THE MECHANISMS AND ENERGETICS OF NITRATE UPTAKE
BY MARINE PHYTOPLANKTON

by

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We accept this thesis as conforming
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Date 18 June 1975
ABSTRACT

The results of this study suggest that $\text{NO}_3^-$ uptake in many (but not all) species of marine phytoplankton is mediated by a membrane-bound ($\text{NO}_3^-$, $\text{Cl}^-$)-activated adenosine triphosphatase. In the presence of $\text{NO}_3^-$ and $\text{Cl}^-$, semi-purified membrane preparations exhibit enhanced adenosine triphosphatase activity. The enzyme has characteristics common to other membrane-bound proteins: a break in the Arrhenius plot of 30.9 Kcal/mole at 2.9 °C, parallel purification with the ($\text{Na}^+$ + $\text{K}^+$)-activated transport adenosine triphosphatase, and activation of catalytic activity by non-ionic and anionic detergents.

It is inferred from parallel purification of the ($\text{NO}_3^-$, $\text{Cl}^-$)-activated adenosine triphosphatase and the physiological kinetics of $\text{NO}_3^-$ uptake by intact cells, that the enzyme translocates $\text{NO}_3^-$ across the cell membrane, into the cytoplasm, against the chemical concentration gradient of the ion. The half-saturation constants for activation of the adenosine triphosphatase by $\text{NO}_3^-$ are less than 1 $\mu$M for most species tested and correlate with half-saturation constants for $\text{NO}_3^-$ uptake by whole cells. The three dinoflagellates tested did not exhibit any relationship between $\text{NO}_3^-$ concentrations and ATP hydrolysis, and it is inferred that the ($\text{NO}_3^-$, $\text{Cl}^-$)-activated adenosine triphosphatase is probably absent from this group.

Results of metabolic inhibitor studies (including KCN, 2,4-dinitrophenol, dichlorodimethylurea, and carbonyl
cyanide m-chlorophenylhydrozone) imply that the source of ATP for the NO\textsuperscript{3} transport is primarily cyclic photophosphorylation \textit{in vivo}. These results are consistent with observations of selective inhibition of NO\textsuperscript{3} uptake in unialgal cultures as well as in natural populations.

Field studies with natural phytoplankton communities from Knight Inlet, B. C., suggest a physiological adaptation to external nitrogen concentrations may occur. This adaptation is characterized by increased intracellular chlorophyll \textit{a} synthesis in response to 10–15% nitrogen enrichment over a 6–8 hr period. During the adaptive period carbon fixation is temporarily suppressed, apparently due to competition between inorganic carbon and inorganic nitrogen for high-energy nucleotides from the light reactions.

The results of this study are related to previous proposals for the metabolic pathway of nitrogen in marine phytoplankton. In conclusion, a modified pathway is proposed stressing (1) group differences, in that nitrogen assimilation in dinoflagellates appears different from other groups, and (2) the energetics and biochemical feed-back controls of nitrogen assimilation.
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"Every theory of the course of events in nature is necessarily based on some process of simplification and is to some extent, therefore, a fairy tale."

Sir Napier Shaw

**Introduction**

Photosynthetic marine organisms, like their terrestrial counterparts, require light, carbon dioxide, certain inorganic ions, and often organic compounds for growth and reproduction. In most marine environments, the inorganic nitrogen sources, ammonium, nitrite, nitrate (and possibly hydroxylamine) appear to be in limited supply to primary producers relative to the demand. Most natural oligotrophic conditions are encountered in such areas as the Eastern Tropical Pacific (Thomas, 1969, 1970; Thomas and Owen, 1971), the Sargasso Sea (Hulburt, 1960; Riley et al, 1949), and perhaps the oceanic province in general (Parsons and Takahashi, 1973). Even in neritic waters, total inorganic nitrogen is usually less than $10^{-5}$ M (Harvey, 1960), and phytoplankton appear to thrive in such a dilute nutrient medium. Increasingly, it is becoming clear, that the major limiting factor controlling phytoplankton growth in the seas is most frequently a suitable nitrogen source (Corner and Davies, 1971).

Of the naturally occurring nitrogen sources, ammonium appears to be the most energetically favorable form for amino acid anabolism. It has been observed that more oxidized forms of nitrogen, such as nitrite or nitrate, must be
enzymatically reduced to ammonium prior to incorporation into amino acids (Eppley et al, 1969; Lui and Roels, 1972; Packard and Blasco, 1974). The stepwise reductions to ammonium are endergonic reactions, requiring specific enzyme cofactors (e.g. NAD(P)H; FADH; reduced ferredoxin). Thus, it is not surprising that most phytoplankton assimilate ammonium more readily than nitrite or nitrate. Despite this apparent energy "barrier" to oxidized nitrogen assimilation however, these oxidized nitrogen molecules are more readily available in most oceanic environments. Ecologically, nitrate may represent the most important single nitrogen source in the sea.

In order to utilize nitrate as a substrate for cellular anabolism, phytoplankton must transport the ion across the cell membrane, reduce the substrate in a series of reactions to ammonium, and incorporate the ammonium into primary amino acids (notably glutamate) (fig. 1). Of these three processes (transport, reduction, and incorporation), transport across the plasmalemma, presumably often against the negative chemical potential of the ion (Eppley and Rogers, 1970), is the least understood.

The mechanism of passage of nitrate across the cell membrane has been hypothesized by some investigators. Allusions to "permeases" and facilitated diffusion have been made by Packard and Blasco (1974) and Platt and SubbaRao (1973);
Fig. 1. Nitrogen assimilation in marine phytoplankton (after Packard and Blasco, 1974). Nitrate, nitrite, and ammonium are transported across the cell membrane via hypothesized permeases. Once in the cell, nitrate and nitrite are reduced stepwise to ammonium, which is then incorporated into α-keto glutarate to form glutamic acid. This amino acid, at the major branch-point, may either transaminate the amino nitrogen to other carbon skeletons, or undergo further modifications, to form new amino acids.
Fig. 1.

2. proline
3. arginine
4. glutamine

proline dehydrogenase
ornithine-citrulline cycle

5. alanine
6. asparagine
7. aspartate
8. cysteine
9. glycine
10. histidine
11. isoleucine
12. leucine
13. lysine
14. methionine
15. phenylalanine
16. serine
17. threonine
18. tryptophane
19. tyrosine
20. valine

transaminase
α-keto acids

1. glutamate
NAD(P)+
NAD(P)H

α-keto glutarate

6. glutamate dehydrogenase

nitrite reductase

NH₄⁺

nitrate reductase

NO₂⁻

NO₃⁻

NH₄⁺

NO₂⁻

NO₃⁻

AMINO ACID SYNTHESIS
AMMONIA ASSIMILATION
NITRATE REDUCTION
ENVIRONMENTAL NUTRIENTS

NITROGEN ASSIMILATION
however, no direct evidence has been published to support these speculations. The phospholipid bilayer, thought to be the major constituent of the plasma membrane, is not very permeable to charged molecules like nitrate, so absorption of the ion must follow other routes into the cell. An alternative possibility might be a membrane-bound protein in the phospholipid bilayer that mediates the translocation of nitrate. This "ionophore" might simply allow diffusion, in which case it would be a true permease, following the original definition of the term by Monod (1942); or it may "pump" nitrate against the negative gradient of the chemical potential of the ion.

If the nitrate-accepting protein simply allowed diffusion of nitrate, the internal concentration of the ion should equal the external concentration, providing no other process intervened. This hypothesis is difficult to test directly because in the steady-state, intracellular nitrate is continuously being reduced to ammonium; diminishing the intracellular nitrate concentrations. This type of diffusion has been called "trapping diffusion" (Wilson, 1962), the driving force being provided by the biochemical reduction of nitrate within the cell. This model implies that the nitrate uptake rate is a function of nitrate reduction, or specifically the enzyme nitrate reductase (E.C. 1.6.6.1 and E.C. 1.6.6.3).

Nitrate uptake by whole cells can be described adequate-
ly by Michaelis-Menten kinetics (e.g. Caperon and Meyer, 1972; MacIsaac and Dugdale, 1972). This type of kinetics relates the velocity of uptake to the concentration of the nutrient by the expression:

$$V = \frac{V_{\text{max}} \cdot S}{K_s + S}$$

where: $S$ is the extracellular nutrient concentration
$V$ is the velocity of nutrient uptake
$K_s$ is the nutrient concentration supporting $\frac{1}{2}$ maximum uptake velocity ($\frac{1}{2}V_{\text{max}}$).

If trapping diffusion were responsible for nitrate uptake by the cell, it should follow that the half-saturation constant for nitrate uptake should be of a similar order of magnitude as the half-saturation constant for nitrate reductase ($K_m$). Determinations of $K_m$ for nitrate reductase indicate that this value is in excess of the concentrations of nitrate usually present in natural seawater by about a hundredfold (Eppley and Rogers, 1970). Thus it is improbable that nitrate reductase serves to provide a chemical gradient across the cell membrane; in fact, the cell must "pump" nitrate into the cytoplasm in order to reach effective substrate concentrations for a high degree of reducing efficiency.

Another possible mechanism for nitrate uptake is that of so-called "active transport". This process requires that nitrate uptake be coupled directly to an exergonic
chemical reaction. Here the internal nitrate concentration may be relatively insignificant; nitrate may be accumulated against its concentration gradient, providing that a suitable energy source is available to drive the ion across the membrane. The obvious candidate for this chemical energy is ATP, which, through hydrolysis, liberates enough energy for the translocation of nitrate. In this model the nitrate-accepting protein is an adenosine triphosphatase (or some analogous enzyme), catalyzing the hydrolysis of ATP in the presence of external nitrate ions. An analogous situation is the \((Na^+ + K^+)\)-activated transport adenosine triphosphatase (E.C. 3.6.1.3), first described by Skou in 1957, and found in many animal and plant tissues (c.f. Askari, 1974; Balke and Hodges, 1975; Karlsson and Kylin, 1974; Maslowski and Komoszynski, 1974).

At the outset this thesis was an attempt to determine some aspects of the physiological role of ATP in marine phytoplankton. This goal was not reached however, because of the surprise discovery of the nitrate-activated adenosine triphosphatase. The result was more involvement with the kinetics and physiological role of the enzyme, rather than with ATP per se. It is hoped however, that the research documented here will contribute to knowledge of the physiological role of ATP in nutrient assimilation processes, as well as adding to the understanding of nitrogen metabolism of marine phytoplankton.
Materials and Methods

A. Laboratory Studies

I. Cultures

In all, nine species of marine phytoplankton were examined for the presence of a (NO\textsuperscript{3}, Cl\textsuperscript{-})-activated adenosine triphosphatase. Most of the enzyme kinetics were determined from membrane preparations from *Skeletonema costatum* (Grev) Cleve (Bacillariophyceae), as this diatom had the greatest enzyme activity per unit carbon and was easiest to culture. Caution should be stressed however, about over-generalizing the characteristics and kinetics of the (NO\textsuperscript{3}, Cl\textsuperscript{-})-activated adenosine triphosphatase from *S. costatum* to other species. The eight other species were: *Ditylum brightwellii* (West) Grunow (Bacillariophyceae), *Dunaliella tertiolecta* Butch (Chlophyceae), *Eutreptiella gymnastica* Schiller (Euglenophyceae), *Amphidinium carterae* Hulb. (Dinophyceae), *Gonyaulax polyedra* Stein (Dinophyceae), *Gonyaulax tamarensis* var. *excavata* Braarud (Dinophyceae), *Isochrysis galbana* Parke (Haptophyceae), *Chroomonas salina* (Crytophyceae).

With the exceptions of *Isochrysis galbana*, *Gonyaulax polyedra*, and *Dunaliella tertiolecta*, the cultures were isolated in this laboratory by Ms. R. Waters and maintained as part of the University of British Columbia Northwest Pacific Culture Collection. *I. galbana* and *D. tertiolecta* were originally obtained from Dr. M. Parke and the Woods Hole
Oceanographic Institution respectively. *G. polyedra* was obtained from R. W. Eppley at Scripps Institute of Oceanography.

Each species was grown in unialgal, but not axenic, batch cultures at 16-19 °C in sterile 2-6 l. Erlenmeyer flasks. The two diatoms, *Skeletonema costatum* and *Ditylum brightwellii* were grown on medium "f", diluted to "f/2" with autoclaved seawater (Gullard and Ryther, 1962). The other species were grown on medium "J" (Table 1), developed in this laboratory. Except where indicated, NaNO₃ was the sole inorganic nitrogen source added to the culture media and atmospheric contamination of the media by ammonia was prevented by prebubbling air entering the culture vessels through a saturated solution of ZnCl₂ (Caperon and Meyer, 1972).

Light was supplied by banks of 40 Watt cool white fluorescent tubes. The maximum light intensity was determined in lux from a Photovolt Corporation light meter (Model 511 M), and converted to langley/min using the conversion factor $5 \times 10^{-6} \text{ly/min} = 1 \text{lux}$ (Westlake, 1965). Using this conversion factor, the maximum light intensity was approximated at 0.12 ly/min. Cultures were maintained in a 12:12 light/dark cycle.

II. Preparation of membrane "vesicles"

After the cultures reached the stationary phase of growth (as determined by daily chlorophyll a determinations)
**TABLE 1 - Medium "J"**

1,000 ml seawater  
(filtered through 0.45μ Millipore)

<table>
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<th>Component</th>
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<tr>
<td>100 ml distilled water</td>
<td>2.5x10^-6 g Cu (as Cl^-)</td>
</tr>
<tr>
<td>0.25 g Tris buffer</td>
<td>1x10^-5 g Na_2MoO_4</td>
</tr>
<tr>
<td>0.03 g Na_2SiO_3</td>
<td>1x10^-6 g vitamin B_12</td>
</tr>
<tr>
<td>0.1 g NaN_3O_3</td>
<td>5x10^-4 g thiamin HCl</td>
</tr>
<tr>
<td>0.01 g K_2HPO_4</td>
<td>3x10^-5 g nicotinic acid</td>
</tr>
<tr>
<td>6x10^-3 g Na_2EDTA</td>
<td>3x10^-5 g Ca pantothenate</td>
</tr>
<tr>
<td>2x10^-3 g FeCl_3·6H_2O</td>
<td>3x10^-5 g p-aminobenzoate</td>
</tr>
<tr>
<td>2x10^-4 g Mn (as SO_4^-)</td>
<td>1x10^-6 g biotin</td>
</tr>
<tr>
<td>2x10^-5 g Zn (as Cl^-)</td>
<td>5x10^-4 g inositol (meso)</td>
</tr>
<tr>
<td>2.5x10^-6 g Co (as Cl^-)</td>
<td>6x10^-7 g folic acid</td>
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the cells were harvested in 250 ml centrifuge buckets at 5000 g in a refrigerated centrifuge at 5 C. The pellets were collected in 50 ml centrifuge tubes and sonicated in an Ultrasonic T30C1 sonicator for ca. 10-20 min at 0-2 C. The exact length of time for the sonication period varied from species to species, depending upon the cell wall morphology, composition, and size. When no intact cells could be observed under phase contrast microscopy at 400 X magnification, the fragments were suspended in 5 mM Tris-acetate buffer, pH 7.9 at 0 C. These were then centrifuged at 5000 g for 60 min. The supernatant was collected, sonicated for 10 min, and centrifuged at 25,000 g for 30 min. This sonication/centrifugation procedure was repeated up to three times for those species with a thick wall (e.g. diatoms). A white, amorphous, flocculent material was observed at the bottom of the tubes after the 25,000 g spin and the supernatant was clear and uncoloured.

The supernatants were decanted and the pellets were then suspended in a solution of 0.01% Triton X-100 and 0.01% deoxycholate (DOC) in 5 mM Tris-acetate buffer. This suspension was incubated in a shaking water bath at 25-30 C for 15 min. The supernatant was treated in the same manner as the pellet. The pellet suspension was then centrifuged at 40,000 g for 60 min and the supernatant was centrifuged at 100,000 g for 60 min. Supernatants from these latter two
centrifugations were assayed for \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase activity.

III. Enzyme assays

\(a)\) \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase

Three methods were used to determine the activity of the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase. They are described in increasing order of their sensitivity to detect adenosine triphosphatase activity.

1. Inorganic phosphate method \((\text{Fiske-SubbaRow})\)

This method (Fiske and SubbaRow, 1925 as adapted by Lindeman, 1958) has the advantage of being fast and relatively easy, although its sensitivity depends upon high specific activity of the enzyme preparation. It was the assay originally used here, and is suitable for semi-purified membrane preparations from \(S. \text{costatum}\).

The activity of the enzyme was determined by following the hydrolysis of ATP and measuring the inorganic phosphate released (Fiske and SubbaRow, 1925). Five \(\mu\)g of protein from the Triton extracts (ca. 200 \(\mu\)l) were incubated in 1.5 ml of solution containing 10 mM Tris-acetate buffer, pH 7.9; 10 \(\mu\)M \(\text{NaNO}_3\); 50 \(\mu\)M \(\text{MgCl}_2\); 50 \(\mu\)M Tris-ATP, and 10 \(\mu\)M dithiothreitol (DTT). The reaction mixture was incubated for 30 min at 20 C, after which 1.0 ml of 60% trichloracetic acid (TCC) was added. The precipitated protein was centrifuged at 10,000 g for 10 min. Two ml of the supernatant was
collected and assayed for ATP hydrolysis; the precipitate was assayed for protein against bovine serum albumin standards (Sigma Chemical Co.) (Lowry et al, 1951).

2. Coupled enzyme assay (pyruvate kinase - lactate dehydrogenase)

The adenosine triphosphatase may be coupled in vitro to pyruvate kinase (E.C. 2.7.1.40) and lactate dehydrogenase (E.C. 1.1.1.27); the activity of the enzyme can be determined by following the change in absorption at 340 nm, due to the stoichiometric oxidation of NADH. Thus:

\[
\begin{align*}
\text{ATP} + \text{Mg}^{2+} &\rightarrow \text{ADP} + \text{P}_i + \text{PEP} \rightarrow \text{pyruvate} + \text{NADH} + \text{NAD}^+ + \text{ATP} \\
\end{align*}
\]

The substrate concentrations used were the same as in the Fiske-SubbaRow method (1 above) with the addition of 5 mM PEP, 0.2 mM NADH and excess lactate dehydrogenase (Sigma Chemical Co.; Type 1 with pyruvate kinase). The adenosine triphosphatase activity was followed from 0-0.2 OD units in a recording spectrophotometer.

3. \((\gamma-P^{32})\)-ATP method (Isotopic discrimination assay)

The most sensitive assay for determining activity of the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase was found to be with the use of \((\gamma-P^{32})\)-ATP as a substrate (New England Nuclear).
In this procedure the final concentrations in the assay mixture were: 2 mM Tris-ATP, pH 7.9, 2 mM MgCl$_2$, 10 μM NaNO$_3$, 10 μM dithothreitol, 1.5 mM Tris-acetate buffer, and 2 to 3 x 10$^6$ cpm (γ-P$^{32}$)-ATP. The mixture was preincubated for 10 min and the reaction was initiated by the addition of 5 μg protein. The final volume was 1.25 ml. After 15 min the reaction was terminated by the addition of a 48 solution of (NH$_4$)$_7$Mo$_7$O$_{24}$·4H$_2$O in 10 N H$_2$SO$_4$ (Goldman and Albers, 1973). Five hundred μl of isobutanol was added to each tube and the solutions were mixed in a vortex mixer for 60 sec. A 200 μl aliquot from the isobutanol phase was removed for counting in a dioxane-naphthlene cocktail (Aquafluor; New England Nuclear) in a Nuclear Chicago Isocap/300 liquid scintillation counter.

b) Nitrate reductase (NADH-dependent)

Nitrate reductase was routinely assayed in the Triton extracts prior to determination of the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase. If nitrate reductase was detected, the extract was dialyzed for 2-4 hr (sometimes overnight) against 10 mM Tris-acetate buffer containing 5 mM EDTA and 1 mM dithiothreitol.

The NADH-dependent nitrate reductase was assayed by following the NO$_3^-$-stimulated oxidation of NADH from 0-0.2 OD units in a recording spectrophotometer as described by Hageman and Hucklesby (1971).
c) (Na$^+$ + K$^+$)-activated adenosine triphosphatase

The (Na$^+$ + K$^+$)-activated adenosine triphosphatase was assayed as a marker for the cell membrane fraction according to the methods of Maslowski and Komoszynski (1974).

IV. Determination of Protein and Chlorophyll a

Protein was determined as described by Lowry et al (1951), using bovine serum albumin standards. Protein was used for determinations of the specific activity of the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase in order that comparison between various ion-stimulated adenosine triphosphatases could be made. In addition, the specific activity was used as a parameter of relative enzyme purity.

Chlorophyll a was determined spectrophotometrically at 665, 645, and 630 nm respectively using the equations of Parsons and Strickland (Strickland and Parsons, 1972). Cells were filtered on Whatman GF/C glass fiber filters and coated with a few ml of a 1% MgCO$_3$ suspension. The filters were homogenized by hand in an all-glass tissue grinder (Pyrex) with 10 ml of 90% acetone, centrifuged for 30 min at 2000 x g at 5 C. Activity of the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase was calculated per mg chlorophyll a to compare relative specific activities between species.

V. ATP determinations

Parameters affecting the intracellular ATP pool size were investigated, to determine what, if any, coupling
existed between NO$_3^-$ uptake and ATP.

One hundred ml aliquots of *S. costatum* cultures were taken from log growth phase and incubated at 18 C in 250 ml Pyrex bottles. The incident light, provided by banks of 40 Watt cool white fluorescent tubes, was attenuated by placing neutral density filters (Kahlsico Internation Corp.) over the bottles to produce 60, 30, 15, 1 and 0% illumination. One set remained at the maximum light intensity (i.e. 100%) of ca. 0.12 ly/min. After a 2 hr incubation, the cells were filtered on Whatman GF/C glass fiber filters and the ATP was extracted twice in boiling Tris-HCl buffer as described by Holm-Hansen and Booth (1966). Using known amounts of ATP, the efficiency of the extraction technique was estimated as ca. 80%. The samples were assayed for ATP with luciferin-luciferase preparations (Sigma Chemical Co.) and the photon emission was detected in a Unicam liquid scintillation counter employing only one photomultiplier. Standards and background counts were performed in conjunction with each sample. Samples were assayed in triplicate and the mean of the three counts was used to calculate the net intracellular ATP pools.

To determine the effects of temperature, the method was performed at 8, 18, and 28 C (±2 C) at each of the 6 light intensities.

The effects of metabolic inhibitors and extracellular
nutrients on the ATP pool was determined by the addition of the inhibitors or nutrients at varying concentrations either during the incubation period or immediately after, but always prior to the ATP extractions.

B. Field Study at Knight Inlet, B. C.

To gain some understanding of the parameters controlling the physiological role of ATP in NO$_3^−$ and NH$_4^+$ uptake by whole, natural phytoplankton communities, two cruises were made on the C. S. S. Vector to Knight Inlet, B. C. This inlet is a glacier-fed estuarine fjord ca. 250 miles north of Vancouver, B. C., and has been under intensive, continuous study by A. G. Lewis and D. P. Stone (Institute of Oceanography, University of British Columbia). The two cruises, in July and September 1974, corresponded to the recession of the spring bloom and peak of the secondary fall bloom respectively, as determined by in situ chlorophyll a concentrations (fig. 2). A control station was established in the adjacent Queen Charlotte Strait for comparison with the estuarine Inlet stations (fig. 3). Table 2 lists the coordinates of the sample stations and their acronyms.

To determine primary productivity, hydrocast samples were made at each station during daylight hours from the 2 m depth with 2 l. Van Dorn bottles. Each sample was split into three equal parts. One part was enriched with 3 μM NO$_3^−$/l and one with 3 μM NH$_4^+$/l; the remaining sample was not en-
Fig. 2. Seasonal variation in chlorophyll a in Knight Inlet, B. C. in 1973 at the surface (▲) and 5 m (●). Samples for the determination of primary productivity were taken in July and September.

Fig. 3. The west coast of British Columbia and northwest Washington showing the study area (insert). Above, Knight Inlet, B. C., indicating the positions of the sample stations.
Fig. 2.
**TABLE 2**

Coordinates and depths of the stations sampled in Knight Inlet, B. C.

<table>
<thead>
<tr>
<th>Station</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Depth (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q. C.</td>
<td>50°39.1'N</td>
<td>126°48.5'W</td>
<td>254</td>
</tr>
<tr>
<td>Kn 3</td>
<td>50°39.2'N</td>
<td>126°10.9'W</td>
<td>203</td>
</tr>
<tr>
<td>Kn 5</td>
<td>50°41.7'N</td>
<td>125°47.0'W</td>
<td>406</td>
</tr>
<tr>
<td>Kn 7</td>
<td>50°48.4'N</td>
<td>125°37.3'W</td>
<td>523</td>
</tr>
<tr>
<td>Kn 11</td>
<td>51°02.6'N</td>
<td>125°34.0'W</td>
<td>199</td>
</tr>
</tbody>
</table>
The subsamples were preincubated in a simulated-
in\textit{ situ} deck incubator for 0-6 hr in duplicate 125 ml Pyrex bottles equipped with ground-glass stoppers. Banks of 40 Watt fluorescent tubes provided an incident light intensity of 0.09 ly/min at maximum. Flow-through seawater kept the samples at approximately sea surface temperature. The design of this incubator is described by Doty and Oguri (1958).

After the desired preincubation the samples were inoculated with 2 \( \mu \text{Ci} \) \( \text{Na}_2\text{C}^{14}\text{O}_3 \) and incubated for 2-4 hr. Neutral density filters were used to attenuate the light to 60, 30, 15, 1 and 0\% of the incident intensity. One set remained at the maximum intensity. After the incubation period, the cells were filtered on 0.45 \( \mu \) Millipore filters at less than 12 cm vacuum pressure, fumed over concentrated HCl for 15 sec and dried in a dessicator containing 30\% soda lime. The filters were counted for radioactivity ashore using the channels ratio method. The inorganic carbon fixation was determined from the equations of Strickland and Parsons (1972).

Chlorophyll \( \text{a} \) was determined in each sample prior to and after the incubation period as described in sec. A-IV. Estimates of the 1\% light depth were made with a Secchi disc. In addition, vertical profiles of nitrate, phosphate, dissolved oxygen, chlorophyll \( \text{a} \), temperature and salinity were made at each station. Whole water samples were taken from
the 2 m hydrocast and preserved in Lugol's solution for cell identification and counting (Banse, 1974).

Cell counts were made in 10 cc chambers after allowing the cells to settle overnight. A Zeiss inverted microscope, fitted with phase contrast objectives, was used to identify and count each sample. Depending on the cell density, 50 to 100 random fields were counted at 250 X and final cell counts were expressed on a per liter basis (Banse, 1974). In addition, aliquots were preserved from the experimental samples after the incubation period and counted as described.
Results

A. Laboratory Studies

I. Characteristics and kinetics of the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase

a) Effects of hydrogen ion concentration

The effects of hydrogen ion concentration on the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase are shown in fig. 4 for *Skeletonema costatum* and *Chroomonas salina*. In both species the enzyme exhibited a major activity peak at ca. pH 7.8-8.1 with a secondary optimum at pH 6.9. Although bimodal pH profiles for a single enzyme are not uncommon (Dixon and Webb, 1964), the possibility that this pH profile represents the presence of more than one enzyme may be assumed, due both to the heterogeneity of the preparation and the lack of uniformity between the pH 7.9/6.9 ratio in both species. [Two major proteins might be inferred: one with an optimum at pH 7.9 and a secondary enzyme with an optimum at pH 6.9.] As the hydrogen ion concentration may alter substrate binding characteristics of enzymes, kinetic studies were carried out at pH 7.9 for all species.

Karlsson and Kylin (1974) have described the properties of a \(\text{Mg}^{2+}\)-stimulated \((\text{Na}^+ + \text{K}^+)\)-activated adenosine triphosphatase from sugar beet cotyledons and indicated a similar pH profile for enzyme activity. The pH profile was independent of ionic strength and suggests the titration of at
Fig. 4. The pH profiles for the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase from Skeletonema costatum and Chroomonas salina. Both profiles show a bimodal curve with maxima at pH 7.8 - 8.2 and a secondary peak at ca. 6.9.
least two amino acid residues involved in catalytic activity. The possibility that ion transport adenosine triphosphatases may be conservative in primary amino acid structure, and perhaps share a common mechanism, if not similar catalytic binding site(s), is suggested.

b) Effects of temperature on enzyme activity

Changes in the lipid phase-state are known to affect the Arrhenius plot of membrane-bound enzymes in vitro (Labrooke and Chapman, 1969; Raison et al., 1971). The lipid phase-state change is reflected by a "break" in the slope at a transition temperature characteristic of the lipids associated with the enzyme (Barnett and Grisham, 1973a, 1973b; Raison et al., 1971). In fig. 5 the Arrhenius plot for a crude enzyme preparation from Skeletonema costatum is shown from 0-25 C at pH 7.9 (the buffer pH was adjusted at each temperature to maintain a constant hydrogen ion activity). A difference in the activation energy of 30.9 Kcal/mole was extrapolated to 2.9 C for the (NO₃⁻, Cl⁻)-activated adenosine triphosphatase when the preparation was assayed without the addition of either Triton X-100 or DOC to the membrane preparation. Upon addition of detergents however, the slope of the curve shifted to 16 Kcal/mole and was uniform throughout the temperature range examined (fig. 6). These results strongly suggest the (NO₃⁻, Cl⁻)-activated adenosine triphosphatase is associated with a lipid moiety and at tempera-
Fig. 5. Arrhenius plot of (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase from *Skeletonema costatum* membrane vesicles without the addition of detergents. A break in the slope, corresponding to a change in activation energy of 31.9 Kcal/mole, was extrapolated to 2.9°C.
Fig. 6. Arrhenius plot of the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase from \textit{Skeletonema costatum}. The enzyme was extracted from membrane vesicles with 0.1% Triton X-100 and 0.05% DOC. The slope corresponds to an activation energy of ca. 16 Kcal/mole.
tures below ca. 3°C the activation energy is so great as to repress enzyme activity by about 50% in vitro.

c) Determination of the enzyme locus

Data for the parallel purification of the (Na\(^+\) + K\(^+\))-activated adenosine triphosphatase (Table 3), and the Arrhenius plots (figs. 5 and 6) suggest the (NO\(_3^-\), Cl\(^-\))-activated adenosine triphosphatase is bound to, or associated with a membrane. As nitrate reductase (associated with both the cytosol and outer chloroplast membrane) was not detected when the (NO\(_3^-\), Cl\(^-\))-activated adenosine triphosphatase exhibited the greatest specific activity, association of the (NO\(_3^-\), Cl\(^-\))-activated adenosine triphosphatase with the chloroplast membrane was not probable. Although it is difficult to demonstrate a specific enzyme marker for the plasmalemma, the (Na\(^+\) + K\(^+\))-activated adenosine triphosphatase has not been demonstrated in endoplasmic reticulum or mitochondria (c.f. Askari, 1974) and may in fact be a reliable enzyme marker for the plasmalemma. To date, it has been difficult to obtain purified membrane preparations of tonoplasts (vacuolar membranes); the possibility remains that the (Na\(^+\) + K\(^+\))-activated adenosine triphosphatase may be associated with vacuolar membranes as well (Hodges, 1972). Despite this possibility, it is thought probable that the (NO\(_3^-\), Cl\(^-\))-activated adenosine triphosphatase is primarily associated with the plasmalemma.
TABLE 3

Parallel purification of the (NO₃⁻, Cl⁻)-activated adenosine triphosphatase with the (Na⁺ + K⁺)-activated adenosine triphosphatase.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Nitrate Reductase (μM NAD⁺/mg protein-hr⁻¹)</th>
<th>(NO₃⁻, Cl⁻) ATPase (μM PO₄/mg protein-hr⁻¹)</th>
<th>(Na⁺ + K⁺) ATPase (μM PO₄/mg protein-hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000 x g spin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>supernatant</td>
<td>30.0</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>pellet</td>
<td>7.9</td>
<td>16.0</td>
<td>7.2</td>
</tr>
<tr>
<td>100,000 x g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>supernatant</td>
<td>N.D.</td>
<td>210</td>
<td>19.0</td>
</tr>
<tr>
<td>pellet</td>
<td>N.D.</td>
<td>15</td>
<td>2.1</td>
</tr>
</tbody>
</table>

The activities of three enzymes are indicated from a representative experiment from four fractions. The (NO₃⁻, Cl⁻)-activated adenosine triphosphatase appears to purify in parallel with the (Na⁺ + K⁺)-activated adenosine triphosphatase, an enzyme marker of the plasma membrane. (ND=not detected).
d) Divalent cation requirements

Two divalent cations, Ca$^{2+}$ or Mg$^{2+}$, were found to fulfill the cation requirements for enzyme activity. Of these, Mg$^{2+}$ had a lower half-saturation constant ($K_m = 17 \mu M$ as opposed to $52 \mu M$ for Ca$^{2+}$), and was used in virtually all the assays. Mg$^{2+}$ stimulated adenosine triphosphatase activity without the addition of any activating anions however. In some enzyme preparations the Mg$^{2+}$-stimulated adenosine triphosphatase represented over 90% of the total adenosine triphosphatase activity of the unpurified enzyme preparations (Table 4). As Mg$^{2+}$ was required for full (NO$_3^−$, Cl$^−$)-activated adenosine triphosphatase activity, and was added in excess of the calculated $K_m$ for the latter enzyme, the Mg$^{2+}$-stimulated adenosine triphosphatase activity was subtracted from the (NO$_3^−$, Cl$^−$)-activated adenosine triphosphatase.

e) Interference from (Na$^+$ + K$^+$)-activated adenosine triphosphatase

The (Na$^+$ + K$^+$)-activated adenosine triphosphatase was detectable in the membrane preparations, and purified in parallel with the (NO$_3^−$, Cl$^−$)-activated adenosine triphosphatase (Table 3). As NO$_3^−$ was usually added as the Na$^+$ salt, the effects of 1 mM Na$^+$ (as NaCl) on the (Na$^+$ + K$^+$)-activated adenosine triphosphatase were determined without the addition of K$^+$. These data are shown in fig. 7, and suggest that low concentrations of Na$^+$ have little effect on ATP
Fig. 7. The effects of varying Na$^+$ on the activity of the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase from Skeletonema costatum.
TABLE 4

The per cent adenosine triphosphatase activity for three ion-stimulated adenosine triphosphatases in six preparations. The enzymes were assayed from the 100,000 x g supernatant using the isotopic discrimination assay method.*

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>(NO&lt;sub&gt;3&lt;/sub&gt;-, Cl&lt;sup&gt;-&lt;/sup&gt;)</th>
<th>(Na&lt;sup&gt;+&lt;/sup&gt; + K&lt;sup&gt;+&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>81</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>79</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>84</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>67</td>
<td>21</td>
<td>12</td>
</tr>
</tbody>
</table>

* Final ion concentrations were:

Mg<sup>2+</sup> - 10 mM

NO<sub>3</sub> - 50 µM; Cl<sup>-</sup> - 1 mM

Na<sup>+</sup> - 100 mM; K<sup>+</sup> 10 mM
hydrolysis without the addition of K⁺.

f) Anion requirements and specificity

The addition of Cl⁻ to the (NO₃⁻, Cl⁻)-activated adenosine triphosphatase greatly enhances the activity of the enzyme in vitro. The possibility that a separate adenosine triphosphatase, stimulated by Cl⁻ alone, and independent of NO₃⁻, was not inferred because no significant increase in adenosine triphosphatase activity could be observed without the addition of NO₃⁻ (in the presence of excess Mg²⁺). These data suggest a loose coupling between Cl⁻ and NO₃⁻, and are consistent with the hypothesis that two separate anion binding sites are present on the enzyme(s). The two anions (NO₃⁻ and Cl⁻) activate adenosine triphosphatase according to Michaelis-Menten kinetics (fig. 8a); the calculated $K_m$ for Cl⁻ being 11 μM in S. costatum at 1mM NO₃⁻ concentration. In addition, the Lineweaver-Burk plot (1/V vs. 1/S) (fig. 8b) indicates the two anions are not competitive, as the curves for each anion do not intercept at 1/Vₘₐₓ.

Nitrate activation of the adenosine triphosphatase is depicted graphically in fig. 9 for seven species. These curves are summarized in Table 5 for all species. The dinoflagellates, Amphidinium carterae, Gonyaulax tamarensis var. excavata, and Gonyaulax polyedra failed to demonstrate any NO₃⁻ activation of ATP hydrolysis; the enzyme may not be present in these species. The other species represented all
Fig. 8a. Michaelis-Menten plot of the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase from *Skeletonema costatum*. The inorganic phosphate method (Fiske-Subbarow) was used in these assays.

Fig. 8b. Lineweaver-Burk transformation of the Michaelis-Menten plot (8a). The half-saturation constants for NO$_3^-$ and Cl$^-$ were 0.9 and 11 µM respectively.
Fig. 8a.

\[ \mu M \text{PO}_4/\text{mg protein - min}^{-1} \]

- \( \bullet - \text{NO}_3^- \)
- \( \square - \text{Cl}^- \)

Fig. 8b.

\[ \frac{1}{V} \]

\[ - \frac{1}{S} \]
had adenosine triphosphatase activity, although the relative specific activity (ATP hydrolysis per mg chlorophyll a) varied widely (Table 5). The calculated $K_m$ values for $\text{NO}_3^-$ activation of the adenosine triphosphatase at 20 mM $\text{Cl}^-$ (saturating $\text{Cl}^-$ concentration) were 0.37 $\mu$M for *Skeletonema costatum*, 0.7 $\mu$M for *Ditylum brightwellii*, 0.23 $\mu$M for *Dunaliella tertiolecta*, 0.13 $\mu$M for *Isochrysis galbana*, 0.12 $\mu$M for *Eutreptiella gymnastica*, and 0.40 $\mu$M for *Chromononas salina*. These values were calculated from computerized linear-regression analysis of the Woolf plots ($S/V$ vs. $S$) and are significantly different from each other and from zero at the 95% confidence level.

The binding specificity of the ($\text{NO}_3^-$, $\text{Cl}^-$)-activated adenosine triphosphatase for other anions was interpreted from analysis of half-saturation constants. The assays for ion selectivity were made at pH 7.9 and 20°C. Half-saturation constants were calculated from computerized linear-regression analysis of the Lineweaver-Burk plots. $\text{I}^-$, $\text{Br}^-$, and $\text{F}^-$ ($\text{Na}^+$ salts) had $K_m$ values of 106 ± 22, 74 ± 16 and 92 ± 25 respectively ($N = 3$). Maximum enzyme activity was not suppressed by non-chloride halides, indicating a competitive type of inhibition, or common halide binding site(s). $\text{NO}_3^-$ did not alter the $K_m$ of $\text{Cl}^-$, up to 1 mM $\text{NO}_3^-$. These results imply a separate binding site(s) for $\text{NO}_3^-$ (fig. 10). Furthermore, the specific activity of the enzyme was not
### TABLE 5

Activity of the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase

<table>
<thead>
<tr>
<th>Species</th>
<th>$V_{\text{max}}$ (µM PO$_4$/mg protein-hr$^{-1}$)</th>
<th>$V_{\text{max}}$ (µM PO$_4$/mg chlorophyll $a$-hr$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$K_S$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonyaulax polyedra</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Gonyaulax tamarensis var. excavata</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amphidinium carterae</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>?*</td>
</tr>
<tr>
<td>Dunaliella tertiolecta</td>
<td>3.17</td>
<td>1.9 x 10$^6$</td>
<td>0.21</td>
<td>1.4</td>
</tr>
<tr>
<td>Eutreptiella gymnastica</td>
<td>8.74</td>
<td>3.1 x 10$^7$</td>
<td>0.12</td>
<td>-</td>
</tr>
<tr>
<td>Chroomonas salina</td>
<td>5.66</td>
<td>1.1 x 10$^7$</td>
<td>0.40</td>
<td>-</td>
</tr>
<tr>
<td>Isochrysis galbana</td>
<td>6.12</td>
<td>4.4 x 10$^6$</td>
<td>0.13</td>
<td>0.1</td>
</tr>
<tr>
<td>Skeletonema costatum</td>
<td>22.02</td>
<td>4.6 x 10$^7$</td>
<td>0.37</td>
<td>0.5, 0.4</td>
</tr>
<tr>
<td>Ditylum brightwellii</td>
<td>4.60</td>
<td>6.1 x 10$^7$</td>
<td>0.69</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Grant and Turner (1969)
+all others Eppley et al., (1969)
Fig. 9. Michaelis-Menten and Woolf plots for the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase from seven species. Each point is the mean of three determinations at the corresponding substrate concentration. Assays were done using the isotopic discrimination method.
Fig. 10. Effects of varying NO$_3^-$ concentrations on the half-saturation constant for Cl$^-$ in the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase from Skeletonema costatum at 20 °C and pH 7.9.
statistically altered whether NaNO$_3$, KNO$_3$ or NH$_4$NO$_3$ was the source of NO$_3^-$ (P>0.05). Ionic strength was kept constant within each assay series by the addition of MgSO$_4$; SO$_4^{2-}$ does not affect ATP hydrolysis under the conditions of the assay and was assumed not to be an activator of the enzyme.

**g) Nucleotide specificity**

Determinations of the nucleotide specificity on the (NO$_3^-$, Cl$^-$)-activated, enzymatic hydrolysis of the terminal phosphate from various nucleotides were made using semi-purified membrane preparations from *S. costatum*. The results of these experiments are summarized in Table 6. GTP and ITP were hydrolyzed in the presence of 1 mM NO$_3^-$ and 10 mM MgCl$_2$, however the activity of the enzyme was only 32% and 25% respectively of the hydrolysis observed when ATP was the substrate. CTP, TTP, PEP, and ADP did not show detectable inorganic phosphate (Fiske and SubbaRow technique) in response to NO$_3^-$ activation, under identical assay conditions. These results suggest ATP is the primary substrate under physiological conditions (i.e. the enzyme is primarily an adenosine triphosphatase); and GTP and ITP may support some enzyme activity, but the velocity of hydrolysis of these latter nucleotides (and presumably NO$_3^-$ transport) is much lower than with ATP.

**h) Velocities of the reaction(activity)**

The specific activities of the (NO$_3^-$, Cl$^-$)-activated
The nucleotide specificity of the (NO₃⁻, Cl⁻)-activated adenosine triphosphatase from *Skeletonema costatum* membrane preparations. Enzyme activity was assayed with the inorganic phosphate technique.

<table>
<thead>
<tr>
<th>Nucleotide*</th>
<th>% Activity (relative to ATP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>ND</td>
</tr>
<tr>
<td>GTP</td>
<td>32 ± 11</td>
</tr>
<tr>
<td>ITP</td>
<td>25 ± 8</td>
</tr>
<tr>
<td>CTP</td>
<td>ND</td>
</tr>
<tr>
<td>TTP</td>
<td>ND</td>
</tr>
<tr>
<td>PEP</td>
<td>ND</td>
</tr>
</tbody>
</table>

*For list of abbreviations see Appendix II.

(ND = not detected)
adenosine triphosphatase are shown in Table 5. To compare interspecific activities of the enzyme at saturating substrate concentrations ($V_{\text{max}}$), independent of purification, the specific activities were calculated on a unit chlorophyll a basis. These data may not be physiologically valid (i.e. they may not be the activity in vivo), as detergents are known to enhance the activity of some membrane-bound adenosine triphosphatases (Nakao et al., 1974). Despite the possible inadequacy of the technique, the relative specific activity may reflect $V_{\text{max}}$ if the degree of enzyme activation is linearly proportional to detergent concentrations (the final concentration of detergent was constant for each assay). The values ranged from $6.1 \times 10^7 \mu\text{M ATP/mg chl a - hr}^{-1}$ in *Ditylum brightwellii* to $1.9 \times 10^6 \mu\text{M ATP/mg chl a - hr}^{-1}$ in *Dunaliella tertiolecta*. Experiments to determine the stoichiometric ratio of NO$_3^-$ transported per ATP hydrolyzed were not successful primarily due to the difficulty in purifying the enzyme. The possibility remains that this ratio may be species specific; it is difficult to relate ATP hydrolysis directly to NO$_3^-$ transport at this time.

i) Activity of the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase in *Skeletonema costatum* grown on NH$_4^+$

To determine whether the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase was induced by NO$_3^-$ during cell growth or was a constitutive enzyme, *Skeletonema costatum* was grown on
"f/2" with 28 μg-atoms NH$_4^+$/l as the sole inorganic nitrogen source (substituting for 28 μg-atoms NO$_3^-$/l). Under these conditions, neither nitrate reductase nor nitrite reductase were detectable. However, the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase was found in the cell membrane fractions, at activities as great (per mg chlorophyll a) as when NH$_4^+$ was excluded from the growth media. Under identical assay conditions, the NH$_4^+$-grown cells demonstrated 4.37 x 10$^7$ μM ATP/mg chlorophyll a - hr$^{-1}$ as compared to 4.6 x 10$^7$ μM ATP/mg chlorophyll a - hr$^{-1}$ when grown on NO$_3^-$.

The aberrance between these two data may be attributable to experimental error, and are the mean values of four determinations on each preparation. These results strongly imply that the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase is constitutive.

II. NO$_3^-$ uptake and the intracellular ATP pools

in Skeletonema costatum

a) Effects of light and temperature on the intracellular ATP pool

The results of the effects of light and temperature on the net intracellular ATP pool in S. costatum cultures are shown in fig. 11. The intracellular ATP pool varies directly with light intensity, presumably due to the contributions of the light reactions. As the high-energy triphosphate pool size was calculated per mg chlorophyll a, the effects of short-term light adaptation were minimized, specifically
Fig. 11. Effects of light and temperature on the ATP pool in *Skeletonema costatum.*
those due to increased chlorophyll a synthesis per cell in the light attenuated samples (Jørgensen, 1964; Ryther and Menzel, 1959; Steeman-Nielsen et al., 1962; Uribe and Li, 1973). In addition, due to the short incubation period before the ATP extraction (2 hr), only a 5% increase in chlorophyll a was detected in the 100% light bottle at 18 C. Increased ATP pools were observed at 8 and 18 C, relative to the level observed in cells incubated at the higher temperature, 28 C. As the ATP pool is not a metabolic rate itself, but merely represents the difference between rate of input and rate of output of the nucleotide, calculations of the Q₁₀ (a rate dependent coefficient) would not be physiologically valid. It would seem however, that decreased temperature lowers the catabolism of ATP at a greater rate than the temperature independent synthesis of ATP during photophosphorylation. This overall difference (between enzymatic, temperature dependent, ATP catabolism and photochemical, temperature independent ATP anabolism) is reflected by a greater ATP pool at lower temperatures at corresponding light intensities.

b) Effects of metabolic inhibitors

The ATP pool of a photosynthetic organism is filled from four distinct sources, namely substrate phosphorylation, oxidative phosphorylation (including chemiosmotic gradients), non-cyclic photophosphorylation (photosystem II), and cyclic photophosphorylation (photosystem I). It is
possible to semi-selectively distinguish the contributions of each of these ATP sources to the net intracellular ATP pool by incubating the organisms with a "selective" metabolic inhibitor prior to ATP extraction.

To determine the qualitative contribution of oxidative phosphorylation to the ATP pool in the light and dark, KCN and 2,4-dinitrophenol were added separately to 125 ml aliquots of Skeletonema costatum cultures. The samples were incubated in duplicate for 2-4 hr at 18 C in the light and dark and the ATP was extracted as described. In the light, neither inhibitor effectively reduced the ATP concentration per mg chlorophyll a by more than 10%, as indicated in fig. 12a, b. In the dark however, the ATP pool was depleted by 71% with KCN and over 88% with 2,4-dinitrophenol. These results imply the contributions of oxidative phosphorylation to the ATP pool is relatively minor in the light, but not in the dark, as suggested by Arnon (1963).

Selective inhibition of non-cyclic photophosphorylation with $10^{-4}$ M 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Boardman, 1971) was used to approximate the contributions of PS II to the ATP pool. The results of this experiment are shown in fig. 12c, and indicate that DCMU reduced the ATP pool by about 47% in the light and 12% in the dark (N=3).

Inhibition of cyclic photophosphorylation with metabolic inhibitors is not selective. Raven (1974) has inter-
Fig. 12. Effects of $10^{-4}$M KCN (a), DNP (b), DCMU (c), and CCCP (d) on the ATP pool of *Skeletonema costatum* at 20°C in the light and dark.
interpreted the relative importance of cyclic photophosphorylation in phosphate uptake by inhibiting the process with the uncoupler, carbonyl cyanide m-chloro phenylhydrozone (CCCP). Despite the possible multiple effects of such an uncoupler (Boardman, 1971), attempts were made to determine the role of PS I in filling the ATP pool. CCCP, at $10^{-4} M$, reduced the ATP pool by 84% in the light and 29% in the dark (fig. 12d), indicating a major source of ATP in the light is due to cyclic electron flow in PS I.

Although it is not possible to precisely determine the rate of ATP output from each of the four possible sources from these data, the results suggest that oxidative and substrate phosphorylations are relatively minor sources of ATP in the light, or at least the ATP pool is not preferentially filled from these two processes. In the dark however, neither photosystem I or PS II is operative, causing a reduction in the overall ATP pool (fig. 11), and a dependence of the ATP pool on oxidative phosphorylation primarily (fig. 12a, b).

c) Effects of metabolic inhibitors on NO$_3^-$ uptake

To determine the effects of metabolic inhibitors on NO$_3^-$ uptake, 100 ml samples of _S. costatum_ (containing ca. 2 x 10$^7$ cells) were inoculated into 1 l. of artificial seawater (Kessler, 1967) enriched with f/2 nutrients (Guillard and Ryther, 1962). Initial NO$_3^-$ concentrations were adjusted
Fig. 12-2. Effects of $10^{-4}$ M KCN, DCMU, and CCCP on nitrate uptake velocities in *Skeletonema costatum* at 20°C and 0.09 ly/min.
to 28 μg-atoms/l. The cell suspension was subsampled into 125 ml Erlenmeyer flasks, and incubated with either 10⁻⁵ M KCN, 10⁻⁵ M DCMU or 10⁻⁵ M CCCP in the light at 18°C. NO₃⁻ uptake was followed by measuring the decrease in extracellular NO₃⁻ (Eppley et al., 1971; Ahmad and Morris, 1967). Only CCCP inhibited NO₃⁻ uptake in the light (fig. 12-2), while KCN and DCMU had no appreciable effect on uptake per se. These results are consistent with those reported by Eppley and Coatsworth (1968), and support the suggestion of Healy (1973) that cyclic photophosphorylation is a major energy source for NO₃⁻ uptake in the light.

d) Effects of extracellular NH₄⁺, NO₃⁻, and PO₄³⁻ on intracellular ATP pools

The addition of extracellular NH₄⁺ or NO₃⁻ to cultures of S. costatum (grown on NO₃⁻) within 60 sec of ATP extraction caused a marked reduction in the ATP pool. The decrease in ATP was non-linearly dependent upon the concentration of the extracellular nitrogen source, and not demonstrable in the dark (Table 7). These results appear to be at variance with those reported by Ullrich-Eberius (1973), who found NO₃⁻ had no appreciable effect on the ATP pool of Ankistrodesmus braunii (see discussion). In contrast to the effect of the nitrogen ions however, 3 μg-atoms PO₄³⁻/l enrichments increased the ATP level over a 60 sec period, suggesting the flux of PO₄³⁻ into the nucleotide pool is rapid.
Table 7

Effects of $\text{NO}_3^-$, $\text{NH}_4^+$, and $\text{PO}_4^{3-}$ on the ATP pool of *Skeltonema costatum*. Cells were grown at 18 C and incubated for 1 min with the nutrient concentrations indicated under identical light conditions (0.09 ly/min).

<table>
<thead>
<tr>
<th>Condition</th>
<th>N</th>
<th>nM ATP/mg chlorophyll a*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>17</td>
<td>247 ± 34</td>
</tr>
<tr>
<td>900 µM $\text{NO}_3^-$</td>
<td>3</td>
<td>120 ± 19</td>
</tr>
<tr>
<td>9 µM $\text{NO}_3^-$</td>
<td>6</td>
<td>163 ± 28</td>
</tr>
<tr>
<td>2.5 µM $\text{NO}_3^-$</td>
<td>6</td>
<td>221 ± 30</td>
</tr>
<tr>
<td>900 µM $\text{NH}_4^+$</td>
<td>3</td>
<td>149 ± 31</td>
</tr>
<tr>
<td>9 µM $\text{NH}_4^+$</td>
<td>3</td>
<td>194 ± 16</td>
</tr>
<tr>
<td>2.5 µM $\text{NH}_4^+$</td>
<td>3</td>
<td>224 ± 23</td>
</tr>
<tr>
<td>8 µM $\text{PO}_4^{3-}$</td>
<td>3</td>
<td>284 ± 46</td>
</tr>
<tr>
<td>0.8 µM $\text{PO}_4^{3-}$</td>
<td>3</td>
<td>261 ± 17</td>
</tr>
</tbody>
</table>

*Mean of N determinations ± standard deviation*
It seems reasonable to suggest that the addition of either extracellular $\text{NO}_3^-$ or $\text{NH}_4^+$ causes increased hydrolysis of ATP and thereby reduces the intracellular ATP pool. This process appears analogous to the addition of extracellular $\text{K}^+$ to the $(\text{Na}^+ + \text{K}^+)$-activated transport adenosine triphosphatase (Glynn, 1962).

B. Field Studies on Natural Phytoplankton Communities in Knight Inlet

I. Morphometric and hydrographic considerations

Knight Inlet is divided into two main basins, separated by shallow sills. One sill, at 63 m, separates station QC from Kn 3 and a second sill at 65 m separates Kn 3 from the headward stations (fig. 13). The consequences of this bottom topography are complicated and not fully understood, but essentially the sills restrict the deep water circulation throughout most of the year (Stone, personal communication). Due to summer entrainment and offshore upwelling, deep water exchanges slowly from May to December with high salinity water coming into the estuary from 10 m to the bottom. The less dense, low salinity surface waters, contained in the upper 10 m, flow seaward.

The surface waters are separated from the subsurface water masses by a strong halocline from Kn 7 to the head (Table 8). The picnocline does not appear to lead to nutrient impoverishment however, as both nitrate and phos-
TABLE 8

Salinity, nitrate and the estimated 1% light depths at the sample stations in July and September 1974. A pronounced halocline is observable at all stations in the Inlet, especially in the upper 5 m.

<table>
<thead>
<tr>
<th>Station</th>
<th>Depth (m)</th>
<th>Salinity °/oo</th>
<th>NO3 (µg-at/l)</th>
<th>1% light depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC</td>
<td>0</td>
<td>31.06</td>
<td>31.02</td>
<td>20.21</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>31.08</td>
<td>31.09</td>
<td>20.21</td>
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<td></td>
<td>10</td>
<td>31.27</td>
<td>31.39</td>
<td>19.49</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>31.68</td>
<td>31.73</td>
<td>19.97</td>
</tr>
<tr>
<td>Kn 3</td>
<td>0</td>
<td>16.70</td>
<td>22.19</td>
<td>16.64</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>24.48</td>
<td>27.62</td>
<td>13.91</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>29.23</td>
<td>29.73</td>
<td>15.18</td>
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<tr>
<td></td>
<td>30</td>
<td>30.22</td>
<td>20.44</td>
<td>19.28</td>
</tr>
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<td>11.28</td>
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<td></td>
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<td>3.27</td>
<td>3.82</td>
<td>1.09</td>
</tr>
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<td></td>
<td>5</td>
<td>20.92</td>
<td>24.59</td>
<td>14.08</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>26.99</td>
<td>27.91</td>
<td>19.62</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>29.86</td>
<td>30.11</td>
<td>24.11</td>
</tr>
<tr>
<td>Kn 11</td>
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<td>0.57</td>
<td>0.89</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20.43</td>
<td>20.59</td>
<td>12.35</td>
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<tr>
<td></td>
<td>10</td>
<td>27.87</td>
<td>26.97</td>
<td>19.30</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>29.93</td>
<td>30.03</td>
<td>22.20</td>
</tr>
</tbody>
</table>
Fig. 13. Bottom topography and generalized circulation pattern in Knight Inlet, B. C. and adjacent Queen Charlotte Straits.
phorus are relatively abundant in the euphotic zone throughout most of the year. Nitrate averages ca. 25-30 μg-atoms/l in the estuary, far in excess of some reported values of nitrogen limitation (Glooschenko and Curl, 1971; Thomas, 1971).

II. A brief description of the phytoplankton community

The seasonal variations in chlorophyll a (fig. 2) indicate a bimodal bloom, characteristic of many temperate waters (Parsons and Takahashi, 1973; Raymont, 1962; Smayda, 1973). A spring bloom in March-April was observed in 1974 at QC, Kn 3, Kn 5, Kn 7, and Kn 11 and a secondary fall bloom was seen in August-September at QC, Kn 3, Kn 5, and Kn 11 for surface waters (0-5 m).

On all cruises, phytoplankton populations were relatively low in the inlet and high in the adjoining Strait, possibly due to the steep salinity gradients upstream. The major species represented in the Strait were characterized by diatoms, especially Chaetoceros debilis, Thalassiosira nordenskoldi, Skeletonema costatum, Asterionella japonica, and Thalassionema nitzschioides. The inlet stations were not dominated by a single species, although all the net plankton (>25 μ) were marine, and undoubtedly were carried into the estuary from the adjoining Straits. Skeletonema costatum appeared abundant at Kn 3 and Kn 5, but was virtually absent from Kn 11 in July. At that time nanophyto-
plankton were predominantly observed in the preserved samples.

III. Experimental results

a) Effects of nitrogen enrichment on inorganic carbon fixation

Field studies with whole, natural phytoplankton communities from Knight Inlet indicate a brief, transitory decrease in inorganic carbon fixation per unit chlorophyll a (i.e. assimilation ratio) at all light intensities tested with 3 µg-atoms N/l enrichments (fig. 14). The enrichments (either NO$_3^-$ or NH$_4^+$ as the nitrogen source) only represent 10-15% of the dissolved in situ nitrogen concentrations (Table 8); hence excess nutrient repression was not considered to be a cause of the decreased productivity over a 2 hr incubation period (MacIsaac and Dugdale, 1972).

The assimilation ratios ranged from 3 to 7 in the un-enriched samples at maximum light intensity (i.e. 100%). At Kn 11 however, light inhibition was observed at intensities over 60%, implying the cells were shade adapted (Ryther and Menzel, 1959). At this latter station, particulate suspended material decreased the light penetration so severely that the 1% light depth was estimated at less than 1 m. Enrichment with nitrogen during the first 2 hr resulted in 60-70% decrease in assimilation ratios (fig. 14 A and E). The inorganic carbon fixation during this period did not vary
Fig. 14. Comparison of the effects of $\text{NO}_3^-$ (●) and $\text{NH}_4^+$ (■) with unenriched samples (●) on photosynthetic assimilation ratios. At QC, a 2 hr incubation with either nitrogen source resulted in decreased assimilation ratios (A), but this effect was diminished after a 6 hr preincubation (B). At Kn 3 (C) and Kn 5 (D) 6 hr preincubation with $\text{NO}_3^-$ or $\text{NH}_4^+$ indicated a difference in the rate at which the two communities responded to nutrient conditions. Kn 3 showed less suppression of carbon fixation after 6 hr, possibly due to increased adaptation to external nutrients. At Kn 7 (E) a 2 hr incubation caused an altered light response; the enriched samples responded linearly with light intensity, while the control described a hyperbola. At Kn 11 (F) inhibition at intensities above 60% was observed in all samples after a 2 hr incubation.
Fig. 14.

Assimilation Ratio (mg C/mg Chl a-h⁻¹)

% Light Intensity
hyperbolically with light intensity, but remained linear, suggesting dark reactions were not limiting (Qasim et al., 1969).

Following a 6 hr preincubation with either nitrogen source, addition of $2 \mu$Ci $\text{Na}_2\text{C}^{14}\text{O}_3$ resulted in increased assimilation ratios compared to unenriched samples (fig. 14 B, C, D, F). These results indicate a physiological adaptation to external nutrient concentrations by the cells. The adaptive period appears to be between 2 and 6 hr and is characterized by "reverse kinetic" relationships between inorganic carbon fixation and external nitrogen concentrations. These types of kinetics imply decreasing carbon fixation with increasing extracellular nutrient concentrations. These data are consistent with unpublished observations by Dugdale (Harrison, personal communication).

b) Effects of nitrogen enrichment on chlorophyll a/cell

The physiological adaptation of the phytoplankton communities to 6 hr preincubations with either nitrogen source was accompanied by increased chlorophyll a synthesis per cell (Table 9). At Kn 11 initial chlorophyll a/cell averaged ca. $9.10 \times 10^{-7} \mu g$, representing 1.5 to 2.3 times the intracellular chlorophyll a found in cells from the other four stations (Table 9). This relatively high level of intracellular chlorophyll a is consistent with umbrophillic adaptation (Jørgensen, 1964; Ryther and Menzel, 1959;
Steeman Nielsen et al., 1962). Upon addition of nitrogen, chlorophyll a/cell increased by ca. 10 to 20% within 6 to 8 hr, and was accompanied by increased assimilation ratios (fig. 14), at all stations.
TABLE 9

The effects of nitrate and ammonium in intracellular chlorophyll a. These data are taken from Sept. samples from 2 m after 6 to 8 hr preincubation with either nitrogen source. Cell counts were made on preserved samples within a week of collection. An inverted microscope was used to count 100 random fields at 250 X. Chlorophyll a data are mean values and are intended for intrastation comparison only.

<table>
<thead>
<tr>
<th>Station</th>
<th>Condition*</th>
<th>Cells/l(x10^6)</th>
<th>Chl a/cell (x10^-7ug)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC</td>
<td>A</td>
<td>4.27</td>
<td>4.21</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4.32</td>
<td>4.30</td>
<td>+2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>4.51</td>
<td>4.93</td>
<td>+18</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>4.71</td>
<td>4.87</td>
<td>+13</td>
</tr>
<tr>
<td>Kn 3</td>
<td>A</td>
<td>2.11</td>
<td>8.06</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>2.19</td>
<td>7.66</td>
<td>-5</td>
</tr>
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<td>C</td>
<td>2.40</td>
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<td></td>
<td>B</td>
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<td></td>
<td>C</td>
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<td>6.48</td>
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<td>D</td>
<td>3.26</td>
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<tr>
<td>Kn 11</td>
<td>A</td>
<td>0.33</td>
<td>9.10</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.41</td>
<td>8.72</td>
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</tr>
<tr>
<td></td>
<td>C</td>
<td>0.37</td>
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</tr>
<tr>
<td></td>
<td>D</td>
<td>0.37</td>
<td>10.31</td>
<td>+13</td>
</tr>
</tbody>
</table>

*A- original samples
B- unenriched samples
C- nitrate enriched
D- ammonium enriched
Discussion

A. Nitrate Uptake in Marine Phytoplankton

The results of the laboratory studies with membranes isolated from six species of marine phytoplankton (excluding the dinoflagellates\(^1\)), indicate the presence of an enzyme that hydrolyzes ATP in the presence of Mg\(^{2+}\), Cl\(^-\), and NO\(_3^-\). The physiological function of the (NO\(_3^-\), Cl\(^-\))-activated adenosine triphosphatase cannot be inferred from the biochemical studies alone because (a) the protein is no longer oriented \textit{in vitro} on the plasmalemma, and (b) none of the activating ions are truly substrates for the reaction (i.e. the only true substrate apparently is ATP). To date however, analogous membrane-bound enzymes, such as the (Na\(^+\) + K\(^+\))-activated adenosine triphosphatase (Skou, 1957), the Ca\(^{2+}\)-activated adenosine triphosphatase (Schatzmann and Vincenzi, 1969), the HCO\(_3^-\)-activated adenosine triphosphatase (Narumi and Kanno, 1973), and the Si(OH)\(_4^-\)-stimulated, Mg\(^{2+}\)-activated adenosine triphosphatase (Hemmingsen, 1971), have been implicated in the translocation of their respective activating ions across various membranes. Mainly because of the difficulty in understanding the molecular mechanism(s) of these enzymes (or more specifically, the difficulty in explaining the coupling between scalar energy liberated from

\(^1\)see Appendix I
ATP hydrolysis to the vectoral flux of ions), the roles of membrane-bound adenosine triphosphatases in regulating intracellular ion concentrations has to be determined by an examination of both physiological and biochemical properties, since neither is adequate to serve this purpose alone.

A major feature of the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase, isolated from both *Skeletonema costatum* and *Chroomonas salina*, is the bimodal pH profile. If the adenosine triphosphatase was solely responsible for NO$_3^-$ transport, NO$_3^-$ uptake by whole cells should exhibit a similar hydrogen ion dependence. This correlation is difficult to obtain from the literature because of the lack of published data on the pH dependence of NO$_3^-$ uptake by marine phytoplankton. Ullrich-Eberius (1973) has provided however, a pH profile for NO$_3^-$ uptake in *Ankistrodesmus braunii* (Chlorophyceae) which exhibits a similar bimodal pH dependence *in vivo* to that observed here for the isolated (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase *in vitro*. *Ankistrodesmus braunii* was found to have an optimum NO$_3^-$ uptake velocity at ca. pH 8.0 and a secondary optimum at ca. pH 6.0. These results are comparable to the optima of 7.8-8.2 and 6.9 found for the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase (fig. 4). It may be possible to attribute the slight shift in the secondary pH peak to interspecific variation, or perhaps physiological conditions that were not duplicated (e.g. the addition of detergents).
A second physiological (and perhaps more ecologically significant) feature implicating the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase with \(\text{NO}_3^-\) uptake, is the correlation between their half-saturation constants. Table 5 summarizes the comparison between \(K_m\) values calculated for \(\text{NO}_3^-\) activation of the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase and \(K_s\) values reported for \(\text{NO}_3^-\) assimilation by whole cells (Eppley et al, 1969). *Skeletonema costatum, Ditylum brightwellii,* and *Isochrysis galbana* half-saturation constants agree, within experimental variance, with reported \(K_s\) values for \(\text{NO}_3^-\) uptake. The results for *Dunaliella tertiolecta* are significantly different, although activation of the enzyme by anionic detergents during the extraction procedure (Nakao et al, 1974), clonal variations (Hecky and Kilham, 1974), and variations in culturing conditions cannot be ruled out. In addition, Grant and Turner (1969), using \(5 \times 10^7\) to \(5 \times 10^8\) cells/ml, reported that the \(\text{NO}_3^-\) uptake velocity in *Amphidinium carterae* was "too low to measure", supporting the observation that the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase is apparently absent from this species. Eppley et al (1969) reported that the \(K_s\) for \(\text{NO}_3^-\) uptake in *Gonyaulax polyedra* is ca. 10 \(\mu\text{M}\). This value is about tenfold greater than for species with the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase (i.e. diatoms, euglenoids, etc.). The apparent variations in \(K_s\), thought to be associated with cell
size and habitat (Eppley et al, 1969; Parsons and Takahashi, 1973) have not fully explained the abilities of dinoflagellates to reach the dominant population densities sometimes observed in "red tides" (e.g. Pincemin, 1969). Although the three dinoflagellates tested do not appear to have the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase to mediate the transport of NO$_3^-$ across the plasmalemma, their undoubted capability to utilize NO$_3^-$ indicates there is another mechanism for its uptake. For example, dinoflagellates may utilize a coupled transport phenomenon: the maintenance of an electrochemical potential across the cell membrane, allowing NO$_3^-$ to be transported along the electrochemical gradient of the ion (Katchalsky and Curran, 1967).

Ion uptake kinetics in photosynthetic organisms are often characterized by complicated, multiphasic functions (Nissen, 1973, 1974). Essentially these kinetics can be resolved into two major components: a high capacity, low affinity system (i.e. high $V_{\text{max}}$, high $K_s$), and a low capacity, high affinity system (i.e. low $V_{\text{max}}$, low $K_s$) (e.g. Azam and Volcani, 1974; Hellebust and Lewin, in press). The ability to distinguish between these two components kinetically is a function of substrate concentration. The kinetics of the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase suggest that the enzyme has a relatively high affinity for NO$_3^-$ uptake (i.e. $K_m < 1 \mu$M NO$_3^-$ for most species), however, due to the large energy of activation (fig. 5) and the direct
dependence of \( \text{NO}_3^- \) transport on ATP hydrolysis (fig. 8a), this system may not have a high capacity. Photosynthetic dinoflagellates, while capable of utilizing \( \text{NO}_3^- \) for amino acid synthesis (Packard and Blasco, 1974), have, on the whole, a lower affinity for the ion than that of other groups. These observations suggest that dinoflagellates may possess a low affinity, but high capacity system, for \( \text{NO}_3^- \) uptake, while other species, with the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase, utilize primarily a high affinity, low capacity system.

Eppley et al (1969) and Parsons and Takahashi (1973) have suggested the variations in \( K_s \) for \( \text{NO}_3^- \) uptake may be due to variations in cell size, and perhaps habitat of the species (e.g. oceanic vs. neritic). Hecky and Kilham (1974) have disputed the hypothesized relationship between \( K_s \) and cell size, by pointing out that variations in half-saturation constants are sometimes observed in various strains of the same species, grown under identical culture conditions. (Presumably cell sizes are uniform between strains.) As \( K_s \) values for \( \text{NO}_3^- \) uptake in marine phytoplankton are calculated over relatively broad ranges of the external \( \text{NO}_3^- \) concentrations (e.g. \( \text{NO}_3^- \) may typically vary from 0 to 50 \( \mu \text{M} \)), it may be difficult to resolve the various components of the uptake kinetics. Thus, species, and perhaps even clonal variations, may be due to kinetic interactions between the high capacity,
low affinity system and the low capacity, high affinity system, operating in different relative proportions under variable external nutrient concentrations. In addition, it should be stated that both the high capacity, low affinity system and the low capacity, high affinity system apparently exhibit Michaelis-Menten kinetics individually (e.g. Hellebust and Lewin, in press). This observation makes it especially difficult to resolve the two systems from kinetic experiments where the concentrations range over two orders of magnitude, and few determinations of nutrient uptake velocities are made at high concentrations.

A third feature implicating the \((\text{NO}_3^-, \text{Cl}^-)\)-activated adenosine triphosphatase with \text{NO}_3^- transport is the results of physiological experiments suggesting that the primary energy source for the uptake process is due to cyclic photophosphorylation \textit{in vivo}. As \text{NO}_3^- uptake is not severely inhibited by respiratory inhibitors, such as KCN or DNP, or by DCMU (an inhibitor of non-cyclic photophosphorylation), but is affected by CCCP, the correlation between \text{NO}_3^- uptake and cyclic photophosphorylation is apparently not due to induction of the carrier system, but rather to increased substrate (i.e. ATP) concentrations in the light. These results are consistent with the observations that increasing light intensities result in increasing ATP concentrations (fig. 11).
Grant and Turner (1969) and MacIsaac and Dugdale (1972) have demonstrated that NO$_3^-$ uptake is light dependent and can be described by Michaelis-Menten kinetics with respect to light intensity (i.e. NO$_3^-$ uptake velocities vs. light intensities describe a rectangular hyperbola). If the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase were solely responsible for NO$_3^-$ uptake, and primarily dependent on cyclic photophosphorylation for ATP, NO$_3^-$ uptake should undergo diel periodicity in situ. This suggestion is consistent with observations made by Eppley et al (1971), who reported that NO$_3^-$ uptake in Skeletonema costatum exhibited light/dark periodicity. Furthermore, not only does NO$_3^-$ uptake exhibit diel periodicity, but the substrate-inducible enzyme, nitrate reductase, has been observed to be light dependent as well (Eppley et al, 1971; Packard, 1973; Packard and Blasco, 1974). The diel periodicity observed for nitrate reductase may be attributed to light intensity per se (Beavers and Hageman, 1969), as well as the induction of the enzyme by NO$_3^-$. As the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase is apparently constitutive (see Results section A.I.i), NO$_3^-$ uptake may occur at low levels in the light, even in the presence of NH$_4^+$ (a repressor of nitrate reductase). Under these conditions, the intracellular NO$_3^-$ concentration would not be affected by nitrate reductase, as presumably the enzyme would be repressed by NH$_4^+$ (Rigano and Violante, 1973). Consequent-
ly, NO\textsubscript{3} uptake, but not reduction or assimilation, may occur in the presence of NH\textsubscript{4}\textsuperscript{+}. This phenomenon would cause a reduction in the extracellular NO\textsubscript{3} concentrations, but would not be reflected by NO\textsubscript{3}\textsuperscript{-}-dependent growth. This type of interaction may be termed "luxury consumption" (Ketchum, 1939), and is suggested to be due to inhibition of NO\textsubscript{3} reduction and incorporation, but not NO\textsubscript{3}\textsuperscript{-} uptake. In addition, ATP is apparently the rate limiting substrate in the dark at non-limiting NO\textsubscript{3}\textsuperscript{-} concentrations. Thus the induction of nitrate reductase in the light may be due to increased ATP supply to the (NO\textsubscript{3}, Cl\textsuperscript{-})-activated adenosine triphosphatase, resulting in increased NO\textsubscript{3} uptake. The increased NO\textsubscript{3} uptake may stimulate the synthesis of nitrate reductase, resulting in a more classical type of induction of nitrate reductase.

A fourth feature suggesting that NO\textsubscript{3} uptake is correlated to ATP hydrolysis may be inferred from the results of extracellular NO\textsubscript{3} enrichments on the intracellular ATP pool. These data (Table 7) imply that extracellular NO\textsubscript{3} reduces the ATP pool rapidly and non-linearly, presumably due to increased ATP hydrolysis. This phenomenon appears to be analagous to the addition of extracellular K\textsuperscript{+} to the (Na\textsuperscript{+} + K\textsuperscript{+})-activated adenosine triphosphatase (Glynn, 1962) and supports the hypothesis that the physiological role of the (NO\textsubscript{3}, Cl\textsuperscript{-})-activated adenosine triphosphatase is to mediate the transport of external NO\textsubscript{3} into the cytoplasm. In addition, the
data from Table 7 also suggest that extracellular NH$_4^+$ stimulates ATP hydrolysis. As this cation is not an activator of the (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase, these results suggest that NH$_4^+$ uptake may also be due to an active transport process, but via a different pathway than for NO$_3^-$.

Ullrich-Eberius (1973) has observed that NO$_3^-$ addition does not cause a reduction in the ATP pool (in the light) in Ankistrodesmus braunii. Although these results appear at variance with the data from Table 7, the time interval between addition of NO$_3^-$ and determination of the ATP pool in A. braunii was about 10 min. This relatively long interval may allow the organism to restore the ATP charge (i.e. reach a new equilibrium) (e.g. Holm-Hansen, 1973), possibly leading to the conclusion that NO$_3^-$ uptake was not directly ATP dependent.

In 1939 Ketchum pointed out the importance of PO$_4^{3-}$ in NO$_3^-$ uptake. The results of PO$_4^{3-}$ enrichments on the ATP pool (Table 7) imply that PO$_4^{3-}$ is rapidly incorporated into organic phosphates (notably ATP). It is suggested that PO$_4^{3-}$ may partially limit NO$_3^-$ uptake if PO$_4^{3-}$ concentrations are limiting, or the flux of PO$_4^{3-}$ in the adenylate pool is low. The indirect effect of PO$_4^{3-}$ limitation under these conditions is suggested to be a reduction in the ATP pool (but not necessarily ADP or AMP), thereby limiting such ATP
dependent processes as $\text{NO}_3^-$ uptake. This phenomenon has been observed by Sakshaug (personal communication) in chemostat cultures. In addition, Raven (1974) has suggested that $\text{PO}_4^{3-}$ uptake is coupled to ATP hydrolysis (i.e. the anion is actively transported across cell membranes). The source of ATP for this process is apparently cyclic photophosphorylation; it is inhibited by CCCP. As external $\text{PO}_4^{3-}$ is apparently related to increased ATP pools in *Skeletonema costatum*, the stoichiometric ratio of $\text{PO}_4^{3-}$ per ATP hydrolyzed is probably greater than 1. (If the ratio were less than 1 net intracellular ATP would decrease in the presence of increased $\text{PO}_4^{3-}$.)

Nitrate uptake in most species of marine phytoplankton is apparently mediated by a constitutive, membrane-bound adenosine triphosphatase, and not inhibited by $\text{NH}_4^+$. Field studies with natural phytoplankton communities were carried out to determine what physiological feedback mechanisms control $\text{NO}_3^-$ uptake *in vivo*. Firstly, it was observed that $\text{NO}_3^-$ enrichments (as well as $\text{NH}_4^+$ enrichments) inhibited inorganic carbon fixation for ca. 2 to 6 hr (fig. 14). This suppression of carbon fixation was not dependent on the previous light history of the cells, as the samples were obtained from the same absolute depths at each station despite extreme changes in the light extinction coefficients.
As both NO$_3^-$ uptake and inorganic carbon fixation are light dependent processes, the inhibition of carbon fixation in the presence of increased external nitrogen concentrations implies a competition between the two elements for either light itself, or a product of light reactions. After a 6 hr preincubation with either nitrogen source however, carbon fixation was not affected by external nitrogen concentrations, although the light intensities were unaltered. These results suggest that an adaptive period of between 2 and 6 hr is required by natural populations of phytoplankton after alterations in the external nitrogen concentrations. The ecological significance of this adaptation may not be crucial under most natural circumstances, because extracellular nitrogen concentrations probably rarely increase with such rapidity.

The adaptive period observed in Knight Inlet, following nitrogen enrichments, was accompanied by increased chlorophyll a/cell (Table 9). As external NO$_3^-$ may increase ATP hydrolysis, thereby reducing the intracellular ATP pool (Table 7), it is inferred that increased chlorophyll a synthesis relieves this energy drain by providing a greater intracellular light-trapping area. Thus ATP dependent processes, preferentially driven by light reactions, such as carbon fixation, NO$_3^-$, NH$_4^+$, and PO$_4^{3-}$ uptake, may be supplied with more substrate (i.e. ATP) as the result of increased
light-dependent ATP synthesis. This suggestion has also been made, to some extent, by Bates (1974), who observed increased chlorophyll a/cell following NO$_3^-$ enrichments in Skeletonema costatum (Bates, personal communication).

The reverse kinetic interaction between inorganic carbon and inorganic nitrogen suggests that one major physiological control of the utilization of these two elements involves chlorophyll a/cell. Biochemically however, chlorophyll a/cell appears to be regulated, at least in part, by feedback from ATP pools (or perhaps more precisely the relative proportions of ATP, ADP, AMP, and PO$_4^{3-}$). This reverse kinetic pattern has been observed for other nutrients, including Si(OH)$_4$ and carbon, Si(OH)$_4$ and nitrate, and NH$_4^+$ and carbon (Dugdale, personal communication). In all cases where the reverse kinetic phenomenon exists, the nutrients are apparently transported either by an adenosine triphosphatase, or in the case of carbon, coupled in some way directly to ATP (e.g. in the intermediary metabolism). In addition, the primary ATP source for the uptake and assimilation of these nutrients is apparently from the light reactions, with the exceptions of the apochloritic (colourless) organisms, such as Nitzschia alba, that require Si(OH)$_4$, but cannot utilize inorganic carbon (e.g. Goering, 1974; Hemmingsen, 1971).

Based on the results of these experiments, a modified
pathway of nitrogen metabolism in diatoms was constructed (fig. 15). The principal modification in the pathway (as opposed to the one presented in fig. 1) is the addition of the (NO\textsuperscript{-3}, Cl\textsuperscript{-})-activated adenosine triphosphatase. It should be noted however, that the sources of energy for NO\textsuperscript{-3} uptake, NO\textsuperscript{-3} reduction, and NH\textsubscript{4}\textsuperscript{+} incorporation are not identical to those proposed by Packard and Blasco (1974) (fig. 1).

Ahmad and Morris (1967) have indicated that NO\textsuperscript{-3} reduction is sensitive to DCMU, suggesting that the reductants are derived from non-cyclic photophosphorylation. The reductants (either NADH or NADPH, depending on the species) are suggested to be supplied specifically from Photosystem II, while the primary ATP source for NO\textsuperscript{-3} uptake is generated from Photosystem I.

Dinoflagellates appear to deviate from this proposed pathway in a number of important aspects. Firstly, the uptake of NO\textsuperscript{-3} is not directly coupled to ATP hydrolysis in the species examined here. Hence, it would appear that NO\textsuperscript{-3} uptake in dinoflagellates is mediated by some mechanism(s) other than the (NO\textsuperscript{-3}, Cl\textsuperscript{-})-activated adenosine triphosphatase. Secondly, observations of nitrate reductase activity in a *Gonyaulax polyedra* bloom off the coast of Baja California by Packard and Blasco (1974) indicate that this enzyme does not appear to undergo diel periodicity, typically observed in other species. These workers have suggested that NO\textsuperscript{-3} reduc-
Fig. 15. A generalized scheme of NO$_3^-$ metabolism in some marine phytoplankton. Nitrate uptake is suggested to be mediated by a membrane-bound (NO$_3^-$, Cl$^-$)-activated adenosine triphosphatase. The primary (but not sole) ATP source for this process appears to be cyclic photophosphorylation. Once inside the cell, NO$_3^-$ may be sequestered in a vacuole, or reduced via nitrate reductase (NR) to NO$_2^-$. Nitrite is further reduced to ammonium, utilizing ferredoxin as an electron donor. The ammonium is probably incorporated into $\alpha$-keto glutarate to form glutamate. Regeneration of $\alpha$-keto glutarate may occur if the amino nitrogen of glutamate is transaminated to other keto acids. Indirect evidence appears to suggest that ammonium uptake is ATP dependent as well.
NITRATE METABOLISM
(GENERALISED SCHEME)
tion may be coupled to respiratory, rather than photosynthetic, reductants. It is suggested that nitrate reductase in dinoflagellates is substrate inducible (Eppley et al., 1973), implying that even in the dark $\text{NO}_3^-$ uptake may occur in this group. Thirdly, the major enzyme implicated in $\text{NH}_4^+$ assimilation, namely glutamate dehydrogenase, appears to be absent from some species, notably *Amphidinium carterae* (Falkowski, unpublished data; Ahmed, personal communication). In order to incorporate $\text{NH}_4^+$ into amino acids, at least some species must utilize an alternative pathway(s) than indicated in either fig. 1 or fig. 15. The proposed nitrogen pathway in dinoflagellates (fig. 16) indicates that the probable route of $\text{NH}_4^+$ incorporation is through glutamine synthetase. Transaminations from the amido-nitrogen of glutamine to other amino acids may be catalyzed by glutamine (amide): 2-oxoglutarate amino transferase oxido-reductase (also known as glutamate synthetase) (Dainty, 1972). This pathway may also be present in blue-green algae (Dharmawadene et al., 1973; Haystead et al., 1973). The enzyme glutamine synthetase requires ATP for $\text{NH}_4^+$ incorporation, but has a lower $K_m$ for $\text{NH}_4^+$ than glutamate dehydrogenase. In addition, transaminations from glutamine to $\alpha$-keto glutarate results in the formation of 2 molecules of glutamate. This latter amino acid is a substrate for glutamine synthetase. Thus, the synthesis of 2 glutamate molecules may allow one to act as an inter-
Fig. 16. A postulated pathway for nitrogen assimilation in dinoflagellates. Nitrate uptake is suggested to be thermodynamically coupled to a Na\(^+\)/K\(^+\) pump on the cell membrane. The reduction of NO\(_3^-\) is mediated by an NADH-dependent nitrate reductase (NR), which probably derives electrons from respiration. Once NO\(_3^-\) is reduced to NO\(_2^-\), nitrite reductase (electron source in vivo unknown) further reduces the nitrogen to NH\(_4^+\). The NH\(_4^+\) is incorporated into glutamate to form glutamine. This latter step, mediated by glutamine synthetase (GS), is probably followed by the addition of the amido nitrogen to \(\alpha\)-keto glutarate to form two moles of glutamate. The reaction is catalyzed by glutamate synthetase (G1S). The regeneration of \(\alpha\)-keto glutarate is achieved by the transamination (TR) of the amino nitrogen from one glutamate to other keto acids. In this group (dinoflagellates), amino acid metabolism is suggested to occur in both the light and dark, as electron sources for reduction, and high energy nucleotides do not appear to be light dependent. It is suggested that the major function of the chloroplast is to generate carbon skeletons for amino acid synthesis.
Fig. 16.

POSTULATED NITROGEN ASSIMILATION in DINOFLAGELLATES

\[
\begin{align*}
\text{NO}_3^- & \xrightarrow{\text{ATP, NADH}} \text{NO}_3^- \\
\text{NH}_4^+ & \xrightarrow{\text{ATP, NADH}} \text{NH}_4^+ \\
\text{NH}_4^+ & \xrightarrow{\text{glutamate}} \text{ATP, NADH} \\
\text{ADP} & \xrightarrow{\text{NAD}} \text{NADH} \\
\text{glutamine} & \xrightarrow{\text{glutamate}} \text{ATP, NADH} \\
\text{amino acids} & \xrightarrow{\text{glutamate}} \text{ATP, NADH} \\
\end{align*}
\]
mediary nitrogen source (as in fig. 1), while the other is recycled through glutamine synthetase. This conservation of carbon skeletons may reduce the carbon drain from the tricarboxylic acid cycle.

B. A Model for NO\textsubscript{3}\textsuperscript{−} Uptake Based on Bisubstrate Kinetics

The isolated (NO\textsubscript{3}\textsuperscript{−}, Cl\textsuperscript{−})-activated adenosine triphosphatase is identified in vitro by NO\textsubscript{3}\textsuperscript{−}-stimulated (and by Cl\textsuperscript{−}, but to a lesser extent) ATP hydrolysis. While the enzyme is biochemically an adenosine triphosphatase, the physiological role of the membrane-bound protein is essentially the active transport of NO\textsubscript{3}\textsuperscript{−}. Presently NO\textsubscript{3}\textsuperscript{−} uptake kinetics are determined directly by measuring the disappearance of NO\textsubscript{3}\textsuperscript{−} from the medium, as it is not possible to directly relate the activity of the (NO\textsubscript{3}\textsuperscript{−}, Cl\textsuperscript{−})-activated adenosine triphosphatase to NO\textsubscript{3}\textsuperscript{−} uptake at this time. In vitro therefore the anion (NO\textsubscript{3}\textsuperscript{−}) functions as an enzyme activator and may not be considered a true biochemical substrate. From a physiological or ecological standpoint however, NO\textsubscript{3}\textsuperscript{−} may be considered a true substrate in vivo.

It has generally been accepted that NO\textsubscript{3}\textsuperscript{−} uptake kinetics may be approximated by the Michaelis-Menten expression (e.g. Caperon and Meyer, 1972; Eppley and Rogers, 1970). Grant and Turner (1969) and MacIsaac and Dugdale (1972) have pointed out that NO\textsubscript{3}\textsuperscript{−} uptake velocities also appear to be a function of light intensity. The latter two authors have
indicated that light (acting as a physiological substrate) is related to $\text{NO}_3^-$ uptake velocities by Michaelis-Menten kinetics as well. The effects of light are probably indirect; the results of this study suggest that light increases intracellular ATP, a substrate necessary for the $(\text{NO}_3^-, \text{Cl}^-)$-activated adenosine triphosphatase. For the purposes of a descriptive model of $\text{NO}_3^-$ uptake however, light may be considered to be a physiological substrate required by many species for $\text{NO}_3^-$ uptake.

Briefly, both extracellular $\text{NO}_3^-$ concentrations and light may be considered substrates affecting the velocity of $\text{NO}_3^-$ uptake in situ. Both substrates may obey Michaelis-Menten kinetics individually (when the other substrate is saturating). Ecologically neither light nor extracellular $\text{NO}_3^-$ is often saturating however. It is probable that $\text{NO}_3^-$ uptake kinetics may not obey true Michaelis-Menten functions when both substrates are limiting simultaneously.

Before considering the mathematical functions necessary to describe bisubstrate uptake kinetics, attention should be drawn to the treatment of light and extracellular $\text{NO}_3^-$ uptake as independent variables affecting $\text{NO}_3^-$ uptake velocities. Three possible relationships between $\text{NO}_3^-$ uptake velocities ($v$) and both light ($L$) and extracellular $\text{NO}_3^-$ ($N$) are shown in fig. 17. These three-dimensional profiles may be obtained by saturating the uptake system with one substrate.
either light or extracellular $\text{NO}_3^-$) and varying the second substrate accordingly. Consequently, two maximum uptake velocities may be determined, one corresponding to light ($V_{\text{max}}^L$) and the other to extracellular $\text{NO}_3^-$ ($V_{\text{max}}^N$).

Of the three possibilities, (a) represents the optimal steady-state system in that the ATP output generated from the light reactions is balanced by the rate of ATP hydrolysis stimulated by extracellular $\text{NO}_3^-$. The system depicted in (c) is unstable because light limitation would eventually limit ATP, the energy source ultimately responsible for $\text{NO}_3^-$ uptake (except in dinoflagellates). As the system approaches the steady-state the kinetics would be more accurately described by (a). The conditions represented by (b) are stable, in that intracellular ATP (i.e. light reactions) is not limiting $\text{NO}_3^-$ uptake, although the $\text{NO}_3^-$-stimulated ATP hydrolysis is less than ideal (possibly because of limiting uptake sites).

The three conditions represented in fig. 17 do not adequately resolve the $\text{NO}_3^-$ uptake velocities in the maximum response portions of the curve (i.e. the regions where zero order kinetics are operative). Descriptions of these regions could be obtained by including half-saturation constants. As half-saturation constants cannot be solely derived from $V_{\text{max}}$ values, and must be calculated from experimental data, a matrix might be set up to include $V_{\text{max}}$ and half-saturation
Fig. 16. Three dimensional profiles of NO$_3^-$ uptake velocities as a function of light and extracellular NO$_3^-$. These profiles may be obtained by holding one substrate constant (either light or NO$_3^-$) and varying the second substrate accordingly. Individually, two families of curves may be obtained, one corresponding to light and the other to extracellular NO$_3^-$. 
Fig. 17.

(a) \( v_{\text{max}}^N = v_{\text{max}}^L \)

(b) \( v_{\text{max}}^L > v_{\text{max}}^N \)

(c) \( v_{\text{max}}^L < v_{\text{max}}^N \)
constants. Thus:

\[
\begin{pmatrix}
V^L_{\text{max}} & K_L \\
V^N_{\text{max}} & K_N
\end{pmatrix}
\]

(1)

where: \( K_L \) is the light intensity supporting half-maximum 
\( \text{NO}_3^- \) uptake velocities at \( \text{NO}_3^- \) saturation.

\( K_N \) is the \( \text{NO}_3^- \) concentration supporting half-maximum 
\( \text{NO}_3^- \) uptake velocities at light saturation.

Functionally it is possible to construct a curve for 
\( \text{NO}_3^- \) uptake from two points, \( V_{\text{max}} \) and the half-saturation constant, by fitting a line through these points in a linear transformation of the Michaelis-Menten expression. The matrix in (1) can be easily rearranged to fit a linear transformation, for example the Lineweaver-Burk, double reciprocal relationship.

\[
\begin{pmatrix}
1 & -1 \\
\frac{L}{V_{\text{max}}} & K_L \\
1 & -1 \\
\frac{N}{V_{\text{max}}} & K_N
\end{pmatrix}
\]

(1a)

When neither substrate is saturating however, the affinity of the phytoplankton cells for \( \text{NO}_3^- \) may not be adequately described by either \( K_L \) or \( K_N \) (i.e. Michaelis-Menten kin-
etics). If the cells are considered to be uptake sites requiring two substrates (light and \( \text{NO}_3^- \)), a hypothetical tertiary intermediate complex is formed.

\[
(L) + (N_e) + (C) \xrightarrow{k_1} (L-N_e-C) \xrightarrow{k_2} (L-N_e-C) \xrightarrow{k_3} (C_N) \]

(2)

The affinity constant \( (K_{LN}) \) is defined as:

\[
K_{LN} = \frac{[L-N_e-C]}{[L][N_e][C]} = \frac{k_1}{k_2 + k_3}
\]

(3)

In the steady-state condition (e.g. fig. 17a), \( K_{LN} \) is a product of the substrate concentrations \( (L \text{ and } N_e) \) supporting half-maximum uptake velocities (determined when both substrates are saturating).

By incorporating the matrix given in (1) into bisubstrate enzyme kinetics (e.g. Alberty, 1956) and solving for \( K_{LN} \), the following equation is generated:

\[
K_{LN} = -LN(1 + \frac{K_L}{L} + \frac{K_N}{N} - \frac{V_{\text{max}}}{V})
\]

(4)

In the steady state \( K_{LN} \) is a function of \( V_{\text{max}}/2 \) (i.e. the function \( \frac{V_{\text{max}}}{V} \) may be approximated as 2).

\( K_{LN} \) represents a physiological parameter inversely proportional to the affinity of the phytoplankton for \( \text{NO}_3^- \) but also dependent on light, and may be useful in predicting the
integrated effects of these two variables on $\text{NO}_3^-$ uptake. The values of $K_{\text{LN}}$ indicate the relative affinity of a phytoplankton community for $\text{NO}_3^-$ under ecologically realistic conditions.

In conclusion, the model presented here attempts to deal with $\text{NO}_3^-$ uptake as a two substrate system. The $K_{\text{LN}}$ values that may be calculated attempt to predict the effective assimilatory ability in situ from knowledge of the light intensity and extracellular $\text{NO}_3^-$ concentrations. The application of this model to field conditions is straightforward, if the kinetic parameters in (1) are determined. In addition, as light intensities are seldom reported along with $K_s$ values, clarification of real or apparent half-saturation constants may be resolved through the use of $K_{\text{LN}}$. The values of $K_{\text{LN}}$ do not indicate the relative importance of light or extracellular $\text{NO}_3^-$, but describe the effective affinity of the cells for extracellular $\text{NO}_3^-$ at a given light intensity. This latter information is a potentially crucial parameter in modelling primary productivity in the sea.
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Appendix I

Preliminary Experiments on Dinoflagellates
The apparent lack of the (NO\textsuperscript{3}, Cl\textsuperscript{−})-activated adenosine triphosphatase in the three dinoflagellates examined suggests that the mechanism for NO\textsuperscript{3} uptake may be different from the other organisms studied. The membrane fractions isolated from the three species, especially *Gonyaulax polyedra* did exhibit (Na\textsuperscript{+} + K\textsuperscript{+})-activated adenosine triphosphatase activity. This latter enzyme was assayed as a marker for the plasma-lemma. From the above it was evident that there was no direct coupling between ATP hydrolysis and NO\textsuperscript{3} uptake. However, the accumulation of ions against their negative chemical gradients can occur without such direct coupling of the process to an exergonic chemical reaction. One such process, known as secondary transport, requires that the uptake of one nutrient (against its negative chemical gradient) be thermodynamically coupled to the active transport of some other ion. For example, Sprott et al (1975) have recently suggested that a marine pseudomonad (recently designated as an *Alteromonas haloplanktis*) transports some amino acids into the cytoplams by simultaneously transporting Na\textsuperscript{+} outward. In such a case the inward gradient for the uptake of the amino acids is dependent upon an outward gradient for Na\textsuperscript{+}. Here it is hypothesized that a similar transport phenomena may occur in dinoflagellates; that NO\textsuperscript{3} uptake is coupled to the energy expended in maintaining an asymmetrical distribution of monovalent ions. The following is a brief description of some preliminary experi-
ments designed to test this hypothesis.

To determine the intracellular concentrations of Na$^+$, K$^+$, and Cl$^-$ in *Gonyaulax polyedra*, 10 ml samples of culture were sedimented at 5000 x g in a refrigerated centrifuge at 5°C, washed once with 0.5 M Tris-acetate buffer (pH 7.9), sonicated for 5 min at 0°C, and the final suspension was centrifuged at 20,000 x g for 10 min. The supernatant was assayed for Na$^+$ and K$^+$ in a Techtron AA 120 atomic absorption spectrophotometer at 589.0 m$\mu$ and 766.5 m$\mu$ respectively. Sea-water of 32°/oo was used as a reference and 0.5 M Tris-acetate buffer as the control blank. Intracellular Cl$^-$ was measured by micro-titration with AgNO$_3$ using a Radiometer (Model 25b) pH meter equipped with a scale expander. The results of these determinations are shown in Table A-10, along with reported ion compositions of *Ditylum brightwellii* (Anderson and Sweeney, 1974), and sea water (Riley and Chester, 1971). The data from *G. polyedra* indicate that intracellular Na$^+$ is slightly greater than K$^+$, the opposite of the usual condition.

Blinks (1940) observed high intracellular Na$^+$ relative to K$^+$ in one species of *Valonia*. In the latter case the resting potential of the cells was 10-20 mv positive to the outside. The unusual positive resting potential in this species, correlated with the distributions of Na$^+$ and K$^+$ suggests that the resting potential may be partially propagated by these ions.
TABLE A-10

The sodium, potassium, and chloride concentrations of seawater and some marine phytoplankters (mM).

<table>
<thead>
<tr>
<th></th>
<th>Na</th>
<th>K</th>
<th>Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>seawater (30°/oo)</td>
<td>414</td>
<td>90</td>
<td>482</td>
</tr>
<tr>
<td><em>Ditylum brightwellii</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>101</td>
<td>126</td>
<td>156</td>
</tr>
<tr>
<td>Dark</td>
<td>118</td>
<td>64</td>
<td>117</td>
</tr>
<tr>
<td><em>Gonyaulax polyedra</em></td>
<td>317</td>
<td>119</td>
<td>408</td>
</tr>
<tr>
<td><em>Amphidinium assymetricum</em></td>
<td>148</td>
<td>78</td>
<td>190</td>
</tr>
</tbody>
</table>
Unfortunately, due to the small cell size and thecal plates in *G. polyedra*, it is difficult to measure the resting potential in their species. *Amphidinium assymetricum* Kof. et Swezy, a large (70 μ length), naked dinoflagellate was available for study. Individuals, taken from the middle of the light cycle (L/D:12/12), were placed on a Fuchs-Rosenthal hemocytometer slide with ca. 1 ml medium J (Table 1) in a Faraday cage at room temperature. Glass microelectrodes, filled with 2 M KCl, and with a tip diameter of less than 1 μ, were inserted into apparently healthy, motile cells. A silver-silver chloride reference electrode was placed in the medium droplet. The membrane potential was amplified 10 times by a D.C. amplifier and measured from a Techtronix D-15 storage oscilloscope. The mean of 10 individual recordings was calculated to be +7.6 mv relative to the medium. One individual had a negative potential of 5.2 mv, but the remainder of the recordings were all positive. These results indicate that *A. assymetricum* can be positively charged relative to the extracellular fluid, at least during part of its light cycle. This potential is probably correlated to a reversed distribution of intracellular Na⁺ and K⁺ (Table A-10).

In the absence of direct recordings of membrane potentials from other photosynthetic dinoflagellates, it is difficult to assess the consequences of the positive membrane potential in *Amphidinium assymetricum* from these data alone;
it is especially tenuous to generalize to other species of dinoflagellates. In addition, diel periodicity in ionic composition may influence membrane potentials. This latter complication has been found to influence the bioluminescence in *G. polyedra* (Sweeney, 1974). This process appears to be related to the membrane potential.

If it is found that other species of dinoflagellates have slight positive membrane potentials, it would appear that the driving force for the uptake of small negatively charged ions, such as NO$_3^-$, NO$_2^-$, and PO$_4^{3-}$ would be propagated by the electrical gradient. Such a model would further require an ion receptor site bound to the membrane to aid in the permeation of the charged ions across the nonpolar phospholipid bilayer, in addition to enhancing ion selectivity. These preliminary observations in dinoflagellates suggest that NO$_3^-$ uptake in dinoflagellates may not be a consequence of primary active transport, but is due to a secondary coupled transport mechanism. Consequently, it may be feasible to selectively inhibit anion uptake in dinoflagellates by absorbing a K$^+$ ionophore, such as valinomycin, onto the cell membranes, thereby hypopolarizing the membrane potential and inhibiting cell growth.
Appendix II

Abbreviations
ATP, ADP, AMP - adenosine tri, di, and monophosphate

CTP - cytosine triphosphate

DR - photosynthetic dark reactions

ETS - electron transport system

FDX<sup>red</sup>, FDX<sup>OX</sup> - reduced and oxidized ferredoxin

GTP - guanosine triphosphate

ITP - inosine triphosphate

α-KG - "alpha" keto glutarate (= 2 oxoglutarate)

LR - photosynthetic light reactions

NADH - nicotinamide adenine dinucleotide

NADPH - nicotinamide adenine dinucleotide phosphate

PEP - phospho(enol)pyruvate

P<sub>i</sub> - inorganic phosphate

TCA - tricarboxylic acid cycle

TTP - thymidine triphosphate
Publications


