Regulation of Phosphate Accumulation in the Unicellular Cyanobacterium *Synechococcus*

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The phosphorus contents of acid-soluble pools, lipid, ribonucleic acid, and acid-insoluble polyphosphate were lowered in *Synechococcus* in proportion to the reduction in growth rate in phosphate-limited but not in nitrate-limited continuous culture. Phosphorus in these cell fractions was lost proportionately during progressive phosphate starvation of batch cultures. Acid-insoluble polyphosphate was always present in all cultural conditions to about 10% of total cell phosphorus and did not turn over during balanced exponential growth. Extensive polyphosphate formation occurred transiently when phosphate was given to cells which had been phosphate limited. This material was broken down after 8 h even in the presence of excess external orthophosphate, and its phosphorus was transferred into other cell fractions, notably ribonucleic acid. Phosphate uptake kinetics indicated an invariant apparent *Km* of about 0.5 μM, but *Vmax* was 40 to 50 times greater in cells from phosphate-limited cultures than in cells from nitrate-limited or balanced batch cultures. Over 90% of the phosphate taken up within the first 30 s at 15°C was recovered as orthophosphate. The uptake process is highly specific, since neither phosphate entry nor growth was affected by a 100-fold excess of arsenate. The activity of polyphosphate synthetase in cell extracts increased at least 20-fold during phosphate starvation or in phosphate-restricted growth, but polyphosphatase activity was little changed by different growth conditions. The findings suggest that derepression of the phosphate transport and polyphosphate-synthesizing systems as well as alkaline phosphatase occurs in phosphate shortage, but that the breakdown of polyphosphate in this organism is regulated by modulation of existing enzyme activity.

The importance of phosphate supply in regulating the growth of aquatic organisms is widely recognized and has prompted a number of investigations into the uptake and distribution of phosphate in eucaryotic (1, 10, 11, 34, 35) and procaryotic (4, 5, 13, 20, 21, 37) microorganisms. Such studies have emphasized that the phosphate content of whole cells and of individual cell components can be varied over quite wide limits and also that phosphate uptake capacity depends on the previous history of the cells (4, 13, 38, 42). This is also true of polyphosphate, which can be accumulated to the extent of 50% or more of the total cell phosphorus if cells are provided with energy and phosphate in excess but are restricted by some other nutrient, such as nitrogen or sulfate (16, 18), or if the cells are supplied with phosphate after a period of deprivation (1, 13, 40).

Cyanobacteria are frequently prominent members of undesirable blooms in natural waters, in which phosphate availability is a common limiting factor, and their phosphate relationships have been studied by a number of workers. Although continuous culture methods were employed in some of these investigations (5, 21), the physiological state of the cells was less well defined in others (4, 20, 41), so that it is not always clear whether the observations made relate specifically to phosphate starvation or to nutrient restriction in general. The work described here made use of balanced exponential cultures growing without nutrient restriction and also of nitrate- and phosphate-limited continuous cultures for investigations into the regulation both of phosphate uptake and of its intracellular fate in the unicellular cyanobacterium *Synechococcus*. The main findings are that enhanced uptake and potential for polyphosphate formation are similarly regulated, but are distinct processes.

**MATERIALS AND METHODS**

The strain of *Synechococcus* and the general culture conditions and media used have been described previously (23). Water-jacketed vessels (200- or 400-ml working volume), fitted with inlet ports for medium

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and for the CO₂-air mixture and with a single outlet port for gases and culture (25), were used for continuous culture, and all flexible connections were made with surgical-grade silicone tubing. The nitrate-limited medium contained 0.25 mM KNO₃ and 1 mM potassium phosphate, pH 8; 15 μM potassium phosphate and 2.5 mM KNO₃ were used for phosphate-limited cultures. Both media also contained 15 mM triethanolamine brought to pH 8 with H₂SO₄. Each vessel was illuminated by two 100-W incandescent bulbs at a distance of approximately 7 cm. Flow rates were maintained by Harvard peristaltic or syringe pumps. Approach to a steady state was followed by monitoring optical density at 750 nm in a Zeiss PMQ II spectrophotometer and by protein determination (29); a steady state was assumed when these parameters had remained constant for five volume changes in the culture vessel. A similar rule of thumb was followed after the dilution rate was changed. At steady state, the specific growth rate (In 2/doubling time) equals the dilution rate (22). Photosynthetic pigments were estimated as described previously (23).

**Phosphate distribution and estimation.** Pᵢ was estimated as described by Dryer et al. (7). Total phosphate was measured by using the same method after digestion of cell material by autoclaving in 10% (wt/vol) K₂S₂O₈ in tightly closed screw-capped tubes for 45 min at 121°C (4). Radioactively labeled cells were fractionated as described by Roberts et al. (36), except that CHCl₃-CH₂OH (3:1, vol/vol) was used for lipid extraction and RNA was solubilized by incubation in 0.3 N KOH for 16 h at 37°C before DNA was hydrolyzed in hot 5% trichloroacetic acid. Polyphosphate and nucleic acid-derived phosphate in these fractions were separated by charcoal adsorption of nucleotides. Total phosphate in each fraction was obtained by K₂S₂O₈ digestion of an appropriately sized portion. As used here, the term polyphosphate refers only to cold-insoluble polymer.

**³²P uptake.** In long-term uptake experiments, washed cell suspensions from batch or continuous cultures were transferred to washed medium pre-equilibrated with 1% CO₂-air and containing 0.2 to 0.15 mM phosphate (0.2 to 0.7 μCi/μmol). Cultures were diluted with temperature- and gas-equilibrated medium at intervals so that cell densities were kept between the limits of 30 and 100 μg of protein per ml throughout the growing periods.

Short-term uptake was measured at 30°C or as given in individual experiments. Cells were washed and diluted in 10 mM triethanolamine sulfate, pH 8.0. Neither rates nor affinities were affected by the addition of 10 mM KCl or 5 mM MgCl₂ or by changing the pH of the assay mixture to 7.5. Radioactive phosphate (1 to 50 μCi/μmol) was added to initiate uptake, and five or six 0.5-ml samples were taken in each measurement at intervals of 10 s to 2 min, depending on the experiment. Each sample was filtered rapidly through a 0.45-μm pore size membrane filter which had been boiled previously in 0.5 M lithium chloride–1 mM phosphate, pH 9, washed twice with 0.5-ml portions of the same solution, and dried at 70°C. For experiments in which the proportion of total counts taken up recoverable as Pᵢ were to be measured, alternate filtered samples were transferred as rapidly as possible to weighing dishes containing 2-ml portions of 5% trichloroacetic acid or 0.5 N perchloric acid at 0°C. After 30 min, the samples were centrifuged, and portions of the supernatant were taken for total counts and for determination of Pᵢ by precipitation of the phosphomolybdate complex with triethylenamine (43, 46) or extraction into isobutanol–benzene (1:1, vol/vol) (3). The remaining samples, filtered, washed, and dried as usual, served as controls for checking total uptake in these experiments. Uptake was linear with time in each experimental series.

Radioactivity was usually measured either on dried membrane filters or on samples of radioactive solution dried on 2.5-cm disks of Whatman no. 3 filter paper in 0.4% (wt/vol) 2,5-bis(5'-t-butyl-2-benzoxazolyl)thiophene in toluene. Counts in butanol–benzene extracts were measured in 0.2 N NH₄OH by Cherenkov radiation. Specific activities were determined from samples extracted and counted in the same way.

**Cell breakage and enzyme assays.** Suspensions containing 5 to 20 mg of cell protein per ml in G buffer [0.02 M Tris-sulfate, pH 8, 0.2 M (NH₄)₂SO₄, 1 mM EDTA, 1 mM β-mercaptoethanol; modified from Mührlardt (32)] were passed twice through a chilled French pressure cell at 12 to 14,000 lb/in.² All subsequent steps were carried out at 0 to 4°C. Unbroken cells and large debris were removed by centrifugation at 11,000 × g for 30 min. The supernatant was centrifuged again for 90 min at 96,000 × g, and the precipitate of green membranes was discarded. Solid (NH₄)₂SO₄ (2.25 g/10 ml) was dissolved in the supernatant, and the whole solution was allowed to stand in ice for 30 min. The heavy precipitate, which contained more than 90% of the polyphosphate synthetase and polyphosphatase activities of the extract, was collected by centrifugation at 11,500 × g for 30 min. It was dissolved in 1 to 3 ml of G buffer and assayed at once. In one preparation 1 mM phenylmethyl sulfonflyl fluoride was included in the buffer in which the cells were broken to inhibit protease activity which might be responsible for releasing membrane-bound proteins into the soluble portion. This did not affect the distribution of either enzyme activity after high-speed centrifugation.

Although polyphosphatase activity was unchanged after 3 weeks of storage in ice, about 30% of the polyphosphate synthetase activity was lost in 1 day, either in ice or frozen at −20°C. Inactivation was faster if (NH₄)₂SO₄ or β-mercaptoethanol was omitted from the suspending buffer. Neither enzyme activity decreased by more than 15% if whole cells were kept frozen at −60°C for 6 weeks.

**Polyphosphate synthetase.** Polyphosphate synthetase was assayed by determining the rate of formation of acid-insoluble radioactive material from γ-3²P]ATP (36, 32), using 0.2 to 2 μg of enzyme protein in a reaction volume of 0.5 ml. Preliminary experiments established that Mg²⁺ was required for the reaction and that 5 mM ATP, with a ratio of Mg²⁺ to ATP of at least 1:1, was optimal. Increase in acid-insoluble radioactivity was linearly related to time and to enzyme concentration under these conditions. Addition of polyphosphate as a primer for the reaction was not required and did not alter the rate of the reaction. ADP (2 mM) added to the reaction mixture reduced the counts in acid-precipitable material by 85%, suggesting that the reaction was reversible.
The reaction product was characterized by heating for 20 min at 100°C in 1 N HCl (14), after protein and nucleic acid were removed by adsorption to charcoal in the presence of 0.5 m NaCl (32); 87% of the total counts in the original preparation were recovered as Pi, after this treatment. The deproteinized product was also used for estimating metachromasy in toluidine blue O (45) and gave the same absorbance shifts at 530 and 630 nm expressed in terms of phosphate content as a sample of polyphosphate isolated chemically from *Synechococcus*, as described below.

**Polyphosphatase.** Polyphosphatase was assayed by following release of P from chemically isolated polyphosphate (12) in 1-m reaction mixtures containing 0.3 to 1.4 mg of extract protein and polyphosphate equivalent to 1.5 to 2 μmol of acid-releasable phosphate. Preliminary experiments showed that Mg<sup>2+</sup> required was in this reaction.

**Preparation of polyphosphate.** Phosphate-free medium (3 liters) was inoculated with cells from an exponential culture to a protein concentration of 175 μg/ml. After 72 h of starvation in light and with air-CO<sub>2</sub> gassing, P<sub>i</sub> to a final concentration of 5 mM was added, and the accumulation of polyphosphate was allowed to proceed for 5 h. The whole culture was then chilled, and the cells were collected by centrifugation and stored at -60°C until used. Radioactively labeled polyphosphate was prepared from a 200-ml culture starved as described above and then supplied with 0.135 mM phosphate (12 μCi/μmol) and incubated for an additional 5.5 h. Only 3% of the counts added were left in the medium at this time. Polyphosphate was extracted from hypochlorite digests of cells as described by Harold (14). The final preparation was faintly opalescent and contained no detectable P<sub>i</sub> before acid hydrolysis. The material was completely excluded from Sephadex G-50 and 85% was excluded from Sephadex G-75, suggesting that the average molecular weight was more than 50,000.

**Internal water space.** Internal water space was measured as previously described (23).

**Radioactive materials.** <sup>32</sup>P, carrier-free in 0.1 N HCl was obtained from New England Nuclear Corp. or from International Chemical and Nuclear Co. The [γ-<sup>32</sup>P]ATP used in the enzyme assays was a gift from Ray Wu, Cornell University.

## RESULTS

**Cell composition: steady states.** When the composition of cells grown either without restriction or with limiting nitrate is compared with the composition of phosphate-restricted cells (Table 1), it is apparent that most alterations in phosphate content can be attributed specifically to phosphate restriction rather than to changed growth rate. The RNA content is exceptional in varying directly with growth rate in both types of restriction, even though the absolute level was consistently lower in the phosphate-limited cultures. The P<sub>i</sub> pool (Table 2) was reduced almost to one-tenth of that found in unrestricted cells at the lowest phosphate-limited specific growth rate investigated. It was, however, still more than 1 mM, although the culture outflow phosphate was below 5 μM, the limit of detection with the method used. Cultures whose growth rate was restricted by the availability of either phosphate or nitrate were yellow-green and had greatly reduced phycocyanin contents, as previously described (2, 24); phycocyanin content varied directly with specific growth rate over the range of 0.025 h<sup>-1</sup> (50 μg of phycocyanin per mg of total protein) to 0.163 h<sup>-1</sup> (200 μg of phycocyanin per mg of total protein).

### Table 1. Phosphate content of *Synechococcus* cell fractions

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Specific growth rate (h&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Cold acid soluble&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chloroform-methanol soluble&lt;sup&gt;b&lt;/sup&gt;</th>
<th>DNA</th>
<th>RNA</th>
<th>Polyphosphate&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balanced batch culture</td>
<td>0.14</td>
<td>0.136 (17.7)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.038 (4.9)</td>
<td>0.051 (6.6)</td>
<td>0.42 (54.4)</td>
<td>0.081 (11)</td>
</tr>
<tr>
<td>Phosphate-limited continuous culture</td>
<td>0.115</td>
<td>0.094 (16.8)</td>
<td>0.031 (5.5)</td>
<td>0.055 (9.8)</td>
<td>0.317 (56.7)</td>
<td>0.05 (8.9)</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
<td>0.075 (16)</td>
<td>0.028 (6.0)</td>
<td>0.05 (10.7)</td>
<td>0.241 (51.5)</td>
<td>0.058 (12.4)</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.065 (18)</td>
<td>0.024 (6.6)</td>
<td>0.05 (13.9)</td>
<td>0.2 (55.4)</td>
<td>0.043 (11.9)</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.063 (16.4)</td>
<td>0.016 (4.2)</td>
<td>0.061 (15.9)</td>
<td>0.181 (47.1)</td>
<td>0.04 (10.4)</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>0.054 (21)</td>
<td>0.019 (7.4)</td>
<td>0.048 (18.8)</td>
<td>0.096 (37.5)</td>
<td>0.037 (14.5)</td>
</tr>
<tr>
<td>Nitrate-limited continuous culture</td>
<td>0.163</td>
<td>0.087 (21)</td>
<td>0.06 (6.3)</td>
<td>0.051 (6.5)</td>
<td>0.515 (63.3)</td>
<td>0.007 (0.9)</td>
</tr>
<tr>
<td></td>
<td>0.115</td>
<td>0.122 (17.3)</td>
<td>0.042 (6.2)</td>
<td>0.048 (6.8)</td>
<td>0.396 (56)</td>
<td>0.016 (2.3)</td>
</tr>
<tr>
<td></td>
<td>0.064</td>
<td>0.16 (22)</td>
<td>0.044 (5.8)</td>
<td>0.05 (6.9)</td>
<td>0.361 (49.6)</td>
<td>0.063 (8.6)</td>
</tr>
<tr>
<td></td>
<td>0.032</td>
<td>0.173 (22)</td>
<td>0.045 (5.8)</td>
<td>0.052 (6.7)</td>
<td>0.231 (30)</td>
<td>0.158 (20)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Micromoles of phosphate per milligram of total cell protein.

<sup>b</sup> Cold 5% trichloroacetic acid.

<sup>c</sup> Chloroform-methanol, 3:1.

<sup>d</sup> Cold acid (5% trichloroacetic acid)-precipitable polyphosphate.

<sup>e</sup> Numbers in parentheses are phosphate content expressed as percentage of the chemically determined total in the cells.
<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Acid-soluble pool*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific growth</td>
<td>P_i</td>
<td>Other phosphate</td>
</tr>
<tr>
<td></td>
<td>rate (h⁻¹)</td>
<td>Conc</td>
<td>% of total</td>
</tr>
<tr>
<td>Balanced exponential culture</td>
<td>0.14</td>
<td>11.4</td>
<td>42</td>
</tr>
<tr>
<td>Phosphate-limited continuous</td>
<td>0.115</td>
<td>4.2</td>
<td>28</td>
</tr>
<tr>
<td>cultures</td>
<td>0.06</td>
<td>2.4</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.023</td>
<td>1.4</td>
<td>21</td>
</tr>
</tbody>
</table>

* The values given are averages of three or more determinations except for the balanced exponential results, which are averaged duplicate results.

\( ^{a} \) Internal volume, 5.1 µl/mg of protein (23).

\( ^{b} \) Internal volume, 4.9 µl/mg of protein.

\( ^{c} \) Internal volume, 4.6 µl/mg of protein.

\( ^{d} \) Internal volume, 4.2 µl/mg of protein.

Cells growing at very low specific growth rates contained up to 15 times more carbohydrate than cells from unrestricted cultures, based on cell protein (data not shown). Despite such changes, growth could be maintained indefinitely.

**Cell composition: transients.** If balanced exponential cultures which had been uniformly labeled by growth for five doublings in excess P\(_i\) containing \(^{32}\)PO\(_4\)\(^{-3}\) were transferred to medium containing the same concentration of unlabeled P\(_i\), the counts initially in the acid-soluble pools were chased within one doubling time (5 h), mainly into RNA. No loss of counts from RNA, DNA, or the polyphosphate fraction was observed over the subsequent 20 h of continued exponential growth (Fig. 1). No labeled phosphate was found in the medium in these experiments.

When prelabeled cells were transferred to medium without added phosphate, there was a progressive and approximately proportionate loss of phosphate from all cell fractions including polyphosphate (Fig. 2). Again, no label was detected extracellularly. Net protein synthesis stopped after about 30 to 36 h, by which time RNA counts had been reduced to one-quarter of the starting value and the culture color had changed to yellow-green. As had been found previously (24), the culture remained viable, and growth resumed on readdition of phosphate after a lag, the length of which depended on the duration of the starvation period.

Figures 3A and B show that addition of phosphate to starved cultures did not lead to the direct restoration of the normal phosphorus distribution, but rather involved uptake to at least twice the ratios of phosphate to protein found in exponential cells. Between 3 and 7 h after the addition, more than 60% of the newly acquired phosphate was found as polyphosphate, and rapid incorporation into RNA was also seen (Fig. 3B). Net protein synthesis resumed at an exponential rate between 8 and 10 h after phosphate was added, and at the same time the proportion of counts in polyphosphate began to decline toward normal levels, which were reached after about 35 to 40 h. Polyphosphate breakdown occurred despite the continued availability and uptake of P\(_i\) from the medium (Fig. 3A).

Cells from phosphate-limited continuous cultures also showed excess phosphate uptake when transferred to phosphate-containing medium, to an extent that depended on the specific growth rate and initial ratio of phosphate to protein (Table 3). Again, polyphosphate accounted for...
Grillo and Gibson

Protein.

medium. Symbols: phate-free
uptake rates were to 6 h
parably apparent
the of phosphate uptake also depended on the
analog
FCCP was
tions of more than 20
atures
showed Michaelis-Menten uptake kinetics
growth conditions. Cells
phenyihydrazone
inhibited
that of
saturable at low concentrations. Kinetic con-
tions during phosphate starvation of
Synechococcus. Cells were labeled by growth for 24 h in medium
containing 0.163 mM K2HPO4 (2.6 μCi/μmol) to a
final protein concentration of 83 μg/ml. The cells
were washed and transferred to phosphate-free me-
dium; the protein concentration was 16 μg/ml. During
the experiment, the protein content of the culture was
held between 15 and 65 μg/ml by dilution with phosphate-free medium. Symbols: ○, cold acid soluble
(×10^3); ×, RNA (×10^3); △, DNA (×10^3); ■, CHCl3-
CH2OH soluble (×10^3); ●, polyphosphate (×10^3); □, protein.

Phosphate uptake kinetics. The initial rate of
phosphate uptake also depended on the
growth conditions. Cells from all types of cul-
tures showed Michaelis-Menten uptake kinetics
saturable at low concentrations. Kinetic con-
stants obtained from double-reciprocal plots of
experimental results when cells grown under
different conditions were used are summarized
in Table 4. V_{max} was substantially greater in
phosphate-restricted cells, but the apparent K_m
was not significantly affected by growth condi-
tions. The increased maximum uptake rate was
clearly the result of phosphate limitation, since
the apparent V_{max} of nitrate-limited cells of com-
parably low specific growth rates was close to
that of unrestricted, rapidly growing cells. Phos-
phate uptake rates were reduced by 40% if cells
were not illuminated, but virtually abolished if
cells were kept both dark and anaerobic. The
uncoupler carbonyl cyanide-p-trifluoromethoxy
phenylhydrazine inhibited uptake at concentra-
tions of more than 20 μM, and the fluorine
analog FCCP was effective at approximately
one-tenth this concentration. Arsenate at 100-
fold excess over phosphate was without effect
(Table 5).

All of the radioactivity associated with cells
after uptake periods of 1 to 2 min at 15°C was
acid soluble. P_i accounted for 93.3 ± 7.3% of
the total counts in a total of 13 samples in several
experiments taken between 11 and 50 s after
uptake was started. At 20 and 26°C, 85 to 90% of
the extracted counts were P_i in samples taken
within 30 s, but movement both into other com-
ponents of the acid-soluble pool and into acid-
insoluble components was apparent between 1

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Fig. 2. Changes in phosphorus content of cell fractions
during phosphate starvation of Synechococcus.

Fig. 3. (A) Total phosphate uptake and net protein
synthesis after 1 mM P_i was added to 24-h phosphate-
starved cells. (B) Distribution of new phosphate into
cell fractions. An exponential culture was harvested
and used to inoculate phosphate-free medium; the
protein concentration was 14 μg/ml. The cells were
starved for 24 h (final protein concentration, 46 μg/
ml), then harvested and used to inoculate labeled
medium containing 0.17 mM K2HPO4 (0.9 μCi/μmol)
to 23 μg of protein per ml. The protein content of the
culture was maintained between 23 and 88 μg/ml by
dilution when necessary with medium of the same
composition and specific activity. (A) Symbols: ○, cell
protein; ●, ^32P taken up per milliliter of culture; ○,
^32P taken up per microgram of protein. (B) Symbols:
○, cold acid soluble; □, RNA; △, DNA; ▽, CHCl3-
CH2OH soluble; ●, polyphosphate.

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and 2 min. These results suggest strongly that P\textsubscript{i} is brought into the cells as such and moves into other phosphate-containing components after mixing with the intracellular P\textsubscript{i} pool.

**Polypolyposphate formation.** Cells from balanced exponential cultures and from restricted cultures all contained some polyphosphate, although this amounted to a substantial part of the total cell phosphate only in the nitrate-limited continuous culture with the lowest specific growth rate. However, even in very slow phosphate-limited growth, about 10% of the cell phosphate was found as polyphosphate, the amount of this material in the cells presumably reflecting the relative activities of polyphosphate-forming and -degrading enzyme systems. Polyphosphate synthesis could indeed be measured in extracts prepared from all cells, but the rates of formation were substantially greater in preparations from phosphate-restricted or -starved cultures (Table 6). The changed activity could be attributed specifically to phosphate limitation, since extracts from nitrate-limited continuous culture had specific activities close to those from unrestricted cells. In contrast, although the specific activity of polyphosphatase in extracts from cells grown in continuous culture was about twice that of batch cultures, this was true for both phosphate- and nitrate-limited conditions and was also independent of growth rate (Table 6).

Figure 4 shows the changes in specific activity of polyphosphate synthetase in extracts prepared at intervals during phosphate starvation and recovery after addition of 1 mM P\textsubscript{i}. A pro-

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**TABLE 3. Relation between phosphate-restricted specific growth rate, initial ratio of phosphate to protein, and extent of phosphate accumulation.**

<table>
<thead>
<tr>
<th>Specific growth rate (h(^{-1}))</th>
<th>Ratio of phosphate to protein (μmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>0.14(^b)</td>
<td>0.75</td>
</tr>
<tr>
<td>0.115(^c)</td>
<td>0.56</td>
</tr>
<tr>
<td>0.07(^c)</td>
<td>0.47</td>
</tr>
<tr>
<td>0.05(^c)</td>
<td>0.38</td>
</tr>
<tr>
<td>0.025(^c)</td>
<td>0.26</td>
</tr>
</tbody>
</table>

\(^a\) Samples from continuous cultures were transferred to 100 ml of medium containing 0.125 to 0.194 mM phosphate (0.45 to 1.8 μCi/μmol) as described in the text to a final protein concentration of 20 μg/ml. Samples were taken over a 24-h period, initially at 30-min intervals, filtered through membrane filters, washed, and counted.

\(^b\) Balanced exponential culture (control).

\(^c\) Phosphate-limited continuous cultures.

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**TABLE 4. Kinetics of phosphate uptake in Synechococcus.**

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Specific growth rate (h(^{-1}))</th>
<th>Conc of P\textsubscript{i} pool (mM)(^b)</th>
<th>(K_m) (μM)(^c)</th>
<th>(V_{max}) (nmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balanced batch</td>
<td>0.14</td>
<td>11.4</td>
<td>0.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Nitrate limited</td>
<td>0.064</td>
<td>8.6</td>
<td>1.0</td>
<td>2</td>
</tr>
<tr>
<td>Phosphate limited</td>
<td>0.05</td>
<td>2.4</td>
<td>0.3</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>1.4</td>
<td>0.25</td>
<td>48</td>
</tr>
</tbody>
</table>

\(^a\) Phosphate uptake was measured in light at 25°C as described in the text. Cells from balanced batch and nitrate-limited continuous cultures were collected by filtration and washed in 10 mM triethanolamine sulfate, pH 8, before being suspended in the same solution to between 6 and 20 μg of protein per ml. Phosphate-limited cultures were diluted directly into buffer to a protein concentration of 1.3 to 1.7 μg/ml. Uptake was initiated by adding phosphate having a specific activity of 0.05 to 0.25 μCi/nmol.

\(^b\) Determined on separate samples from the same cultures.

\(^c\) From Lineweaver-Burk plots.

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**TABLE 5. Effect of possible inhibitors on phosphate uptake by phosphate-limited chemostat-grown cells.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Addition</th>
<th>Uptake rate (nmol/min per mg of protein)</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light control</td>
<td></td>
<td>32.8</td>
<td>100</td>
</tr>
<tr>
<td>Dark, aerobic</td>
<td></td>
<td>18.3</td>
<td>56</td>
</tr>
<tr>
<td>Dark, anaerobic</td>
<td></td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>Arsenate (100 μM)</td>
<td></td>
<td>31.2</td>
<td>95</td>
</tr>
<tr>
<td>CCCP (25 μM)</td>
<td></td>
<td>28.8</td>
<td>70</td>
</tr>
<tr>
<td>CCCP (50 μM)</td>
<td></td>
<td>12.8</td>
<td>39</td>
</tr>
<tr>
<td>CCCP (100 μM)</td>
<td></td>
<td>0.7</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) Phosphate concentration, 1 μM; specific growth rate, 0.025 h\(^{-1}\). Inhibitors were added 5 min before uptake was started.

\(^b\) CCCP, Carbonyl cyanide-m-chlorophenyl hydrazone.

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**TABLE 6. Specific activities of polyphosphate synthetase and polyphosphatase in Synechococcus grown under different conditions.**

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Specific growth rate (h(^{-1}))</th>
<th>Sp act of polyphosphate synthetase(^a)</th>
<th>Sp act of polyphosphatase(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balanced exponential culture</td>
<td>0.14</td>
<td>1.4</td>
<td>8.7</td>
</tr>
<tr>
<td>Continuous culture, phosphate limited</td>
<td>0.115</td>
<td>15</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>24.9</td>
<td>22.3</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>34.2</td>
<td>20.5</td>
</tr>
<tr>
<td>Nitrate limited</td>
<td>0.064</td>
<td>2.2</td>
<td>23.9</td>
</tr>
</tbody>
</table>

\(^a\) Enzyme activities are given as nanomoles of product formed per minute per milligram of protein at 30°C.
progressive increase in the activity of the enzyme to about 13 times that found at the start of the experiment was observed, so that its activity rose in parallel with the capacity of the cells to accumulate polyphosphate. After the readdition of phosphate, specific activity declined until the original value was reached about 40 h. The decrease in specific activity was most rapid in the first 8 h after phosphate addition and preceded the resumption of net protein synthesis, which was not observed until between 8 and 16 h. Again, the polyphosphatase activity of extracts did not change significantly during the whole course of the experiment.

The almost constant specific activity of polyphosphatase in all extracts appeared to be inconsistent with the markedly different stability of intracellular polyphosphate observed in the experiments described above. Possible ways in which existing potential enzyme activity could be regulated were therefore investigated. P$_i$ release from radioactively labeled polyphosphate by dialyzed extracts was inhibited by at most 15% by the addition of up to 20 mM P$_i$ to the assay mixture, showing the polyphosphatase was not regulated by variation in the P$_i$ pool within the cells. Although polyphosphatase showed saturation kinetics, maximum activity required high concentrations of substrate, and an apparent $K_m$ of 12 mM (expressed as acid-releasable P$_i$) was obtained from double-reciprocal plots. Even at polymer concentrations which supported only about one-third of the maximum rate, the time course of P$_i$ release was linear until 70 to 80% of the total polyphosphate had been hydrolyzed. This suggests that the substrate concentration was little changed during the course of the hydrolysis, as would be the case if the enzyme were an exophosphatase able to attack the polymer only at chain ends. This suggestion was supported by an experiment in which samples from an enzymatic digestion of $^{32}$P-labeled polyphosphate were removed and chromatographed on polyethylenimine at intervals until digestion was complete. Only radioactive material which stayed at the origin and an increasing amount of P$_i$ could be detected (data not shown).

**DISCUSSION**

Many microorganisms accumulate excess phosphate, generally in the form of polyphosphates, under special conditions (16). The major aims in the present investigation were to examine phosphate distribution in the phototrophic procaryote *Synechococcus* in balanced growth under a range of different conditions and to attempt to account for the findings in terms of three contributing cellular activities: phosphate uptake across the cell membrane, polyphosphate synthetase, and polyphosphatase.

Specific effects of phosphate restriction on the composition of cells grown in continuous culture at similar growth rates become obvious from comparisons between phosphate- and nitrate-limited cultures. Growth rate restriction affects the RNA content with either limitation, but is more obvious when phosphate is limiting. This suggests that ribosome content is less rigorously correlated with growth rate in *Synechococcus* than, for example, in *Escherichia coli* and *Salmonella typhimurium* (8, 30). A significant part of the total cell phosphate was found to be sequestered as polyphosphate even at the lowest specific growth rate studied. Cells from phosphate-restricted growth took up phosphate to well above the levels found in unrestricted cells when P$_i$ was added in excess, and polyphosphate accounted for about 60% of the total in the cells at the time when this total was at its peak. Measurements of individual activities showed that phosphate limitation specifically increased (by factors of 30 to 50 times) both the maximum rate at which phosphate was transported into the cells and the specific activity of polyphos-
phosphate synthetase in extracts in cells from steady states and in cells from unrestricted growth transferred to phosphate-free medium for periods of about 40 h. No change in polyphosphate synthetase activity occurred. An expected consequence under steady-state conditions would be that the efficiency with which cells could acquire phosphate from the medium would be raised, but that at the same time polyphosphate synthesis would compete more effectively with other, functionally more important, phosphate-utilizing reactions in such cells. The increased activities would also account for the rapid phosphate accumulation and polyphosphate formation seen when medium phosphate concentration was raised.

The high transport and polyphosphate synthetase activities characteristic of phosphate restriction were not maintained once Pi was resupplied, and both returned to values characteristic of unrestricted or nitrate-limited balanced cultures within about 6 to 8 h, that is, before significant net protein synthesis had occurred. This suggests either that there is specific inactivation of these cell components or that they have a quite rapid turnover under all conditions. In either case, these activities, like alkaline phosphatase (24) and glucose 6-phosphate dehydrogenase (33) activities, are clearly regulated in response to specific growth conditions.

Alkaline phosphatase was measured with other activities in this study (data not shown). With the exception that the specific enzyme activity appeared to decrease only after the resumption of net protein synthesis in refeeding experiments and could thus be accounted for primarily in terms of dilution of existing stable enzyme, the changes in alkaline phosphatase were parallel to those in transport and in polyphosphate synthetase. These activities thus appear to be coordinately regulated in wild-type cells of Synechococcus, as they are in Aerobacter aerogenes where, however, polyphosphatase is also included (15, 16). The potential for polyphosphate formation is not an essential characteristic (19), and mutants of Synechococcus in which this is absent have been described recently (44). A study of the other phosphate-related activities described in this paper in such strains would be of obvious help in understanding the genetic relationships. The precise nature of the intracellular signal which regulates the expression of these activities is also as yet unknown. Although the size of the intracellular Pi pool can indeed be varied over about a 10-fold range, it seems at least as probable that a more sensitive indicator metabolite, as yet unknown, may be involved, as Willsky et al. (47) have suggested for E. coli.

The almost unvarying activity of polyphosphatase found in these experiments differs from findings with other systems (15, 16, 27, 28). It was also unexpected since polyphosphate does not turn over during unrestricted growth and is not selectively mobilized during phosphate starvation. On the other hand, it is rapidly eliminated after excess formation has occurred after phosphate restriction; this breakdown becomes evident as soon as uptake and synthesis activities return to normal levels. In view of its kinetics, it is possible that substrate concentration may normally restrict the activity of this enzyme, which appears to be an exophosphatase, as it is in Corynebacterium xerosis (31). However, other explanations, such as a different location of the small amount of polyphosphate normally present and excess accumulation, can also be suggested.

Phosphate was transported as Pi against high concentration gradients by Synechococcus in an energy-dependent manner no matter what growth conditions were used. High affinity and energy dependence are in fact generally observed in a number of both procaryotic and eucaryotic microorganisms (1, 4, 6, 9, 17, 20, 21, 34, 38, 41), although the reported values for kinetic constants vary somewhat, and complex kinetics have been observed in multicompartmented eucaryotic cells (35, 39). The apparent $K_m$ for uptake measured here did not change, although $V_{max}$ could be increased by a factor of at least 30-fold by a history of phosphate restriction. This suggests, although it does not prove, that differential expression of a single system is involved. In E. coli, two phosphate transport systems, one inducible and one constitutive, are found (38, 47, 46); these differ also in kinetic constants and in arsenate sensitivity. Phosphate uptake in Synechococcus was virtually unaffected by a 100-fold excess of arsenate, and the cells were found to grow as well in 20 $\mu$M phosphate–50 mM arsenate as in phosphate alone, so that attempts to obtain mutants by using arsenate resistance were not successful.

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**LITERATURE CITED**


44. Vaillancourt, S., N. Beauchemin-Newhouse, and R.