammonium. Both processes serve to halt an unnecessary expenditure of metabolic energy.

When the diatoms are transferred from ammonium media in which they lack nitrate reductase activity to nitrate media, the NADH-nitrate reductase and the three partial activities associated with the enzyme increase steadily over a period of 12 hr. When ammonium and nitrate are present in equal amounts in the media, no nitrate reductase activity appears, showing that

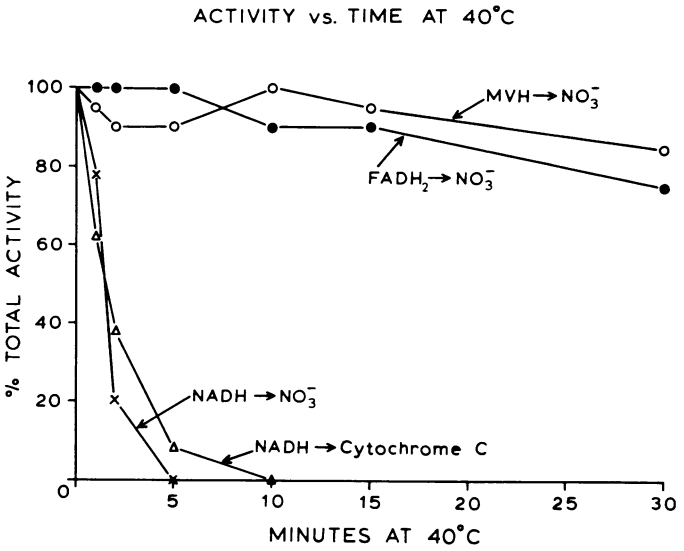


FIG. 7. Effect of mild heat treatment. A 2-ml aliquot of a hydroxylapatite eluate (specific activity 1640) was pipetted into two warmed 13 × 100 mm test tubes in a 40 C water bath. For the first few minutes, the tubes were shaken gently so that the enzyme solution would quickly reach 40 C. At the indicated times, 0.25-ml aliquots were removed and pipetted into an equal volume of cold 0.1 M phosphate buffer. Each heat-treated sample was then assayed by the standard procedures for the various activities and the results are expressed as the percentage of the activity obtained with an unheated enzyme sample. For the NADH-nitrate reductase and $FADH_2$-nitrate reductase assays, 32 μg of enzyme protein were used; 3.2 μg were used for the MVH-nitrate reductase assay; and 16 μg were used for the NADH-Cyt *c* reductase assay.

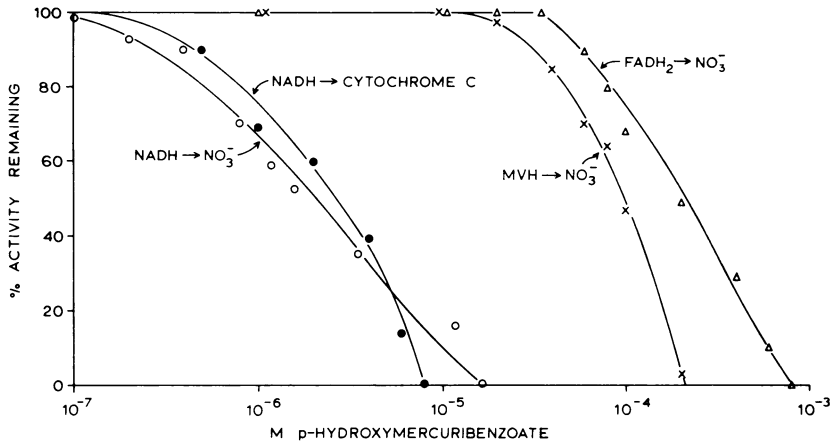


FIG. 8. Effect of increasing concentrations of *p*-hydroxymercuribenzoate on NADH-nitrate reductase and its associated activities. Three to 30 μg of fraction 8 protein were used for the various assays. Results are expressed as the percentage of the activities obtained without added inhibitor, and are presented in a semilogarithmic plot.

Table IV. *Inhibition of NADH-Nitrate Reductase and Its Associated Activities by Metal-binding Agents*

Inhibitor and Final Concn	NADH-Nitrate Reductase	NADH-Cyt *c* Reductase	$FADH_2$-Nitrate Reductase	MVH-Nitrate Reductase
M	% inhibition			
Sodium azide				
10^{-4}	88	0	96	82
10^{-5}	49	0	73	39
10^{-6}	20		24	25
Potassium Cyanide				
10^{-4}	100	0	100	100
10^{-5}	62	0	74	57
10^{-6}	19		35	20
o-Phenanthroline				
10^{-2}	96	88	0	0
10^{-3}	65	23	0	0
10^{-4}	25	0		
Bathophenanthroline				
10^{-3}	73		84	30
10^{-4}	56		50	12
5×10^{-5}		35		

the enzyme is ammonium-repressible, rather than nitrate-inducible. When the diatoms are transferred to media containing no nitrogen source, there is a significant increase in nitrate reductase activity, indicating the nitrate reductase is truly a derepressible enzyme and that nitrate is not required for enzyme synthesis. Nitrate reductase is also derepressed when uric acid is the sole nitrogen source. No nitrate reductase activity is observed in cells which were transferred to media with nitrate plus cycloheximide or uric acid plus cycloheximide, indicating that protein synthesis is required for induction of enzyme activity. Thus, in the absence of ammonium, nitrate reductase is derepressible, allowing the enzyme to be formed even in the absence of nitrate. This diatom, existing in a marine environment, is likely to be dependent on nitrate as its nitrogen source and hence is geared for nitrate assimilation except in those instances when ammonium is available.

Throughout the 200-fold purification, the NADH-nitrate reductase and the associated activities are retained in roughly constant proportion. All the activities elute coincidently during DEAE-cellulose, hydroxylapatite, and Sephadex G-200 gel filtration chromatography. The activities coincide on sucrose density gradient centrifugation and are associated with a single band on polyacrylamide gels. The activities have not been separated by conventional enzyme purification techniques and can be envisioned as manifestations of different parts of the electron transport pathway of nitrate reductase.

The specific activity attained with the *T. pseudonana* nitrate reductase (3120 units/mg protein) is higher than those reported for the purified nitrate reductases from spinach of 523 units/mg (21), *Chlorella fusca*, 405 units/mg (3), *Dunaliella*, 702 units/mg (14), and *Chlorella pyrenoidosa*, 1747 units/mg (23). The *T. pseudonana* nitrate reductase specific activity is comparable to that attained with the activated nitrate reductase from *Chlorella vulgaris*, 3540 units/mg (26).

The low recovery and final purification of the NADH-Cyt *c* reductase activity compared to the other activities can be attributed to other enzymes capable of reducing Cyt *c* present in the enzyme fractions. In fraction 4, there is a marked decrease in the specific activity of the Cyt *c* reductase, suggesting that most of these other Cyt *c* reductase enzymes are removed at this stage. Starting from fraction 4, the purification and recovery of the NADH-Cyt *c* reductase activity is generally proportional to the other activities. Constitutive Cyt *c* reductase enzymes, unrelated to the nitrate reductase, are also present in ammonium-grown cells.

The visible absorption spectrum of the reduced nitrate reductase from *T. pseudonana* indicates that a Cyt may also be present. The observed spectrum is similar to that typically associated with Cyt of the *b*-type which have been identified in *Neurospora* (6) and *Chlorella vulgaris* (23). Spectral measurements of several different purified enzyme preparations revealed identical absorbance peaks. It was not practical to demonstrate a functional role for the Cyt because the large amounts of enzyme protein required could not be obtained from the limited amount of starting material available.

The diaphorase activity is completely inactivated by a 5-min incubation at 40 C, whereas the MVH and $FADH_2$-nitrate reductase activities persist for 30 min. This pattern of inactivation is similar to that observed from heat treatment in *Neurospora* (7) and *Chlorella vulgaris* (23), except that in those organisms, the activity of the MVH-nitrate reductase is initially equal to or less than the NADH-nitrate reductase activity. Upon heat treatment, MVH-nitrate reductase activity from those organisms increases 5- to 10-fold, possibly as a result of

a partial denaturation of the enzyme which renders the substrate binding site more accessible to the dye.

Cyanide and azide exhibit a pattern of inhibition of the activities of nitrate reductase different from *o*-phenanthroline and bathophenanthroline. This data suggests that metal component(s) may be functioning in the nitrate reductase. Molybdenum has been identified in several nitrate reductases (4), and nonheme iron appears in the nitrate reductase from *Neurospora* (7) and *Ankistrodesmus* (27).

Neither the immediate products of nitrate assimilation, nitrite, and ammonium, nor other nitrogenous cellular products such as amino acids, feedback inhibit the nitrate reductase *in vitro*.

Thus, examination of the purified nitrate reductase from *Thalassiosira pseudonana* has revealed that the enzyme has essentially similar properties as the other nitrate reductases which have been examined. The diatom nitrate reductase is a large enzyme complex of 330,000 mol wt, possessing an electron transport pathway of several components. There is a *b*-type Cyt and FAD associated with the enzyme and indirect evidence suggests that metal component(s) are present. From a comparison of the substrate requirements and sensitivity to inhibitors, it can be seen that the electron transport scheme of the *T. pseudonana* nitrate reductase is analogous to that shown in the introduction.

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