Effects of N:P Atomic Ratios and Nitrate Limitation on Algal Growth, Cell Composition, and Nitrate Uptake
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Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake

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Abstract

*Scenedesmus* sp. was grown in chemostats at a fixed growth rate ($\mu$) in an inorganic medium with nitrogen to phosphorus atomic ratios (N:P) ranging from 5 to 80, to investigate the effect of double nutrient limitation. There was no additive or multiplicative effect of the two nutrient limitations: below the optimal cell N:P of 30, growth was determined solely by N limitation and above 30, by P limitation. Cell N remained constant up to the optimal ratio and increased linearly with N:P above it. The level of cell P was high at low N:P (N-limited state) but decreased rapidly until N:P approached the optimal and remained constant at a low level at high N:P (P-limited state).

Protein was the major fraction in which excess N accumulated under P limitation. Cell free amino acids were a constant proportion of cell N at all N:P ratios. RNA concentration was the same regardless of N:P, its level being determined by $\mu$ independent of the type of limiting nutrients. Cell carbon (C) concentration was higher in the P-limited than in the N-limited state. The C fixation rate per unit chlorophyll $a$, however, was constant under both P- and N-limited states because the variation in chlorophyll $a$ content was similar to that of C.

The apparent maximum uptake rate for nitrate ($V$) in N- and P-limited cultures decreased with increasing cell N or N:P. In N-limited cultures the half-saturation constant ($K_a$) also decreased at higher cell N or N:P. The variation of $V$ appeared to be affected by the level of free amino acids.

Transitions between states of nitrogen and phosphorus limitation of phytoplankton growth are common in both lakes and coastal seawaters. Such transitions occur seasonally in eutrophic lakes and may be common in estuarine waters where seawater, which is generally N limited but P sufficient, mixes with freshwater having relatively high N and low P levels. Discharge of wastewaters into a body of natural water may also initiate a transition.

Information on the effects of such a change in nutrient limitation is scarce. Earlier work (Rhee 1974), however, indicated its importance in determining the composition of a phytoplankton community by influencing competition and succession. Although natural phytoplankton contain the two nutrients in an N to P atomic ratio (N:P) of about 15 (Redfield 1958; Ryther and Dunstan 1971), I found the optimal cellular N:P of *Scenedesmus* sp. to be 30; the ratio may vary from species to species.

Baule (1918), Verduin (1964), and Droop (1973) suggested that growth during such transitions was controlled in a multiplicative manner. Later, however, Droop (1974) showed in his experiments on phosphate and vitamin B$_{12}$ limitations with *Monochrysis lutheri* that growth did not follow a multiplicative pattern but was regulated by the single nutrient in shorter supply. In this study, therefore, I have investigated growth during the transition between the N- and P-limited states. Emphasis was placed on changes in cellular composition because the growth rate ($\mu$) of phytoplankton limited by various nutrients is a direct function of the cellular levels of the nutrients (Cape- ron 1968; Droop 1968; Fuhs 1969; Davis 1970; Rhee 1973; Paasche 1973). Since two nutrient-limited states were involved in this study, the kinetics of N-limited growth and N uptake were also investi-
gated to provide baseline information to be used with previous findings on P-limited growth and P uptake (Rhee 1973, 1974).

Materials and methods

Organism and culture medium—An axenic culture of *Scenedesmus* sp. was grown in a defined inorganic medium as reported by Rhee (1973, 1974). Nitrate and orthophosphate were the sole sources of N and P. For growth studies at various N:P, the phosphate concentration was kept constant at 3 μM while the nitrate levels were adjusted between 15 and 240 μM. For the investigation of N-limited growth, nitrate and phosphate concentrations were reduced to 21 and 10 μM.

Cells were counted in a hemacytometer. For the double nutrient experiments cell volume was calculated by the geometric formula relating volume to cell shape.

N-limited growth—Four identical chemostats were made from 1-liter (working capacity) Bellco spinner flasks. Each chemostat had a water jacket through which constant temperature water was circulated at 20° ± 1°C by a Neslab cooler-circulator. Cultures were stirred with a magnetic impeller unit on a Bellco nonheat-generating magnetic stirrer. Air was passed first through a saturated zinc chloride solution to eliminate traces of ammonia, then through distilled water to saturate it with moisture, and finally through a series of bottles filled with sterile glass wool. Cultures were aerated at about 1 liter min⁻¹. Continuous illumination of about 0.082 ly min⁻¹ was provided by white fluorescent lamps coated with Uvinul D-49 (see Rhee 1974). Then cultures were grown at various dilution rates. When a steady state was reached, cells were harvested; the cells and supernatant were stored at −20°C until analysis.

Growth at various N:P—Four Bio-Flo chemostats (model C30), each with a working volume of 600 ml, were used. Continuous illumination at about 0.085 ly min⁻¹ was provided. The alga was grown at a constant dilution rate of 0.59 d⁻¹, or relative growth rate of 0.437 (μ/μₘ, where μₘ is the maximum growth rate). The variation in dilution rate was <4%. Aeration was as for the N-limited culture, except that the rate was about 500 ml min⁻¹.

Nitrate uptake experiments—The N uptake study was carried out in a manner similar to the P uptake experiments (Rhee 1973). Aliquots (50 ml) of the steady state culture from N-limited chemostats were delivered into a series of 125-ml Erlenmeyer flasks that contained various concentrations of nitrate. The flasks were shaken on a rotary shaker under the same illumination as for N-limited growth studies. Uptake was measured at 20-min intervals by filtering 10 ml of suspension through a membrane filter (Millipore, pore size 0.45 μm) that had been washed with 30 ml of double-distilled water and determining the amount of nitrate left in the filtrate. Data were analyzed by a nonlinear regression program (Rhee 1973).

The rate of N uptake under P limitation was calculated from the 1973 data according to

\[ V = (A - B)D \times \text{cell No.}^{-1}, \quad (1) \]

where *V* is the apparent maximum uptake velocity; *A*, the nitrate provided in the inflow medium; *B*, the residual nitrate level at a steady state; and *D*, the dilution rate. This study had been done under a 12:12 light-dark cycle, with the light at 0.084 ly min⁻¹.

Carbon (C) uptake was calculated from cell C concentration by the equation

\[ V_c = \mu q_c, \quad (2) \]

where *V_c* is the C fixation rate, and *q_c*, the cell C content.

Analytical methods—Dissolved organic N was determined by the micro-Kjeldahl method (Strickland and Parsons 1972) and dissolved organic P by the method of Murphy and Riley (1962) after persulfate digestion (Menzel and Corwin 1965). The digestion step was omitted for analysis of orthophosphate. Nitrate, ni-
trite, and ammonia were measured in an AutoAnalyzer (Canelli 1976). Total cell N and P were calculated from the amounts utilized by a steady state culture.

Surplus P was determined as described by Rhee (1973) and total cell C by the method of Menzel and Vaccaro (1964). Chlorophyll a was measured in vivo using a Turner fluorometer according to Strickland and Parsons (1972) because of difficulty in extracting it with solvents. Its concentration was expressed in arbitrary units.

Samples for free cell nitrate and ammonia were prepared by breaking cells in a French Pressure Cell at $1.4 \times 10^7 \text{kg m}^{-2}$ at $5{}^\circ\text{C}$ followed by filtration through a membrane filter (Millipore, pore size $0.22 \mu\text{m}$). The trichloroacetic acid (TCA) extraction method (Epplley and Coatsworth 1968), performed without breaking cells, was found unsatisfactory.

Free amino acids were extracted with boiling water (Dawson 1965) and assayed by the ninhydrin method of Yemm and Cocking (1955). l-Leucine was used as the standard over the range $0.2$–$25 \mu\text{g ml}^{-1}$.

RNA was determined by the orcinol method (Schneider 1960) in a sample prepared as follows: Cells were extracted with 10% cold TCA, and the supernatant was discarded. The residue was suspended in 2 ml of 1 N HClO₄ and subsequently heated at $90{}^\circ\text{C}$ for 30 min. The mixture was centrifuged at $12,100 \times g$ for 30 min and washed twice with HClO₄. The washings were combined with the supernatant, and the mixture was left overnight at $-20{}^\circ\text{C}$. The standard was prepared using yeast RNA hydrolyzed in 1 N HClO₄.

DNA was extracted with 1 N HClO₄ at $70{}^\circ\text{C}$ for 15 min and measured by the col-
Fig. 2. Concentration of cell protein, RNA, free amino acids, lipid N, and DNA as a function of growth rate (μ) in a N-limited culture.

**Results**

*N*-limited growth—Cell numbers at steady states decreased linearly with μ, and no nitrate or dissolved organic N could be detected in the medium. Therefore μ is related to cell N by a saturation curve similar to that found by other investigators (e.g. Caperon 1968).

Cell C concentration was constant regardless of μ and thus the rate of C fixation increased linearly with μ (Eq. 2, Fig. 1A). Since the cellular content of chlorophyll a also increased linearly with μ, the C fixation rate per unit chlorophyll a (assimilation number) was uniform at all dilution rates (Fig. 1B). Therefore the higher rate of C fixation in faster growing cells is related to an increase in the level of chlorophyll a.

Cell protein, lipid N, RNA, and free amino acids measured as amino N were all related to μ by a saturation function (Fig. 2). In P-limited cells the P level in RNA also showed such a relationship (Rhee 1973). DNA, however, appeared to increase linearly with μ (Fig. 2). In studies of P limitation, DNA (measured as P) was invariant with μ when ex-
pressed per unit cell volume (Rhee 1973). In the present study I could not calculate its content per unit volume because of the lack of volume data for the N-limited culture, but microscopic observation during daily cell counts of chemostat samples showed larger cell volumes at higher dilution rates. Since cell nitrate and ammonia levels were too low to be measured accurately because of inadequate sample size, their relation to $\mu$ was difficult to ascertain.

Growth at various N:P ratios—The number of cells in steady state cultures grown in media containing varying N:P ratios increased linearly with N:P up to 30 and then abruptly leveled off (Fig. 3). Since N:P was varied by changing only the nitrate concentrations, the proportional increase indicates N limitation below an N:P of 30, and the leveling off shows P limitation above this ratio. This confirms the optimal ratio of 30 found by P uptake kinetics (Rhee 1974). The transition between N- and P-limited states is quite sharp, indicating that there is no multiplicative or additive effect. A test of the single-nutrient limitation and of the multiplicative model with the data for intracellular P and N contents also confirms this.

The multiplicative model is expressed as

$$\frac{\mu}{\mu_m} = \frac{(q_P - q_{op})}{K_P + (q_P - q_{op})} \times \frac{(q_N - q_{on})}{K_N + (q_N - q_{on})}$$

(3)

Growth under single-nutrient limitation is calculated as

$$\frac{\mu}{\mu_m} = \frac{(q_P - q_{op})}{K_P + (q_P - q_{op})}$$

(4)

when P is limiting, and as

$$\frac{\mu}{\mu_m} = \frac{(q_N - q_{on})}{K_N + (q_N - q_{on})}$$

(5)

when N is limiting, where $K_P$ and $K_N$ are half-saturation concentrations of cell N and P; $q_P$ and $q_N$, cell P and cell N levels; and $q_{op}$ and $q_{on}$, minimum cell P and cell N contents.

The hypotheses were tested by calculating $\mu/\mu_m$ for each of 16 N:P and then determining by a t-test whether the calculated values were significantly different from the experimentally measured value of $0.437 \pm 0.016$ (mean $\pm$ SD).

Since the half-saturation constants are equal to the minimum cell contents of limiting nutrients (Droop 1968; Rhee 1973), the values of $q_{op}$ and $q_{on}$ determined in my previous work (1973, 1974) were used as $K_P(1.64 \times 10^{-9} \text{ umM cell}^{-1})$ and $K_N(45.40 \times 10^{-9} \text{ umM cell}^{-1})$.

The mean value of $\mu/\mu_m$ was estimated to be $0.286 \pm 0.027$ with 95% confidence.
when the multiplicative model was used. With the single-nutrient limitation equations, $0.428 \pm 0.072$ was obtained for N:P \(\leq 30\) and $0.428 \pm 0.013$ for N:P \(\geq 30\). The $t$-test indicates the rejection of the multiplicative hypothesis and the acceptance of a single-nutrient limitation model at the 95% confidence level.

$K_P$ and $K_N$ values determined by curve-fitting Eq. 4 and 5 to the data were $1.62 \pm 0.07 \times 10^{-9}$ $\mu$M cell$^{-1}$ and $45.70 \pm 4.6 \times 10^{-9}$ $\mu$M cell$^{-1}$, not significantly different from the measured $q_{OP}$ and $q_{ON}$ values. This supports the validity of substituting $q_{OP}$ and $q_{ON}$ for $K_P$ and $K_N$ and may also be taken as additional evidence for the single-nutrient limitation model.

**Cell volume**—Cell volume increased with N:P following two different linear functions, one below the optimal ratio and the other above it (Fig. 3).

**Total cell N and P**—No residual inorganic or dissolved organic N or P was detected in the medium at any N:P examined, indicating excess accumulation of both nutrients. Since all nitrate and phosphate in the medium was consumed, the cellular N:P must have been identical to the ratio in the medium.

Cell N concentrations in N-limited states with excess phosphate were always the same, about $86 \times 10^{-9}$ $\mu$M cell$^{-1}$ ($1.2 \times 10^{-6}$ $\mu$g N liter$^{-1}$ cell$^{-1}$) indicating that in this state cell N concentrations are determined by $\mu$ regardless of the N:P of the medium. In P-limited states where N was in excess, the cell P content remained constant at $2.9 \times 10^{-9}$ $\mu$M cell$^{-1}$ ($0.09 \times 10^{-6}$ $\mu$g P liter$^{-1}$ cell$^{-1}$), indicating that in these states cell P concentration was dictated by $\mu$ without respect to the amount of excess N (Fig. 4). The amounts of cell N and P above these constant levels were therefore considered excess accumulation. The ratio of cell N in N-limited states to cell P in P-limited states was also 30, confirming the optimum N:P found above.

**C fixation and chlorophyll a**—The calculated rate of C fixation based on cell C contents was constant at low N:P, but as the ratio approached the optimum the rate increased rapidly and then leveled

![Image](image_url)
off at a higher level at high N:P (Fig. 5A). Chlorophyll a concentrations show similar sigmoid changes (Fig. 5B). Therefore, when the C fixation rate is expressed per unit chlorophyll a, the assimilation number is the same at all N:P (Fig. 5C). As in the N-limited culture experiments, this shows that the faster C fixation rate in the P-limited state is related to the higher concentration of chlorophyll a.

Cell composition—Cells grown at various N:P ratios show protein content changing in a manner similar to that of cell N (compare Fig. 6 with Fig. 4). N accounts for about 16% of protein by weight (Conn and Stumpf 1966) and with
this factor protein N can be calculated. The proportion of protein N to total cell N averages 44% in the N-limited state, while in the P-limited state, the proportion increases with N:P up to 70% or more (Fig. 6). This indicates that in the P-limited state, most excess N accumulates in the protein fraction. In the N-limited state, protein N concentrations are determined by \( \mu \) independent of N:P in the inflow medium or of cell P concentrations.

Lipid N remains constant in the N-limited state and increases in the P-limited state in a manner similar to that of protein (Fig. 7). Lipid N, however, accounts for only a small fraction of the total N, its maximum at N:P = 70 being about 10% (Fig. 6).

DNA concentrations in N-limited states increase slowly in proportion to N:P up to the optimum ratio and more rapidly above it (Fig. 8, upper). As seen in Fig. 8 (middle panel) and reported earlier (Rhee 1973), the DNA per unit cell volume is the same at all N:P. Accordingly, as a proportion of the total cell N, it decreases as N:P becomes larger (Fig. 6). RNA levels were the same at all N:P (Fig. 8, lower). This indicates that cell RNA concentration reflects \( \mu \) independent of the type of limiting nutrient (see discussion).

Free amino acid accumulation follows a pattern similar to that of protein, lipid N, and total cell N (Fig. 9). Unlike protein and lipid N, however, it is a constant proportion of total cell N at a given \( \mu \) at all N:P (Fig. 6). If \( \mu \) is allowed to vary in N-limited growth experiments, the proportion of N that is free amino acid increases with \( \mu \): free amino acid N is only 3.3% of cell N at \( \mu/\mu_m = 0.18 \) but 6.3% at \( \mu/\mu_m = 0.61 \). (The generally low values in N-limited culture are due to culture conditions different from those in N:P experiments.)

The changes in free cell nitrate and ammonia could not easily be observed because the levels were too low to be measured accurately, but the level of each seemed to be <0.8% of total cell N.

The accumulation of surplus P is dramatically higher in the N-limited than in the P-limited state. This is consistent with the results obtained from my previous investigation (Rhee 1974). Surplus P decreases rapidly until N:P approaches the optimum. At ratios higher than the optimum, it remains at a uniform level.
determined by the P-limited growth rate (Fig. 10).

Nitrate uptake—The two maximum uptake velocity curves in Fig. 11 represent the results obtained with N-limited and P-limited cultures. The two curves are not quantitatively comparable because of different growth conditions, as described above. Nonetheless, it is important to note that in both cases the apparent maximum uptake rate (V) is inversely proportional to the cell N:P. The change in N:P in an N-limited culture is caused by higher cell N concentrations at higher \( \mu \) because the cell P level is the same at all \( \mu \). In a P-limited culture, on the other hand, the change in N:P is the result of increasing cell P levels at higher \( \mu \), since in this culture the cell N content remains uniform except at very low dilution rates (see Table 1: Rhee 1974). It is very likely, therefore, that N uptake involves a feedback mechanism by certain N compounds, much as P uptake is affected by acid-soluble inorganic polyphosphates (PP) (Rhee 1974). A plot of V against cell free amino acids gives a negative linear slope (Fig. 12), suggesting that free amino acids are directly or indirectly involved in or reflect the regulatory mechanism of nitrate uptake (see discussion).

The half-saturation constant \( (K_m) \) for nitrate uptake also decreases with N:P (Fig. 11). In contrast, the \( K_m \) for P uptake by the same organism is constant at all \( \mu \) (Rhee 1973). With the present data it is difficult to determine if \( K_m \) values are also related to any intracellular compounds, because of the relatively large standard errors in estimating them (Table 1).

Discussion

N:P and growth—It is clear that there is no growth limitation by N and P simultaneously. Growth is limited by P or N on either side of the optimum N:P. Droop (1974) found no multplicative effect of phosphate and vitamin B_{12} limitations on the growth of _M. lutheri_, which is strictly controlled by the single nutrient in shorter supply. Droop’s experimental approach was different from that used here; he used dilution rates as independent variables in media for four different P:B_{12} ratios. Both his and my results appear to agree with Liebig’s law of the minimum.

The optimal cellular N:P may be species-specific. If this is so, the difference in this value would have great ecological importance. For example, in the same nutrient environment the growth of some species may be limited by one nutrient, while others are regulated by another. The severity of each limitation would determine the outcome of competition, and the interval between the ratios would determine the range in which coexistence is possible (Table 2). Competition models based on the kinetic constants for growth have also been used successfully to predict competitive exclusion and coexistence between two
species (Titman 1976; Tilman 1977). There are various internal nutrient pools; the size of a pool may vary from species to species, restricting the use of models derived from growth kinetics (as also pointed out by Tilman).

Competition and coexistence based on optimal cellular N:P may explain the seemingly synergistic effects of simultaneous N and P addition frequently encountered in bioassay experiments (e.g. Fuhs et al. 1972). If the mixed populations in a bioassay are in the coexistence range in Table 2, addition of P alone to the medium will shift N:P in favor of organism A, thus competitively eliminating B. The addition of N only, on the other hand, will favor B with the elimination of A. The simultaneous addition of both N and P, therefore, would produce more biomass than the sum of the yields produced by the single addition of P or N. Although there is no proof yet of the species-specificity of optimal N:P, Tilman’s data (1977) suggest that the optimal P:Si ratio differs in two competing diatom species.

Determination of optimal N:P may be difficult because of the nutrient pools.

Table 2. Competitive exclusion and coexistence.

<table>
<thead>
<tr>
<th>N:P</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P-limited</td>
<td>A and N-limited</td>
<td>B coexist</td>
</tr>
<tr>
<td>Organism A dominant</td>
<td>→</td>
<td>Organism B dominant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal N:P</td>
<td>Optimal N:P for A</td>
<td>for B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
For the same reason, the interpretation of N:P ratios found in batch-culture work should be made with extreme care. Optimal N:P ratios of ecologically important species of phytoplankton representing various taxa are being investigated in our laboratory.

**N:P and residual N and P**—The minimum cellular N:P observed in *Scenedesmus* sp. was 4 under N-limited conditions, and the maximum was 142 or more under P-limited conditions (Rhee 1974). It is therefore not surprising that there was no detectable residual N or P in the culture medium at the inflow N:P used in the present study (5 to 80). It is probably that residual N or P becomes detectable only when N:P in a medium is lower than the minimum or higher than the maximum cellular N:P. The wide range of cellular N:P indicates that cellular nutrient concentrations are better indicators of nutrient limitation than nutrient levels in the surrounding medium when optimum nutrient ratios are known.

**Protein**—Among the changes in chemical composition measured here, those of protein, RNA, and free amino acids are of special interest. Under conditions of N sufficiency, protein, which accounts for more than 70% of the total cell N, appears to be the major reservoir for excess N. The observation that it is a greater proportion of total cell N in the P-limited state (Figs. 6 and 7) supports this interpretation. Other supporting evidence is that under nutrient stress, or in non-growing bacteria and fungi, the turnover of protein becomes faster (Mandelstam 1963; Pine 1972), and physiological adaptation and growth become dependent upon the turnover rate. For example, in P-starved cells, amino acids produced as the result of protein turnover or breakdown are reincorporated for the synthesis of alkaline phosphatase by increased differential rate of synthesis of this enzyme (Halvorson 1962). In higher plants such amino acids are largely converted to carbon dioxide (Oak and Bidwell 1970).

**RNA**—A close correlation between RNA concentration and μ has been well established for many bacteria (Neidhardt and Magasanik 1960; Maaløe and Kjeldgaard 1966; Maaløe and Kurland 1963; Smith 1969). In a synchronous culture of *Chlorella*, RNA synthesis was also found to control cell division (Lorenzen 1970). Such findings were made mostly under conditions of single-nutrient limitation or in unrestricted medium. It is therefore very interesting to find that RNA concentrations reflect μ independent of N:P in the medium and therefore irrespective of the limiting nutrient. Data from a study of *Enterobacter aerogenes* under double-nutrient limitation (Cooney et al. 1976) showed similar results, although the investigators failed to point it out. At any given μ in that study, RNA concentration appeared unchanged whether C, N, P, or a combination of C, N, and P was limiting. (The criterion of limitation used was not growth but change in macromolecular composition.) Findings such as these may enable us to use measurements of RNA to estimate physiological conditions of natural populations without precise knowledge of the nature of the limiting nutrient. This could be even more significant in light of the possible species-specificity of optimal N:P and the difficulty of determining it.
for a great number of species. RNA can be
determined by either biochemical or
cytochemical methods. A cytochemical
method developed by Fuhs and Chen
(1974) may prove valuable for the mea-
surement of RNA in individual species of
mixed natural populations.

In using RNA as a measure of physi-
ological conditions, one must remember
that it responds in two different ways to
environmental changes, such as tempera-
ture: by changing the rate of protein syn-
thesis without modifying its cellular
levels, or by changing its own cellular
concentrations. The second type of re-
ponse seems to occur under environ-
mental changes which last many genera-
tions. In the steady state cells of a
chemostat, RNA content varies with tem-
perature and pH (Tempest and Hunter
1965). The first type of adaptation has
been observed in batch culture work;
when the \( \mu \) of cells in an unrestricted
medium was altered by temperature, the
cellular RNA level remained constant
(Schaechter et al. 1958; Fuhs and Chen
1974) but the rate of protein synthesis
would probably vary.

**Free amino acids**—The amino acid
pool is a sensitive indicator of cellular
metabolism, particularly of N supply. It is
extremely variable and markedly depen-
dent on the nutritional composition of a
medium, the levels of limiting nutrient,
\( \mu \), and environmental conditions (Cowie
1962; Tempest et al. 1970; Brown and
Stanley 1972; Brown et al. 1972, Drozd et
al. 1972). In protein synthesis the free
amino acids in the pool are obligatory in-
termediate forms of N (Britten and
McClure 1962). Indeed, Cowie (1962)
found a precursor-end product relation-
ship between pool amino acids and cell
protein. The size of the pool, however, is
not related to the rate of protein synthesis
(Dawson 1965). The difference in the
contribution of protein and free amino
acids to the total cell N observed in the
present study (Fig. 6) agrees with these
findings.

The uniform quantitative relationship
of free amino acids to total cell N is of
considerable interest because either the
amino acid pool or the ratio of free amino
N to cell C may be used as a sensitive
indicator of N depletion, if N limitation is
known to exist. One can also substitute
the measurement of free amino acids for
that of total cell N. Such substitution
would be desirable in cases where detri-
tal N prevents an accurate measure-
ment of the particulate N of living or-
ganisms.

In *Escherichia coli* the rate of RNA
synthesis has a direct relationship with
the availability of free amino acids. One
explanation for this mechanism is that
transfer RNA (tRNA) uncharged with
amino acids acts as a repressor of RNA
synthesis. In an N-rich medium tRNA
tends to be saturated with amino acids,
more or less completely derepressing
RNA synthesis, but in an N-limited
medium the relative unavailability of
amino acids leaves a large portion of the
RNA unadenylated (Stent and Brenner
Under conditions of N limitation, there-
fore, free amino acid levels reflect \( \mu \)
and are the equivalent of the levels of PP\(_1\)
or surplus P in P limitation (see Rhee 1973).

**Carbon fixation**—Although cell C con-
centrations were invariant at all dilution
rates in N-limited culture, the C fixation
rate per cell had a positive relationship
with \( \mu \). Such increases in the photo-
synthetic C fixation rate have also been
observed with *Scenedesmus abundans*
(Giddings 1975). Assimilation number in
these organisms and in *Thalassiosira
pseudonana* (Eppley and Renger 1974)
showed no variation with respect to \( \mu \). A
synchronous culture of *Scenedesmus*
(Myers and Graham 1975) showed an es-
sentially unvarying size of the photo-
synthetic unit (the yield of oxygen per
chlorophyll per light flash) throughout its
life cycle. On the other hand, marked
changes of the assimilation number were
reported by Thomas and Dodson (1972),
Caperon and Meyer (1972a), and Cape-
ron and Zieman (1976). Diel variations of
the assimilation number have also been
found in marine diatoms (Eppley et al.
1971; Eppley and Renger 1974).

Growth studies at various N:P ratios
show identical assimilation numbers, but P-limited cells have higher concentrations of chlorophyll \( a \) and thus higher cell C contents than do N-limited cells. This indicates that under conditions of N limitation the proportion of N diverted from chlorophyll \( a \) synthesis to other processes is much greater than the proportion of P diverted under P limitation. Consequently, N limitation impairs chlorophyll \( a \) synthesis much more than P limitation. Bongers (1958) and Fogg (1959) also reported that in N limitation the amount of chlorophyll per cell decreases faster than the total cell N.

**Nitrate uptake**—The variation of \( V \) seems to be related to free amino acid concentrations (Fig. 12) by way of an amination process, or by the rate of pool amino acids utilization, or both. In *Anaabaena cylindrica*, nitrate, nitrite, and hydroxylamine reductase systems are repressed by ammonia and glutamate (Hattori 1962). In certain marine algae, nitrate is quantitatively reduced to ammonia in the absence of carbon dioxide (Brown et al. 1974), and therefore, in the light, the rate-limiting step for nitrate assimilation is ammonia incorporation. In *Chlorella* an exogenous supply of certain amino acids, including glutamate, represses nitrate reductase (Abdullah and Ahmed 1975). Nitrogenase activity in N\(_2\)-fixing organisms appears to be regulated by free cell ammonia (Dharmawardene et al. 1972). Indirect but strong evidence of the dependence of \( V \) on free amino acid concentration is that sulfur uptake in *Chlorella vulgaris* varies positively with the level of sulfur amino acids in the pool (Passera and Ferrari 1975). Conover (1976) and Eppley and Renger (1974) also suggested that a critical rate-limiting step in N uptake is its incorporation into organic compounds. Lui and Roel (1972) suggested that in ammonia utilization the intracellular ammonium level and carbohydrate reserve regulate uptake. In the present study, cell ammonia levels were too low for accurate measurement, and therefore no meaningful relationship between these levels and nitrate uptake can be ascertained.

Two enzyme systems that mediate amination have been reported in a marine bacterium (Brown and Stanley 1972; Brown et al. 1974) and recently in a marine diatom (Falkowski and Rivkin 1976). The two systems differ markedly in their \( K_m \) values. One is glutamic dehydrogenase (GDH) which aminates 2-oxoglutarate to glutamic acid. The other is a two-step process: synthesis of glutamine by glutamine synthetase (GS) and the reductive transfer of amide to 2-oxoglutarate by glutamine (amide)-2-oxoglutarate amino-transferase (GOGAT). In the bacterium the \( K_m \) for GDH is 10 mM; the value for GS is about 20-fold smaller. The values in the diatom are 28 mM for GDH and 29 \( \mu \)M for GS. The two enzyme systems thus probably operate at different levels of free cell ammonia. GS has been reported in blue-green algae (Dharmawardene et al. 1973; Haystead et al. 1973) and in green algae (Loomis 1959). Its existence and possible importance in ammonia incorporation were also reported in *C. vulgaris* and *Chlorella pyrenoidosa* (Reisner et al. 1960; Bassham and Kirk 1964) and in some higher plants (Fowden 1965).

It is not possible to elucidate the mechanism of nitrate uptake in *Scenedesmus* sp. using only the present data. Further investigation on enzymatic and cellular levels is under way to find the mechanism regulating its uptake. Solomonson and Spehar (1977), in an important paper that appeared just as this manuscript was being submitted, proposed a model for feedback control of nitrate uptake by free intracellular ammonia and amino acids.

Variations in \( V \) and \( K_m \) similar to those found here were indicated for *S. abundans* (Giddings 1975), and a decline in \( V \) with increasing \( \mu \) was found in *T. pseudonana* (Eppley and Renger 1974). On the other hand, Caperon and Meyer (1972b) reported a positive slope of \( V \) with \( \mu \) in *T. pseudonana*, and Laws and Caperon (1976) found no variation of \( V \) in *M. lutheri*. It should be noted, however, that the last two results were obtained by the perturbation technique, which disre-
gards the feedback or regulatory effect of intracellular N compounds.

In summary, I have found the growth of Scenedesmus sp. to be limited, not in a multiplicative or additive manner, but in a threshold pattern by the single nutrient in shorter supply. Possible differences in the optimal N:P of various organisms, therefore, may be a basis for coexistence and competitive exclusion. In nutrient limitation not only are the absolute concentrations of limiting nutrients important but also their levels relative to other nutrients. RNA concentrations were independent of which nutrient was limiting, but they were a function of $\mu$ and may therefore be a potentially useful parameter in estimating $\mu$ of natural populations.

In the P-limited state, most excess N accumulated in the protein fraction and increased linearly with N:P. Free amino acids within a cell also increased with N:P under N-sufficient conditions but were a constant proportion of the total cell N. Thus the level of free amino acids appears to be a good potential indicator of N-limited growth rate and might be used to estimate particulate N concentrations of living organisms in cases where detrital N prevents accurate measurement of total cell N. Levels of cell C were higher in the P-limited than in the N-limited state, but because these levels changed in a manner similar to that of chlorophyll $a$, assimilation number remained invariant with respect to the type of limiting nutrient. Assimilation number was also independent of $\mu$. The $K_m$ for nitrate uptake in N-limited cultures appeared to decrease with $\mu$. $V$ also decreased with $\mu$ in both N- and P-limited cultures; the decrease appeared to be related to the level of free amino acids.

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