JOURNAL OF BACTERIOLOGY, Mar. 1988, p. 1143–1147
0021-9193/88/031143-05$02.00/0

Vol. 170, No. 3

Phosphate Transport and Arsenate Resistance in the Cyanobacterium *Anabaena variabilis*

TERESA THIEL

Department of Biology, University of Missouri-St. Louis, St. Louis, Missouri 63121

Received 19 October 1987/Accepted 6 December 1987

Cells of the cyanobacterium *Anabaena variabilis* starved for phosphate for 3 days took up phosphate at about 100 times the rate of unstarved cells. Kinetic data suggested that a new transport system had been induced by starvation for phosphate. The inducible phosphate transport system was quickly repressed by addition of P_i. Phosphate-starved cells were more sensitive to the toxic effects of arsenate than were unstarved cells, but phosphate could alleviate some of the toxicity. Arsenate was a noncompetitive inhibitor of phosphate transport; however, the apparent K_i values were high, particularly for phosphate-replete cells. Preincubation of phosphate-starved cells with arsenate caused subsequent inhibition of phosphate transport, suggesting that intracellular arsenate inhibited phosphate transport. This effect was not seen in phosphate-replete cells.

P_i is the major form of phosphate that is taken up by phytoplankton, including cyanobacteria (13). It appears to be a primary nutrient element that limits freshwater algal growth (7, 20, 24, 25). Uptake of phosphate has been characterized in several strains of cyanobacteria; it is energy dependent (4, 8, 11, 12, 28) and shows saturation kinetics (8, 11, 12, 18, 27). The kinetic parameters for phosphate transport vary considerably among cyanobacterial strains; they are influenced by the composition of the medium (8, 9, 12, 23) and by the nutritional state of the cells (13). In particular, phosphorous-deficient cells have elevated rates of phosphate transport (3, 10–12, 14, 15, 17, 27). The rate of transport is related to the sizes of the internal and external pools of phosphate (10, 12, 14, 22). Phosphate that is taken up by phosphate-starved cells moves rapidly into polyphosphate (11, 19).

Arsenate is an analog of phosphate that is found in many aquatic habitats at concentrations similar to those of phosphate (2, 16, 26). Many cyanobacteria are resistant to concentrations of arsenate that are orders of magnitude greater than those found in natural waters (6, 21). In two strains of *Synechococcus* spp., which are very resistant to arsenate, the analog does not inhibit phosphate transport and is therefore probably not transported by the phosphate transport system (6, 11). *Synechococcus leopoliensis* is more sensitive to arsenite than to arsenate; arsenite interferes with photosynthesis and biosynthesis of amino acids (5).

Little is known for the cyanobacteria about the levels of arsenate resistance, the effect of starvation for phosphate on arsenate resistance, or the mechanism of resistance. In this study I have determined the kinetic parameters relating to the effects of arsenate both on phosphate transport and on growth for phosphate-starved and phosphate-replete cells of the cyanobacterium *Anabaena variabilis*.

MATERIALS AND METHODS

Growth. *A. variabilis* ATCC 29413 was grown in an eightfold dilution of the medium of Allen and Arnon (1) supplemented with 2.5 mM $NaNO_3$, 2.5 mM KNO_3, and 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (pH 7.2). This medium contained 0.33 mM K_2HPO_4, 0.12 mM $MgSO_4$, and 63 μM $CaCl_2$, as well as other nutrients not specifically relevant to phosphate transport. Cells to be starved of phosphate were grown in the same medium with the phosphate omitted. Cultures were incubated at 32°C on a reciprocal shaker at 100 rpm under cool white fluorescent lights at an intensity of approximately 60 microeinsteins m^{-2} s^{-1}. Growth was measured as the optical density at 700 nm.

Transport assay. Transport was measured with cells washed on a filter (mixed cellulose ester, 0.45-μm pore size; Millipore Corp.) four times with 50-ml volumes of phosphate-free medium and suspended in that medium at an optical density at 700 nm of 0.37 (2.2 or 1.7 μg of chlorophyll *a* ml^{-1} for unstarved or phosphate-starved cells, respectively) and warmed to 32°C. In all experiments, cells were incubated at 32°C with shaking under lights, as described above. Experiments were begun by the addition of 3.5 ml of warmed cells to appropriate amounts of $^{32}P_i$ (carrier free; ICN Pharmaceuticals, Inc.), nonradioactive sodium phosphate (pH 7.0), and, in some experiments, sodium arsenate, all in a volume of less than 100 μl. The specific activity of [^{32}P]phosphate was typically 0.5 Ci $mmol^{-1}$ for unstarved cells and 10- to 20-fold less for starved cells. Under these conditions, incorporation was linear for at least 10 min for unstarved cells and at least 5 min for starved cells. Triplicate 1-ml samples of cells were removed from incubation flasks after 1 min (starved cells) or 5 min (unstarved cells), added to 10 ml of 50 mM sodium phosphate (pH 7.0) in the cups of a vacuum filtration apparatus (Hoeffer Scientific Instruments), collected on Millipore filters (mixed cellulose ester, 0.45-μm pore size), and washed three times with 10-ml volumes of 10 mM sodium phosphate (pH 7.0). Dry weight was determined by collecting the cells in 25 ml of the culture used in the experiments on each of six preweighed filters, drying the filters for 3 days in a 70°C oven, and weighing the filters. The weights used for either starved or unstarved cells were 80 to 82 μg ml^{-1}.

Induction and repression of phosphate transport. Cells grown in medium containing 0.33 mM sodium phosphate were washed and suspended in phosphate-free medium. Immediately after being washed and at 24-h intervals, samples of phosphate-starved cells were assayed for phosphate transport at 2.0 and 10 μM phosphate, as described above. Repression of phosphate transport was measured in cells that had been deprived of phosphate for 3 days before the experiment. These phosphate-starved cells were washed in phosphate-free medium and suspended in medium contain-

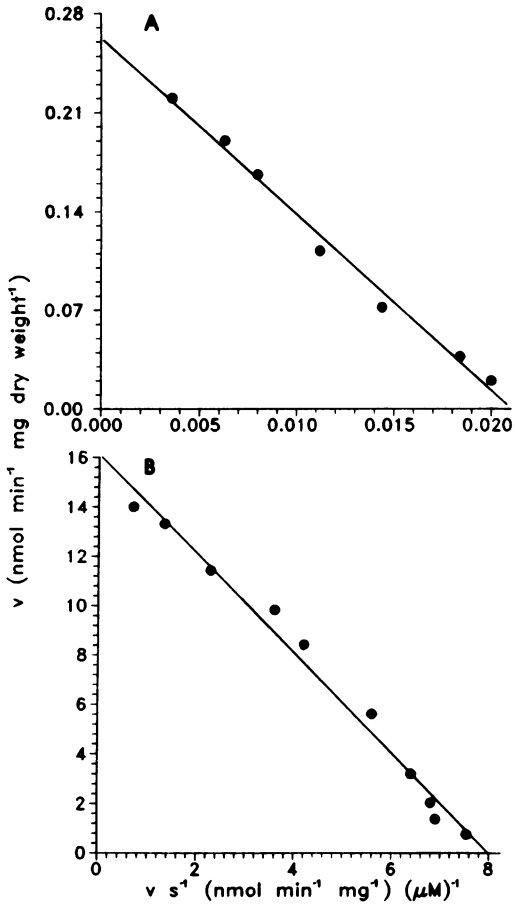

FIG. 1. Hofstee plots for the transport of phosphate in cells grown in medium with 0.33 mM phosphate (A) and in cells grown for 3 days in phosphate-free medium (B). All cells were washed in medium lacking phosphate, and transport of phosphate was measured. Points are the means of at least six determinations of mean uptake velocity (v) for triplicate samples at each concentration of substrate.

ing 100 μM phosphate. Phosphate transport, at substrate concentrations of 2.0 and 10 μM, was measured in samples of cells (washed in phosphate-free medium before the assay) immediately after the addition of 100 μM phosphate and at various intervals up to 48 h after the addition.

Preincubation with arsenate. Unstarved cells and cells cultured for 3 days in the absence of phosphate were harvested, washed and suspended in phosphate-free medium as described above, and warmed to 32°C. Phosphate or arsenate was added to the culture, which was incubated at 32°C for 10 min. A 3.5-ml portion was rapidly collected on a filter and washed six times with 10-ml volumes of phosphate-free medium. The filter containing the washed cells was placed in 3.5 ml of medium with the appropriate amount of phosphate, warmed to 32°C.

RESULTS

Kinetics of phosphate transport. For cells not starved for phosphate, the transport of phosphate showed Michaelis-Menten kinetics, and there was no kinetic evidence for multiple transport systems (Fig. 1A). The K_m for P_i transport was 12 μM, and V_{max} was 0.26 nmol min^{-1} mg (dry weight)$^{-1}$. Starvation of cells for phosphate greatly increased the rate of phosphate transport. Within 48 h after removal of phosphate from the medium, the velocity of transport increased about 100-fold (Fig. 2). For cells starved of phosphate for 3 days, the transport of phosphate still showed Michaelis-Menten kinetics, with a K_m of 2.2 μM and a V_{max} of 18 nmol min^{-1} mg (dry weight)$^{-1}$ (Fig. 1B). Thus, starvation induced a phosphate transport system that completely masked the lower-affinity, low-velocity transport of phosphate by unstarved cells.

Although full induction of the new transport system required 2 to 3 days, repression of the high-velocity system occurred relatively quickly. Addition of 100 μM phosphate to phosphate-starved cells caused a 10-fold reduction in the velocity of transport within 2 h (Fig. 2B). The velocity of transport decreased to nearly that of unstarved cells by 12 h after the addition of phosphate. The addition of chloramphenicol 30 min before and during incubation with phosphate had no effect on this repression, suggesting that protein synthesis is not necessary for inactivation of this transport system (data not shown).

Arsenate resistance and phosphate starvation. Cells previously grown in phosphate-replete medium were resistant to

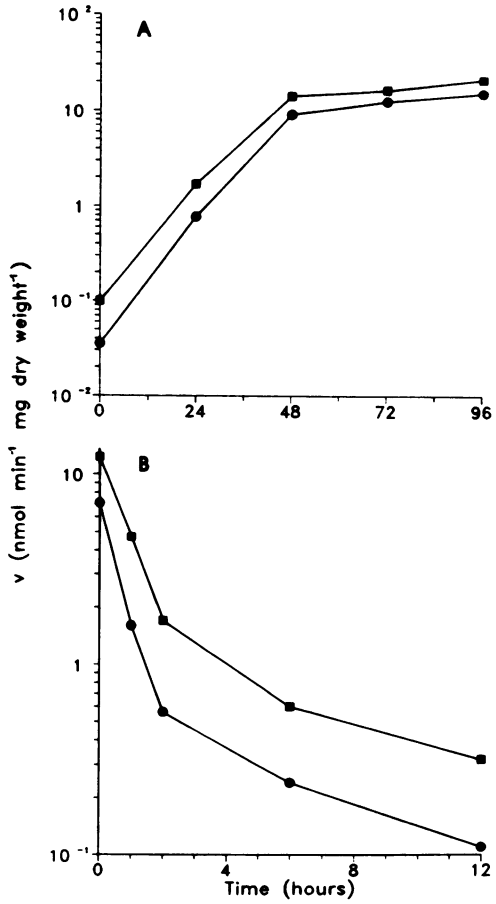

FIG. 2. Induction and repression of phosphate transport. The mean uptake velocity (v) for phosphate was measured for cells that had been grown in medium with 0.33 mM phosphate and then washed and inoculated at the start of the experiment into medium lacking phosphate (A) and for cells that had been grown for 3 days in phosphate-free medium, washed in the same medium, and inoculated into medium containing 100 μM phosphate (B). Points are the means of three determinations of triplicate samples. Phosphate uptake was measured with 2 (●) or 10 (■) μM phosphate.

the toxic effects of arsenate when phosphate was present in the medium (Table 1). These cells were somewhat more sensitive to arsenate in the absence of phosphate (Table 1); however, under these conditions the cells were in fact becoming phosphate starved. The growth of cells previously starved for phosphate was more strongly inhibited by arsenate, but that inhibition was relieved by the addition of phosphate to the medium (Table 1). Both starved and unstarved cells grew very poorly or not at all at concentrations of arsenate exceeding 100 mM (data not shown).

Kinetics of inhibition. The kinetics of inhibition of phosphate transport by arsenate indicated that for unstarved cells, arsenate was a poor noncompetitive inhibitor of phosphate transport, with a K_i of about 1.1 mM (Fig. 3A). In cells starved 3 days for phosphate, inhibition again appeared to be almost completely noncompetitive, with a K_i of about 75 μM; however, there was some suggestion of mixed inhibition (Fig. 3B). There may have been weak competitive inhibition that was masked by the noncompetitive inhibition. At various concentrations of phosphate up to 10 times the K_m for phosphate transport, the ratio of the mean uptake velocity in the presence of arsenate (provided at a concentration of approximately the K_i) to the mean uptake velocity in the absence of arsenate was a constant (data not shown). This was true for starved as well as unstarved cells. Thus, arsenate did not appear to compete significantly with phosphate for transport by either of the phosphate transport systems described here.

Preincubation with arsenate. To determine whether the noncompetitive inhibition of phosphate transport required entry of arsenate into the cell, the effect of preincubation of cells with arsenate on subsequent phosphate transport was determined. Cells were preincubated with arsenate in the presence or absence of various concentrations of phosphate, washed free of arsenate and phosphate, and examined for their phosphate transport capacity. For cells grown in phosphate-replete medium (unstarved cells), preincubation with arsenate did not inhibit subsequent phosphate transport (Table 2). In starved cells preincubated with 100 μM arsenate and 100 μM phosphate, the subsequent transport of phosphate was inhibited more than 80% (Table 2). At higher concentrations of arsenate or lower concentrations of phosphate, transport was inhibited 90 to 95% (Table 2). Similar results were obtained with phosphate concentrations of 25, 50, and 75 μM in the preincubation medium (data not shown). Since phosphate concentrations as high as 100 μM in the preincubation medium had only a slight effect on subsequent phosphate transport after washing of the cells (and that was expected [Fig. 2B]), it seemed unlikely that residual phosphate or arsenate, not removed by washing, could have inhibited transport. In similar experiments the transport of [^{14}C]leucine was not inhibited by preincubation of phosphate-starved cells with 100 or 200 μM arsenate (data not shown). The data suggest that the inhibitory effect of arsenate introduced by preincubation was a result of accumulated arsenate within the cell. This inhibition was not a general effect of arsenate on energy-requiring membrane functions, since the transport of leucine was unaffected.

TABLE 1. Growth of *A. variabilis* with arsenate

Concn (mM) of supplement		Generation time (h) for[a]:	
Arsenate	Phosphate	Unstarved cells[b]	Starved cells[c]
0	0	18.5 ± 2.3	17.4 ± 1.7
0	0.33	18.2 ± 1.2	16.0 ± 2.0
25	0	31.2 ± 1.2	31.8 ± 3.9
25	0.33	24.5 ± 3.8	23.7 ± 2.4
50	0	31.5 ± 2.8	56.4 ± 5.5
50	0.33	21.5 ± 1.7	27.6 ± 1.4
75	0	31.2 ± 1.5	>72
75	0.33	21.1 ± 3.7	31.7 ± 4.1

[a] Generation times are the means ± standard deviations for three experiments in which the generation time for cells in each flask was calculated as the average of three determinations made 24 h apart. Generation times were calculated from the change in A_{700} versus time for cells in exponential growth.

[b] Unstarved cells were grown in medium with 0.33 mM phosphate before being washed in the same medium without phosphate and inoculated into the appropriate media.

[c] Starved cells were grown and washed in medium lacking phosphate for 72 h before inoculation into the appropriate media.

DISCUSSION

A. variabilis cells replete with phosphate took up phosphate at a relatively low rate. As has been demonstrated by others (3, 10–12, 14, 15, 17, 27), starvation of cells for

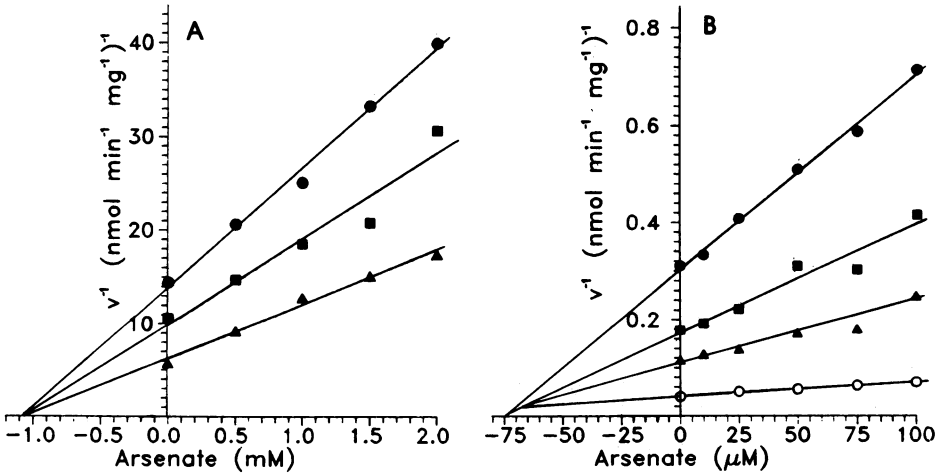

FIG. 3. Dixon plots for inhibition of phosphate transport by arsenate. Points are the means of four to six determinations of mean uptake velocity (v). (A) Unstarved cells, as described in the legend to Fig. 1A, with 5 (●), 10 (■), or 20 (▲) μM phosphate. (B) Starved cells, as described in the legend to Fig. 1B, with 0.5 (●), 1 (■), 2 (▲), or 5 (○) μM phosphate.

TABLE 2. Effect of preincubation of cells with arsenate on the transport of phosphate

Preincubation medium concn (μM) of[a]:		v for[b]:	
Phosphate	Arsenate	Starved cells	Unstarved cells
0	0	11.3	0.125
0	100	0.8	0.135
0	200	0.5	0.113
10	0	10.9	ND[c]
10	100	0.7	ND
10	200	0.6	ND
100	0	9.1	ND
100	100	1.4	ND

[a] Cells, unstarved or starved for phosphate for 3 days before the experiments, were incubated at 32°C in the light in medium containing phosphate and arsenate, as indicated, for 10 min before being washed rapidly and suspended in medium with [^{32}P]phosphate for assay of phosphate transport.

[b] The mean uptake velocities, v (nanomoles per minute per milligram [dry weight]), for three samples were measured at a substrate concentration of 2.0 μM for starved cells and 10 μM for unstarved cells.

[c] ND, Not determined.

phosphate increased the velocity of transport. In this organism, the increase was about 100-fold by 3 days after the onset of growth in the absence of exogenous phosphate. The maximum increase reported for other cyanobacteria is about 40-fold for *Synechococcus* sp. (11). The large increase in the velocity of phosphate transport for phosphate-starved cells of *A. variabilis* was associated with both an increase in V_{max} and a decrease in K_m compared with these values for transport in cells not starved for phosphate. Such changes suggest induction of a phosphate transport system distinct from that present in membranes of phosphate-replete cells. Although it is likely that both transport systems function in starved cells, the inducible system, which had a higher affinity and a faster rate of transport, would have masked the system that is present in unstarved cells. The induction of this relatively high-affinity system was slow, requiring two to three generations for full expression. In contrast, this system was repressed within a few hours after the addition of phosphate to the medium, and the repression did not require protein synthesis. Although multiple transport systems for phosphate have been reported for *Anacystis nidulans* (28), a system that is specifically induced by phosphate starvation has not been reported previously for cyanobacteria.

A. variabilis was very resistant to the toxic effects of arsenate, as are other cyanobacteria (6, 21). Concentrations of arsenate in the 10 to 100 mM range had a toxic effect, but at the lower arsenate concentrations, only cells previously starved for phosphate and then grown with arsenate in the absence of phosphate were particularly sensitive. The ability of phosphate to protect cells from the toxic effects of arsenate may be attributable to competition between phosphate and arsenate in metabolic pathways rather than to competition for transport. Others have reported that arsenate did not compete with phosphate for transport into *Synechococcus* spp. However, they did not try concentrations of arsenate higher than 100 or 200 μM (6, 11). Arsenate did inhibit transport of phosphate into *A. variabilis* (Fig. 3); however, the K_i was high, particularly for unstarved cells, and the inhibition appears to be noncompetitive. In phosphate-starved cells, which were more sensitive to arsenate than unstarved cells, arsenate inhibited phosphate transport with a lower K_i, but the inhibition was again noncompetitive.

Physiological studies suggested that arsenate could enter cells, particularly phosphate-starved cells, and it has been shown that arsenate is taken up, although very poorly, by *S. leopoliensis* (6). The fact that phosphate could protect starved cells from some of the toxicity of arsenate suggested either that phosphate prevented entry of arsenate into the cells or that once in the cells, arsenate caused little damage when phosphate was also present (perhaps to compete with arsenate). The kinetic evidence indicated that phosphate and arsenate did not compete for entry into the cell, yet arsenate did inhibit phosphate uptake. Arsenate could have affected phosphate uptake from inside the cell; preincubation of phosphate-starved cells with arsenate dramatically reduced phosphate transport. This inhibition was specific to starved cells; unstarved cells were unaffected by preincubation with arsenate. This suggests that the transport systems of unstarved and phosphate-starved cells may involve different proteins. Although phosphate itself inhibited subsequent phosphate transport by starved cells, this inhibition was slow compared with the rapid inhibition caused by preincubation of the cells with arsenate (compare Fig. 2B with Table 2). In addition, phosphate could not prevent the uptake of arsenate by starved cells, confirming that arsenate did not enter the cell by the major phosphate transport system of phosphate-starved cells. It is not clear how arsenate entered phosphate-starved cells or whether starvation for phosphate was necessary for transport or arsenate.

The fact that arsenate was transported into starved cells suggests that phosphate protection against arsenate toxicity may be mediated from within the cell. It is possible that in such highly resistant organisms, the mechanism of arsenate resistance includes both selective transport systems that exclude arsenate and intracellular metabolic pathways in which arsenate is a poor competitor for phosphate, provided that phosphate is present.

ACKNOWLEDGMENTS

I am grateful to Jane Starling for stimulating and helpful discussions and to her and Simon Silver for reading the manuscript. I thank Sheri Bonar for excellent technical assistance and Paul Neary for help with preliminary experiments.

This research was supported by National Science Foundation grant DCB-8508542.

LITERATURE CITED

1. **Allen, M. B., and D. I. Arnon.** 1955. Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiol. **30**:366–372.
2. **Andreae, M. O.** 1978. Distribution and speciation of arsenic in natural waters and some marine algae. Deep Sea Res. **25**:391–402.
3. **Batterton, J. C., and C. van Baalen.** 1968. Phosphorus deficiency and phosphate uptake in the blue-green alga *Anacystis nidulans*. Can. J. Microbiol. **14**:341–348.
4. **Bornefeld, T., J. Lee-Kaden, and W. Simonis.** 1974. Lichtabhangige Phosphataufnahme und Metabolismus bei *Anacystis nidulans*. Ber. Dtsch. Bot. Ges. **87**:493–500.
5. **Budd, K., R. Casey, and J. D. MacArthur.** 1986. Arsenite toxicity and arsenite tolerance in the cyanobacterium *Synechococcus leopoliensis*. Can. J. Bot. **64**:2433–2440.
6. **Budd, K., and S. R. Craig.** 1981. Resistance to arsenate toxicity in the blue-green alga *Synechococcus leopoliensis*. Can. J. Bot. **59**:1518–1521.
7. **Dillon, P. J., and F. H. Rigler.** 1974. The phosphorus-chlorophyll relationship in lakes. Limnol. Oceanogr. **19**:767–773.
8. **Falkner, G., K. Werdan, F. Horner, and H. W. Heldt.** 1974. Energieabhangige Phosphataufnahme der Blaualge *Anacystis*

nidulans. Ber. Dtsch. Bot. Ges. **87**:263–266.
9. **Falkner, G., K. Werdan, F. Horner, and H. W. Heldt.** 1976. Energieabhangige Phosphataufnahme der Blaualge *Anacystis nidulans*. Ber. Dtsch. Bot. Ges. **89**:285–288.
10. **Gotham, I. J., and G.-Y. Rhee.** 1981. Comparative kinetic studies of phosphate-limited growth and phosphate uptake in phytoplankton in continuous culture. J. Phycol. **17**:257–265.
11. **Grillo, J. F., and J. Gibson.** 1979. Regulation of phosphate accumulation in the unicellular cyanobacterium *Synechococcus*. J. Bacteriol. **140**:508–517.
12. **Healey, F. P.** 1973. Characteristics of phosphorus deficiency in *Anabaena*. J. Phycol. **9**:383–394.
13. **Healey, F. P.** 1982. Phosphate, p. 105–124. *In* N. G. Carr and B. A. Whitton (ed.), The biology of cyanobacteria. University of California Press, Berkeley.
14. **Healey, F. P., and L. L. Hendzel.** 1975. Effects of phosphorus deficiency in two algae growing in chemostats. J. Phycol. **11**: 303–309.
15. **Healey, F. P., and L. L. Hendzel.** 1979. Indicators of phosphorus and nitrogen deficiency in five algae in culture. J. Fish. Res. Board Can. **36**:1364–1369.
16. **Johnson, D. L.** 1971. Simultaneous determination of arsenate and phosphate in natural waters. Environ. Sci. Technol. **5**:411–414.
17. **Lawry, N. H., and T. E. Jensen.** 1979. Deposition of condensed phosphate as an effect of varying sulfur deficiency in the cyanobacterium *Synechococcus* sp. (*Anacystis nidulans*). Arch. Microbiol. **120**:1–7.
18. **McCarthy, J. J., and E. J. Carpenter.** 1979. *Oscillatoria* (*Trichodesmium*) *thiebautii* (Cyanophyta) in the central North Atlantic Ocean. J. Phycol. **15**:75–82.
19. **Niemeyer, R., and G. Richter.** 1969. Schnellmarkierte Polyphosphate und Metaphosphate bei der Blaualge *Anacystis nidulans*. Arch. Mikrobiol. **69**:54–59.
20. **Owens, O. V. H., and W. E. Esaias.** 1976. Physiological responses of phytoplankton to major environmental factors. Annu. Rev. Plant Physiol. **27**:461–483.
21. **Planas, D., and F. P. Healey.** 1978. Effects of arsenate on growth and phosphorus metabolism of phytoplankton. J. Phycol. **14**: 337–341.
22. **Rhee, G.-Y.** 1973. A continuous culture study of phosphate uptake, growth rate and polyphosphates in *Scenedesmus* sp. J. Phycol. **9**:495–506.
23. **Rigby, C. H., S. R. Craig, and K. Budd.** 1980. Phosphate uptake by *Synechococcus leopoliensis* (Cyanophyceae): enhancement by calcium ion. J. Phycol. **16**:389–393.
24. **Schindler, D. W.** 1974. Eutrophication and recovery in experimental lakes: implications for lake management. Science **184**: 897–899.
25. **Schindler, D. W.** 1978. Factors regulating phytoplankton production and standing crops in the world's freshwaters. Limnol. Oceanogr. **23**:478–486.
26. **Seydel, I. S.** 1972. Distribution and circulation of arsenic through water, organisms and sediment of Lake Michigan. Arch. Hydrobiol. **71**:17–30.
27. **Sicko-Goad, L., T. E. Jensen, and R. P. Ayala.** 1978. Phosphate metabolism in blue-green bacteria. V. Factors affecting phosphate uptake in *Plectonema boryanum*. Can. J. Microbiol. **24**: 105–108.
28. **Simonis, W., T. Bornefeld, J. Lee-Kaden, and K. Majumdar.** 1974. Phosphate uptake and photophosphorylation in the blue-green alga *Anacystis nidulans*. p. 220–225. *In* U. Zimmerman and J. Dainty (ed.), Membrane transport in plants. Springer-Verlag, New York.