NITROGEN METABOLISM IN SCENEDESMUS AS AFFECTED BY ENVIRONMENTAL CHANGES\textsuperscript{1,2,3}

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In recent years investigations into the physiology of green algae have been stimulated by a recognition of their possible commercial use (6) in addition to their common acceptance as research tools for certain fundamental problems. These physiological studies have shown that green algae have a high nitrogen content. A correspondingly high protein content has been inferred (28). Relatively little attention has been given to the utilization of the absorbed nitrogen or to changes in the nitrogen fractions of cells subjected to a changing environment. Comparisons with higher plants (7, 30) have not been possible because of the necessity for large and frequent samples of algae required for reliable analytical determinations. The development of a mass culture technique (14, 16) has made this feasible. This paper reports an extensive study of the nitrogen metabolism of a representative, fresh-water, green alga, Scenedesmus obliquus (Turp.) Kütz., grown in mass culture. Changes in the content of the major nitrogen fractions are correlated with modifications in the environment, and a basis is established for future detailed examination of specific pathways of nitrogen metabolism.

MATERIALS AND METHODS

The strain, Scenedesmus obliquus WH-50, was originally isolated from cultures at the Marine Biological Laboratory at Woods Hole in 1950 and has since been maintained on agar slants. For the nitrogen and dark experiments the cells were subcultured in 500-ml Erlenmeyer flasks containing basal nutrient medium and “aerated” with a mixture of 5% CO\textsubscript{2} in-air. A packed cell volume of 0.10 ml was added to 300 liters of nutrient solution at the beginning of each experiment. As an inoculum for the potassium experiment, an equivalent volume of cells was taken directly from two agar slants. The mass culture obtained at the end of the potassium resupply experiment served for initiation of the study of phosphorus deficiency.

CULTURE CONDITIONS: The mass culture apparatus has been described in detail in a previous paper (16). Briefly stated, the algae were grown in 300-l, glass-covered, polyethylene-lined, plywood vats, having a surface area of 2 m\textsuperscript{2} and a depth of 15 cm. Circulation of the culture was provided by stainless steel stirrers mounted in diagonally opposite corners of the vat. Water for the culture was deionized by an ion exchange column. Five percent CO\textsubscript{2}-in-air was supplied through a porous carbon pipe at a rate of 30 l/hr. The cultures were illuminated by a battery of fluorescent and incandescent lamps mounted on a reflector. Illuminance averaged 1000 fc and irradiance averaged 0.12 gm cal/cm\textsuperscript{2} x sec at the surface of the culture. The temperature of the solution was held between 25 and 29\degree C by means of thermostatically controlled air conditioners mounted in the wall of the culture room.

The shaking apparatus for small cultures, described by Krauss (15), was used for certain experiments. Cell suspensions were placed in 500-ml Erlenmeyer flasks fitted with a ground glass joint containing an inlet tube through which 5% CO\textsubscript{2}-in-air was bubbled. The flasks were rocked in a 25\degree C water bath which was illuminated through a glass bottom by fluorescent lamps.

The basal nutrient solution was that developed in this laboratory (16) containing 100 gm KNO\textsubscript{3}/l, 0.25 gm MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O/l, and 0.25 gm KH\textsubscript{2}PO\textsubscript{4}/l. Micronutrients were supplied as inner complex salts of ethylenediaminetetraacetic acid (EDTA). One hundred ml stock solutions of the complexes were made containing 7.70 gm EDTA-NaFe; 7.70 gm EDTA-NaMn; 9.35 gm EDTA-NaCa; 7.15 gm EDTA-NaZn; 7.70 gm EDTA-NaCu; and 6.87 gm EDTA-NaCo. Sixty ml of the iron stock solution and 30 ml of the stock solutions of the other complexes were added to each 300 l of nutrient solution. This yielded a concentration of 2 ppm of iron and 1 ppm of the other micronutrients in the basal nutrient solution. The pH of this medium was adjusted to 7.0 with KOH.

The cells were harvested for sampling or transfer by pumping the cell suspension through a Sharples centrifuge. Simultaneously the cells were separated and the medium returned to the vat.

Growth was measured by determining the packed cell volume (hereafter referred to as “pvc”). This was accomplished by centrifuging 100 ml of cell suspension in Goetz phosphorus tubes (13). For calculating the weight of cells removed during sampling, the dry weight of cells per 100 ml was determined by transferring the cells to tared aluminum cups and drying at 100\degree C.

During the experiments a culture was grown to a pvc of 0.10 ml/100 ml. This was the point of optimum yield, as determined by Krauss and Thomas.
(16). This level was maintained for several days by periodic harvesting. The pH was controlled and the nitrogen level was maintained by the addition of HNO₃ to the culture. After analytical samples had been taken at this level, the cells were harvested and resuspended in a second vat containing fresh medium deficient in the element concerned. In the N-deficient solution, a chemically-equivalent amount of KCl was substituted for KNO₃; in the K-deficient solution sodium salts were similarly substituted for potassium salts; and P-deficiency was produced by using KCl instead of KH₂PO₄. Deficient elements were resupplied by adding KNO₃ for nitrogen, KCl for potassium, and K₂HPO₄ for phosphorus. Analytical samples were taken periodically during the periods of deficiency and resupply.

**Extraction and Analytical Methods:**Centrifuged cell samples were weighed, and a portion suspended in 1% acetic acid. The remainder was dried in vacuo at 70°C, weighed, and ground in a Wiley mill to pass a 40-mesh screen. Aliquots of the acetic acid suspension were taken for dry weight and total N determinations. The cells in the remainder of the suspension were killed and extracted by heating the suspension for 5 min at 100°C. Following centrifugation, the cells were extracted 3 more times with water at 70°C. The acetic acid and water extracts were combined, filtered, and made to volume. During the nitrogen experiments, the extracts were stored in the refrigerator; in other experiments they were frozen and stored at −21°C. They were thawed just prior to analysis.

Total-N was determined by a micro-Kjeldahl procedure (23) using salicylic acid to include nitrates and a copper sulfate-selenium mixture as a catalyst. Soluble-N was determined by a Kjeldahl analysis of an aliquot of the combined acetic acid and water extract. Protein-N was calculated by difference between the total-N and soluble-N. In the N-deficiency and resupply experiments free amino-N in the combined extract was determined by a manometric ninhydrin procedure (35). In other experiments the more convenient photometric ninhydrin procedure of Troll and Cannon (33) was used. The photometric results were corrected for the presence of free ammonia which yields 25% as much colored complex as the amino acids. Peptide-N was determined as the increase in amino-N after hydrolysis with 6N HCl at 110°C in sealed tubes. The photometric values were corrected for the presence of amide- and ammonia-N. Ammonia, total amide, and glutamine analyses of the combined extract were carried out using the vacuum distillation and hydrolytic procedures of Pucher et al (22). Ammonia in the distillate was determined by Nesslerization. Basic-N was analyzed by phosphotungstic acid precipitation (34) followed by a Kjeldahl determination. This N was further fractionated in one experiment by an amino-N analysis of the supernatant. The amount of amino-N removed is a measure of the concentration of basic amino acids. Arginine was analyzed by a modified Sakaguchi procedure (19). It was separated and identified by paper chromatography with phenol-water, ethanol-water, and butanol-acetic acid-water as solvents followed by spraying the paper with the Sakaguchi reagents (4). Nitrate was analyzed using a phosphotungstic acid procedure (12). Potassium and sodium were determined in ash solutions by flame photometry employing a Beckman DU spectrophotometer. Phosphorus was measured in the ash solution by the AOAC colorimetric method (18).

All determinations were made in duplicate and a set of standards was run with each set of unknowns whenever photometric procedures were used. The deviations from the means were no greater than 5%, unless otherwise noted.

**Results and Discussion**

The high nitrogen content of green algae (8, 16, 28) has led investigators to infer that the protein level is also high. The rapid uptake of nitrate described previously (16) suggested that nitrate might accumulate. This possibility was tested by analyses of the water and ether extracts of dried algal samples which showed that only 0.03% of the dry weight or 0.4% of the total-N was nitrate. Furthermore, analyses of alcohol-extracted dry material showed that 87% of the total-N was insoluble. Additional hot water extraction reduced this value to 74%. These values are lower than those obtained with fresh cells using hot 1% acetic acid followed by hot water as extractants. Apparently drying results in some protelysis. The analyses of fresh material showed that approximately 90% of the nitrogen was insoluble. Analyses of fresh samples from a normal culture showed 15% of the soluble-N was amino-N, 3% ammonia- and amide-N, 60% basic-N, and 0.5% nitrate-N. About one-sixth of the basic fraction was non-amino-N of basic amino acids, i.e., arginine, histidine, lysine, and ornithine. The rest was presumably nucleotide-N although about 2% of the basic-N or 1% of the soluble-N was extractable by chloroform and ether. This treatment is generally regarded as specific for alkaloids (9).

**Nitrogen Deficiency:** Transfer of N-sufficient cells to a medium deficient in nitrogen resulted in a much reduced growth rate. Figure 1 illustrates this effect. The growth rate is expressed in log₂ units per day which is the reciprocal of the doubling time. Growth is markedly reduced during the first 24 hours of deficiency. This early reduction of growth may result from the shock of centrifugation. The slow but continual growth in N-deficient cultures is due to both sharing of nitrogen by new cells and to storage of photoynthate.

Figure 2 shows that the total-N content decreases exponentially as deficiency progresses and levels off after approximately 8 days. This drop could be due to redistribution of nitrogen among newly-formed cells or to excretion of nitrogen into solution. Calculations made from total-N analyses and dry weight determinations indicate that excretion as well as re-
distribution occurred during growth. These results are shown in table 1. About 10% of the cell nitrogen is lost during the first 12 hours but is reabsorbed during the next 12 hours. This loss and uptake is coupled with the decreased growth rate and with variations from the generally exponential character of the N curve. The shift from protein production to fat production reported in Chlorella pyrenoidosa (10, 28) probably occurs during this early period. Production of new material may lag until a different complement of enzymes is formed or activated. After this initial period nitrogen is steadily lost from the cells. This nitrogen may be present in solution in an unavailable form or it may be lost as a gas. The latter seems more likely and could be attributed to the amino acid-nitrite reaction, the loss of ammonia at this neutral pH, or the action of denitrifying bacteria. Similar losses by Chlorella cultures have been reported (20) but have not been substantiated (2). Although the data presented here clearly agree with this earlier report of loss, experiments are planned to reinvestigate the phenomenon in additional mass cultures as well as in small-scale pure cultures.

Nitrogen fraction analyses of progressively deficient cells showed that in relation to the drop in total-N, protein-N remained high, representing 90 to 95% of the total-N. Soluble-N and free amino-N dropped exponentially and remained at a constant level after 3 days. Peptide-N and basic-N remained at a high level for 16 hours and then dropped. The combined amide and ammonia fraction and the nitrate fraction were low. Less than 0.1 mg/gm of each of these fractions was present. Nitrate was not detectable after 16 hours of N-deficiency, but traces of ammonia and amide-N were found in all samples. The analyses of peptide-N, free amino-N, and basic-N reflected the changes in total and soluble-N. A significant difference is not apparent in the proportions of the fractions between normal and deficient cells. This stability in the proportions of fractions indicates a constancy of the mechanism of nitrogen metabolism even under unusual conditions.

Nitrogen Resupply: After 14 days in the N-deficient medium the cells were resupplied with 55.3 gm KNO3. This was equivalent to 7.6 gm of N—an amount calculated to bring the level in the cells from 20 to 85 mg N/gm dry weight. After the first 24 hours, an additional 2.2 gm of nitrogen was added in the form of dilute nitric acid which lowered the pH from 8.3 to 7.6.

Upon the addition of nitrate the cells began to grow and a growth rate of 0.7 log2 unit per day was attained after 24 hours. The cells changed from pale yellow-green color to the deep green characteristic of N-sufficient cultures.

Uptake of nitrogen and protein synthesis are shown in figure 3. There was a lag in uptake and protein synthesis the first 2 hours. The protein level remained proportionally high, protein-N accounting for about 93 to 94% of the total-N. Presumably during this 2-hr period the metabolic apparatus was being reorganized to handle the increase of nitrogen. During this same period slight readjustments in the soluble fractions occurred as shown in figure 4. After one hour, increases in the basic-N and the amide and ammonia fractions were observed. Analytical difficulties were encountered in analyzing the nitrate and the amide and ammonia fractions, and 5 to 20% deviations of duplicates from the mean were found. After this initial 2-hr period, all fractions increased rapidly, but nitrate was taken up more rapidly than protein was synthesized. The amount of protein-N

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**TABLE I**

<table>
<thead>
<tr>
<th>DAYS</th>
<th>NITROGEN DEFICIENT</th>
<th>DRY WT/CULTURE</th>
<th>DRY WT REMOVED</th>
<th>TOTAL N AS % DRY WT</th>
<th>TOTAL CELL-N/CULTURE</th>
<th>N LOST FROM CELLS</th>
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<tr>
<td>0.5</td>
<td>89.0</td>
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<tr>
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<td>21.3</td>
<td>4.00</td>
<td>52.9</td>
<td>0.51</td>
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</tr>
<tr>
<td>4.0</td>
<td>154.5</td>
<td>23.9</td>
<td>2.53</td>
<td>4.76</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>196.0</td>
<td>...</td>
<td>1.49</td>
<td>3.93</td>
<td>1.87</td>
<td></td>
</tr>
</tbody>
</table>

* Corrected for sample removal.
had dropped to 79% of the total-N after 4 and 12 hours. Soluble-N accumulated and was mainly basic-N. Smaller amounts of amino-N and peptide-N were formed. Protein synthesis accelerated during the next 12 hours with protein-N accounting for 86% of the total-N at 24 hours. During the first 12 hours protein synthesis may have been limited by the absence of enough amino acids, peptides and, particularly, basic substances. Alternatively, the accumulation of these substances might be due to some factor which limited protein synthesis. Syrett and Fowden (32) showed that, in the dark, the absence of carbohydrate limited protein formation when ammonia was supplied to N-deficient Chlorella. The present experiments were carried out in the light and pcv values of 0.09 to 0.12 were maintained by sampling. Light intensity per unit cell would be inversely proportional to pcv, and the lowest values were found during the lag of protein formation. Therefore it seems unlikely that light was a limiting factor. After 48 hours protein-N had reached its typical level of 93% of the total-N. Simultaneously, large decrease in the soluble fractions were observed, and presumably this nitrogen was incorporated into protein.

The composition of the basic fraction precipitated from plant extracts by phosphotungstic acid is extremely heterogeneous and can include basic amino acids, peptides, nucleotides, amines, betaines, and alka-

loids. Further fractionation of the basic fraction was carried out by measuring the decrease in amino-N after this precipitation and by analyzing the extract for arginine. These results are shown in table II. During the first 2 hours little change occurred. In all samples taken from 2 to 24 hours most of the basic, non-amino-N consisted of the guanido-N of arginine. However, after 4 hours not all of this nitrogen was in free arginine since multiplying the basic amino-N by 3 yielded results which were about 50% lower than the guanido-N values. An increase in peptide-N during this period suggested that arginine was combined into a peptide or peptides. This was supported by paper chromatography of the extracts. Using 77% ethanol as a solvent and a diethylamine atmosphere the unknown had an Rf value of 0.12 while that of arginine was 0.29. Co-chromatography of arginine and the unknown, using butanol-acetic acid, yielded two spots—arginine at Rf 0.20 and the unknown at Rf 0.05. Co-chromatography of the hydrolyzed unknown and arginine gave a single spot at Rf 0.12. The compounds were detected by spraying the Sakaguchi reagents (19) on the paper. Syrett (31) has also reported an increase in arginine upon hydrolysis of similar Chlorella extracts. After 48 hours the arginine content dropped, but the basic-N remained high, suggesting that other basic amino acids had been formed.

Fig. 3. Increase in the concentrations of total-N and protein-N following the resupply of nitrate to N-deficient Scenedesmus obliquus cells.

Fig. 4. Changes in soluble-N fractions following the resupply of nitrate to N-deficient Scenedesmus obliquus cells.
Under conditions where soluble-N accumulates, one of the responses in higher plants is amide forma-
tion (7, 30). Amide and dicarboxylic amino acid
formation occurred when young excised pea shoots
were supplied with exogenous nitrogen (24), particu-
larly when ammonia rather than nitrate was supplied.
Similar results were obtained when N-deficient yeast
was resupplied nitrate-N or ammonium-N (26, 36).
The rate of protein formation was the same with each
nitrogen source, but much more amide and dicar-
boxylic amino acids were formed in cultures supplied
with ammonia than with nitrate. Syrett (31) and
Syrett and Fowden (32) showed that amides (particu-
larly glutamine) were formed when N-deficient Chlo-
rella cells were resupplied with ammonia. In contrast
to yeast and higher plants, basic amino acids were
also formed. The present results using nitrate are
similar, but much less amide-N was formed in relation
to the other fractions, as was also found with yeast
and peas. In the present experiments about 2 hours
were required before appreciable accumulation of
soluble fractions occurred; responses were immediate
in the experiments of Syrett and Fowden. However,
the duration of deficiency was only three days in their
experiments. Figure 2 shows that in the present ex-
periments a basal level of total-N was not attained
before 5 days. Their experiments also show that soluble-N and the fractions thereof decreased after
2 hrs. This could be attributed to the small amount
of nitrogen resupplied to their cultures.

An attractive hypothesis concerning the formation
of arginine is that the Krebs-Henseleit urea cycle
(17) is operative in green algae. This cycle is shown
in the following diagram:

![Diagram of the Krebs-Henseleit urea cycle]

An experiment designed to test this hypothesis was
conducted using N-deficient cells from the mass cul-
ture vats. Harvested cells were suspended in a N-
deficient basal nutrient solution, and aliquots of this
suspension containing 1.31 gm dry weight were placed
in 500 ml Erlenmeyer flasks. To each flask 2.5 milli-
moles of KNO₃ plus 125 micromoles of a possible
precursor were added. The volume was made to 200
ml with N-deficient medium, and the flasks were
incubated in the shaking apparatus. Fifty-ml samples
taken at periodic intervals were extracted and anal-
alyzed for arginine. The results are shown in table
III. Stimulation of arginine formation by urea and
ornithine was observed after 3 and 6 hrs, but little,
if any, stimulation was found at the later times. The
lack of stimulation by citrulline may have been due to
impermeability of the cells to citrulline as has been
shown for liver slices (17). The stimulation by urea
could be due to a reversal of the arginase system,
since urease is absent from Chlorella (37). Added
ornithine would stimulate citrulline formation and a
possible reverse reaction involving the enzyme form-
ing citrulline from arginine described in Chlorella
by Walker and Myers (38) would in turn result in in-
creased arginine production. In connection with this
experiment, the fact that arginine, citrulline, ornithine,
and urea are much better sources of nitrogen than
ammonium nitrate for the growth of Chlorella (2) is
further evidence for the operation of the cycle.

**Table II**

<table>
<thead>
<tr>
<th>Hours after addition of nitrate</th>
<th>Mg/gm dry wt</th>
<th>Amino-N of arginine</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.46</td>
<td>0.09</td>
</tr>
<tr>
<td>1</td>
<td>0.57</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>0.49</td>
<td>0.30</td>
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<tr>
<td>4</td>
<td>1.46</td>
<td>1.29</td>
</tr>
<tr>
<td>8</td>
<td>4.26</td>
<td>3.45</td>
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<tr>
<td>12</td>
<td>5.98</td>
<td>5.53</td>
</tr>
<tr>
<td>24</td>
<td>6.03</td>
<td>5.04</td>
</tr>
<tr>
<td>48</td>
<td>2.25</td>
<td>1.55</td>
</tr>
</tbody>
</table>

**Table III**

Effect of Possible Precursors on the Synthesis of
Arginine by N-deficient Scenedesmus
obliquus Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Micromoles arginine/gm initial dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃ control</td>
<td>26</td>
</tr>
<tr>
<td>KNO₃ + urea</td>
<td>53</td>
</tr>
<tr>
<td>KNO₃ + ornithine</td>
<td>46</td>
</tr>
<tr>
<td>KNO₃ + citrulline</td>
<td>28</td>
</tr>
<tr>
<td>KCl control</td>
<td>3</td>
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</table>

**Potassium Deficiency and Resupply:** The mean
daily growth rate during 5 days before transfer to
deficient medium was 0.334 log₂ unit; during de-

cency the rate was 0.119 log₂ unit. The culture was
still growing slowly at the end of 21 days in deficient
medium. During the first 24 hours soluble-N in-

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increased slightly, but it was incorporated into protein after this time. Throughout deficiency a progressive increase occurred in the proportion of peptide-N from 20% of the soluble N to 40% after 21 days. Otherwise no significant shifts in the soluble fractions were observed. After the small initial change protein-N continued to make up most of the total-N. Potassium deficiency in Chlorella causes a decrease in photosynthesis and an increase in respiration (22). Presumably just enough photosynthesis was produced in the present experiment to maintain a high protein level, but not enough was formed to enable protein synthesis to continue at a rate sufficient to support normal growth.

After 21 days in deficient medium, the cells were resupplied potassium with 89 gm KCl. The nitrogen fraction analyses during resupply showed that protein synthesis did not start immediately following resupply. A 2% decrease in protein-N was observed in the first hour. After 3 hrs protein synthesis started and continued throughout the 24 hr experimental period. Similar results were obtained by Pison (21), who showed that the immediate response to K resupply was an increase in photosynthesis, and that this was followed by an increase in protein as measured indirectly by chlorophyll analyses. In the present experiment, other than an increase in percentage of protein, the only significant change in the nitrogen fractions after resupply was a decrease in peptide-N. The growth rate increased to 1.22 log_2 units per day during the 24 hrs following resupply.

During these studies of the effect of potassium deficiency and resupply on nitrogen metabolism, K and Na analyses were made on ashed cells. The uptake of Na while K was being lost (fig 5) coupled with the continuing growth suggests that sodium may partially replace potassium in the functioning of the alga, perhaps by maintaining electrolyte balance. In barley when Rb or Ca rather than Na were substituted for K, changes in amino acids were much greater (25). It is possible that greater changes in nitrogen fractions of Scenedesmus would occur if some cation other than Na were substituted for K.

Following resupply of K, rapid uptake of K and Na was observed. After no wise sodium to continued 20 photosynthesis level, 118 normal synthesis to first hour. the potassium with A 2 indirectly continued and period. Similar results was experiment, other who was the only protein, The growth fractions after resupply during the analyses Na of Na by continuing K, when replace of of the time.

**Fig. 5.** Changes in the concentrations of K and Na in *Scenedesmus obliquus* cells during potassium deficiency. The usual concentration of K was found after 6 hrs; Na reached a basal level after 3 hrs, but did not drop during the 24 hr period of resupply to the initial low level found before K-deficiency.

**Phosphorus Deficiency:** Deficiency of P resulted in a significant reduction in growth. The mean growth rate during 3 days prior to deficiency was 0.517 log_2 unit per day, whereas, during 12 days of deficiency the mean growth rate was 0.126 log_2 unit per day. As deficiency progressed the color of the culture became greener, suggestive of phosphorus deficiency symptoms in higher plants.

Nitrogen fractions in deficient cells are shown in table IV. A slight proportional decrease in protein-N was found. In P-sufficient cells 92% of the total-N was protein-N. This decreased to 87 to 89% as P deficiency progressed, but the 11-day sample showed recovery. The increase in each soluble fraction generally paralleled the increase in total soluble-N. Similar increases, particularly of amino-N, have been observed in higher plants (29). However, small increases were observed in the proportion of basic-N expressed as a percentage of the soluble-N, as well as a small relative decrease in free amino-N.

The behavior of the amide and ammonia fraction in early P deficiency was striking, and further frac-

### Table IV

<table>
<thead>
<tr>
<th>DAYS DEFICIENT</th>
<th>PROTEIN-N</th>
<th>SOLUBLE-N</th>
<th>FREE AMINO-N</th>
<th>Peptide-N</th>
<th>BASIC-N</th>
<th>AMIDE AND AMMONIA-N</th>
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<tr>
<td>0.0</td>
<td>89.3 (92)*</td>
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<td>1.84</td>
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<tr>
<td>1.0</td>
<td>85.8 (88)</td>
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<tr>
<td>2.0</td>
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<td>2.85</td>
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<tr>
<td>3.0</td>
<td>77.0 (88)</td>
<td>9.92</td>
<td>1.21</td>
<td>3.94</td>
<td>5.53</td>
<td>0.32</td>
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<tr>
<td>5.0</td>
<td>80.1 (89)</td>
<td>10.24</td>
<td>1.68</td>
<td>2.26</td>
<td>5.32</td>
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</tr>
<tr>
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<td>79.8 (89)</td>
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<td>1.55</td>
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<td>1.52</td>
<td>3.20</td>
<td>0.20</td>
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</table>

* The numbers in parentheses refer to the protein-N as a percentage of the total-N.
tionation into free ammonia-N, unstable amide-N, and stable amide-N was carried out using differential hydrolysis techniques (22). Inasmuch as parallel changes occurred in the basic fraction, analyses for arginine were also made. The results are shown in figure 6. The first effect of deficiency was the formation of basic substances. However, arginine accounts for only approximately 30% of the basic-N. During the first six hours amides and ammonia also increased slightly. These changes were followed by a large increase in ammonia and in stable amide (presumably asparagine). Preliminary experiments had indicated that most of the amide fraction was unstable (presumably glutamine). In this experiment unstable amide was much decreased. After 12 hours arginine again increased and asparagine and ammonia decreased. These changes could be interpreted as follows: Early in deficiency arginine increases due to the incorporation of ammonia derived from protein breakdown, into the Krebs-Henseleit cycle. After 6 hours soluble-P is used up, and the generation of adenosine triphosphate (ATP) via oxidative metabolism ceases. Gest and Kamen (11) showed that 5 washings with fresh medium removed 25% of labeled P from Chlorella and that the exchange took place between acid-soluble-P in the cells and exogenous phosphate. ATP is necessary for the operation of the Krebs-Henseleit cycle (17) and for the formation of glutamine (27). Without ATP, ammonia would increase as observed. If the increase of stable amide is an increase in asparagine, these results support a tentative hypothesis that ATP is unnecessary for its formation. However, amide synthesis from ammonia and the corresponding amino acid requires 3450 cal/mole free energy (5). Alternatively, the stable amide could be urea derived from arginine. It would be measured as a stable amide by the hydrolytic analytical method used (22). The increase of arginine and decrease of ammonia and stable amide after 12 hrs could then be due to an increased supply of soluble-P from decomposition of phosphoproteins or nucleic acids. These changes support the concept of a readjusting metabolism when conditions are radically changed.

Depletion of total cellular phosphorus was gradual, dropping from 8 mg/gm dry weight to 4 mg/gm dry weight in 11 days. Presumably most of the P is combined in insoluble forms, since appreciable loss does not occur before one day of deficiency. This does not

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**Table V**

<table>
<thead>
<tr>
<th>Hrs after addition of phosphate</th>
<th>Protein-N</th>
<th>Soluble-N</th>
<th>Free amino-N</th>
<th>Peptide-N</th>
<th>Basic-N</th>
<th>Amide and ammonia-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>75.1 (92)</td>
<td>6.76</td>
<td>1.08</td>
<td>1.52</td>
<td>3.20</td>
<td>0.20</td>
</tr>
<tr>
<td>0.5</td>
<td>73.1 (92)</td>
<td>6.80</td>
<td>1.38</td>
<td>2.30</td>
<td>3.15</td>
<td>0.19</td>
</tr>
<tr>
<td>1</td>
<td>73.1 (92)</td>
<td>6.34</td>
<td>1.35</td>
<td>0.86</td>
<td>3.10</td>
<td>0.18</td>
</tr>
<tr>
<td>3</td>
<td>70.2 (93)</td>
<td>6.12</td>
<td>1.19</td>
<td>0.42</td>
<td>2.79</td>
<td>0.16</td>
</tr>
<tr>
<td>8</td>
<td>67.8 (94)</td>
<td>4.84</td>
<td>0.94</td>
<td>0.92</td>
<td>2.35</td>
<td>0.14</td>
</tr>
<tr>
<td>18</td>
<td>77.5 (94)</td>
<td>5.07</td>
<td>1.30</td>
<td>1.93</td>
<td>2.43</td>
<td>0.17</td>
</tr>
<tr>
<td>30</td>
<td>80.7 (94)</td>
<td>4.88</td>
<td>1.53</td>
<td>1.21</td>
<td>2.45</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* The numbers in parentheses refer to the protein-N as a percentage of the total-N.

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**Fig. 6.** Changes in cellular ammonia, asparagine, glutamine, and arginine during the early phase of P deficiency in *Scenedesmus obliquus.*

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5 Recent enzymatic studies (39) have established an ATP requirement for asparagine formation, both for synthesis from aspartic acid and ammonia, and for synthesis by amidation of β-alanine followed by carboxylation of the β-α-namylamido. It therefore seems likely that the increase in stable amide is not due to asparagine formation.
mean that the radical changes in soluble-P, postulated in relation to changes in nitrogenous compounds, do not occur. The changes may happen more rapidly than indicated by total-P analyses.

**Phosphorus Resupply:** After 11 days of deficiency 48 gm K$_2$HPO$_4$ was added to the culture. This supplied approximately half as much P as was initially supplied in a P-sufficient medium. Rapid growth of the cells resumed; a rate of 0.690 log unit per day during the first 24 hrs was observed. Changes in the nitrogen fractions are shown in table V. Steady resynthesis of protein occurred. Ninety-two percent of the total-N was protein-N initially, and this increased to 94%. A concomitant decrease in soluble-N was found. The assumption is made that as soluble-P became available ATP necessary for protein synthesis increased. The proportion of amino-N increased from its previously low percentage of the soluble-N. Changes in basic-N followed the changes in soluble-N. Amide and ammonia-N did not change. Peptides increased and then decreased, suggesting a role as intermediates in protein formation.

The changes in total cell phosphorus were rapid. Phosphorus reached the usual level during the first hour; almost doubled in the next 8 hrs; and then decreased to an equilibrium value. Luxury consumption clearly occurred during the early period.

**Dark Experiment:** A mass culture was grown for 6 days in the light. Then the lights were turned out and the algae were cultured for 12 days in the dark. The mean growth rate during the light period was 0.584 log$_2$ unit per day. Apparent growth rates as calculated by pev measurements were 0.325, 0.328, and 0.186 during the respective first 3 days in the dark. After this period no growth was observed.

Changes in the nitrogen fractions during dark culture are shown in table VI. An increase in total-N was observed at the second day and was followed by a decrease. The proportional amount of protein-N remained high until 3 days and was followed by a decrease. The high levels of total-N could be accounted for by the utilization of non-nitrogenous reserves by the respiratory system. Protein breakdown occurred after these reserves were exhausted and protein carbon was utilized to provide energy for the cell. Soluble-N was then formed. Proportions of the soluble fractions remained more or less constant until after the tenth day when the proportion of amino-N decreased. At this time amino acids were being deaminated and respired, thus accounting for the increase in the amide and ammonia fraction. When the experiment was terminated on the 13th day, 1.58 mg NH$_3$-N/l was found in the culture solution, indicating that some excretion had occurred. Only traces of amino-N were found in the solution. In spite of the increase in soluble-N and changes in the fractions, it should be emphasized that the change in protein-N was small and that protein-N accounted for 90% of the total-N at the end of the experiment.

**Conclusions**

All of the factors studied, i.e. nitrogen, phosphorus, potassium, and carbohydrates, are essential for protein synthesis in *Scenedesmus obliquus*. Growth was much reduced or ceased if any one of these was deficient. A striking fact is that only one of these, exogenous nitrogen, was required for the maintenance of a high protein content. The absence of other factors resulted in only a limited amount of proteolysis. Even during nitrogen deficiency, the bulk of the nitrogen in the cell was protein-N. Thus the mechanism for protein formation is not damaged by any of the deficiencies studied. Overflow or shunt metabolism occurs in *Scenedesmus* during nitrogen deficiency. Carbon which ordinarily would be incorporated into protein was formed into fat and carbohydrate (1). However, the present studies show that *Scenedesmus* is predominately a protein-forming organism.

During the first 24 hours of deficiency of any of the factors slight readjustments of nitrogen metabolism occur. In the case of phosphorus, these changes could be correlated with possible changes in phosphorus-containing components of the cell. In nitrogen deficiency, excretion and reuptake occurred. Potas-
sium deficiency resulted in an accumulation of soluble-N fractions without changes in their proportions, and this was followed by a decrease and reincorporation into protein. Darkness resulted in slight changes of the nitrogen fractions.

Following resupply of N, P, or K, protein synthesis resumed. In the case of P, synthesis was resumed almost immediately. Metabolic readjustment occurred during the first few hours following resupply of K and presumably protein synthesis must be preceded by resumption of the photosynthesis. Similar metabolic readjustment occurred when N was resupplied. This was followed, however, by an increase in basic substances, particularly arginine-containing peptides. The accumulation of base-N may be the result of overloading the protein synthesis system with N so that part of it overflows into the basic fraction. Similar shunt metabolism of P-containing compounds may occur since luxury consumption of P was found during resupply. However, with regard to N, all the experimental data indicate that if alternative pathways exist when cells are subjected to peculiar conditions, they are characterized by a low synthetic capacity which prevents major accumulation of any N-fraction other than protein.

**Summary**

*Scenedesmus obliquus*, strain WH-50, was grown in mass culture under conditions of N, P, K, and carbohydrate deficiency. Nitrogen, P, and K were resupplied to the cells. During the experiments samples of cells were harvested and analyzed for nitrogen fractions.

In these experiments, the following results were obtained:

1. During N deficiency the N content of the cells dropped exponentially and reached a basal level after 8 days. This decrease in total-N was due partially to dilution by new cell material and in part to the excretion of N from the cells. During N deficiency the relative proportions of the fractions were little changed. Protein-N continued to account for approximately 90 to 95% of the total-N.

2. Upon resupplying nitrate to N-deficient cells a normal growth rate was resumed during the first 24 hrs. Protein synthesis lagged behind N uptake and soluble-N accumulated in the cells. Most of the soluble-N consisted of base-N; and most of this was tentatively identified as arginine-containing peptides. Precursor experiments indicated that the synthesis of arginine probably occurred through a Krebs-Henseleit urea cycle similar to that found in liver.

3. Potassium deficiency resulted in a reduction in the growth rate of the culture, but growth never ceased during a 21-day K-deficient period. No appreciable shifts in the nitrogenous composition occurred other than an initial small decrease in the protein-N and an increase in the level of peptide-N. The K level in the cells slowly dropped and was replaced by Na. Resupply of K to K-deficient cells resulted in a resumption of normal growth, a decrease in the amount of peptide-N in the cells, and a resumption of protein synthesis. The cells contained a normal amount of K after 6 hours, and a concomitant loss of Na occurred.

4. During P deficiency the amount of protein-N decreased only slightly. Early in the deficient period, changes in free ammonia, stable and unstable amides, and in arginine occurred. The resupply of P to deficient cells enabled the culture to resume normal growth. Steady protein synthesis followed resupply. Except for changes in the peptide fraction, most of the soluble fractions paralleled the behavior of the soluble-N.

5. In darkness an increase in total-N occurred as non-nitrogenous reserves were utilized in respiration. After 2 days in the dark, the total-N decreased. As protein was broken down increased proportions of soluble-N were found. After 13 days of darkness, amino-N had decreased and the amide and ammonia fraction increased. Apparently deamination of amino acids had occurred, for ammonia-N also was found in the culture solution. However, little proteolysis occurred.

It is concluded that while adequate supplies of N, K, P, and carbohydrate are essential for protein synthesis, only N is required for the maintenance of protein once it is formed. Although metabolic readjustments and shunt metabolism do occur under unusual circumstances, protein makes up most of the total-N, and the metabolism of the alga is strongly directed toward the formation and maintenance of protein.

**Literature Cited**


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