Arch. Microbiol. 120, 1–7 (1979)

Archives of
Microbiology
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Deposition of Condensed Phosphate as an Effect of Varying Sulfur Deficiency in the Cyanobacterium *Synechococcus* sp. (*Anacystis nidulans*)

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Abstract. Uptake of orthophosphate and deposition of condensed phosphate were investigated in cells of *Synechococcus* sp. (*Anacystis nidulans*) deficient in phosphorus or sulfur. When phosphorus was restored to phosphorus-starved cells, uptake was rapid and immediate, with the greatest accumulation occurring within the first hour. Uptake was optimum in the pH 7.5–8.5 range. Long-term (6-day) studies of uptake and deposition with cells exposed to a wide range of sulfur deficiency showed that both processes were greatest when the level of exogenous sulfur was reduced to zero. The increase in cellular phosphorus as determined chemically was in agreement with the increased number and size of polyphosphate bodies at the ultrastructural level. Possible mechanisms for the control of phosphorus uptake and condensed phosphate formation by exogenous sulfur are discussed.

Key words: Condensed phosphate – Polyphosphate bodies – Sulfur deficiency – Cyanobacteria – *Synechococcus* – *Anacystis nidulans*.

The deposition of condensed phosphates, most often in the form of linear polyphosphate, is especially prevalent in prokaryotic organisms. Such deposition is generally promoted when the organism's growth is less than vigorous, e.g. during the stationary or plateau phase of normal growth, or when its metabolism reflects a nutrient deficiency or imbalance (Liss and Langen, 1962). Sulfur deprivation has long been known as a prime effector of increased phosphate uptake and polyphosphate deposition in certain heterotrophic bacteria (Smith et al., 1954). Although polyphosphate localizations occur commonly in blue-green bacteria (Drews and Niklowitz, 1956, 1957; Ebel et al., 1958; Jensen, 1968, 1969), the effect of varying degrees of sulfur deficiency on orthophosphate uptake and condensed phosphate deposition has not been demonstrated previously in any of these predominantly obligate photoautotrophs.

Materials and Methods

Anacystis nidulans 625 was obtained from the Indiana University Collection of Algae (now at the University of Texas at Austin) and maintained on Allen's modification of Hughes' medium (Allen, 1968), buffered at pH 8.3 with 1.0 mg/l glycylglycine. Cultures were grown at 39° C under 2600 lux cool white fluorescent illumination supplemented by a 25 W incandescent bulb, on a 16-h light/8-h dark cycle. Both stock and experimental flasks were aerated through cotton-plugged Pasteur pipettes.

Exogenous orthophosphate concentrations were determined colorimetrically by the Murphy-Riley method (Murphy and Riley, 1962; Hayashi, 1976). Concentrations of total cellular phosphorus were measured by autoclaving thrice-washed aliquots of cells with 0.5 g $K_2S_2O_8$ (Batterton and Van Baalen, 1968), then assaying the cooled solutions by the Murphy-Riley technique, and expressing the amount determined on a per-cell basis. Cell numbers were obtained with a Petroff-Hauser counting chamber.

Short-Term Uptake. Cells one week old were washed three times in phosphorus-free medium, then grown for 6 days in P-free medium as described. On day 6 the cells were again washed three times in P-free medium, and resuspended at a density of ca. 94×10^6 cells/ml in medium containing 5 mg/l (0.16 mM) phosphorus as orthophosphate, and buffered variously at pH 7.0, 7.5, 8.0, 8.5, and 9.0. Illumination was increased to 4800 lux. The first samples (0 h) were drawn as quickly as possible after all flasks were inoculated, and thereafter at periodic intervals over a period of 6 h.

Long-Term Uptake. Cells 1 week old were washed four times in sulfur-free medium and resuspended at a density of $28-29 \times 10^6$ cells/cc in cultures containing 10 mg/l (0.32 mM) phosphorus as orthophosphate, and 10, 1.0, 0.1 (0.31 mM, 31 μM, 3.1 μM), and 0.0 mg/l sulfur as sulfate. Appropriate amounts of $Mg(NO_3)_2$ were

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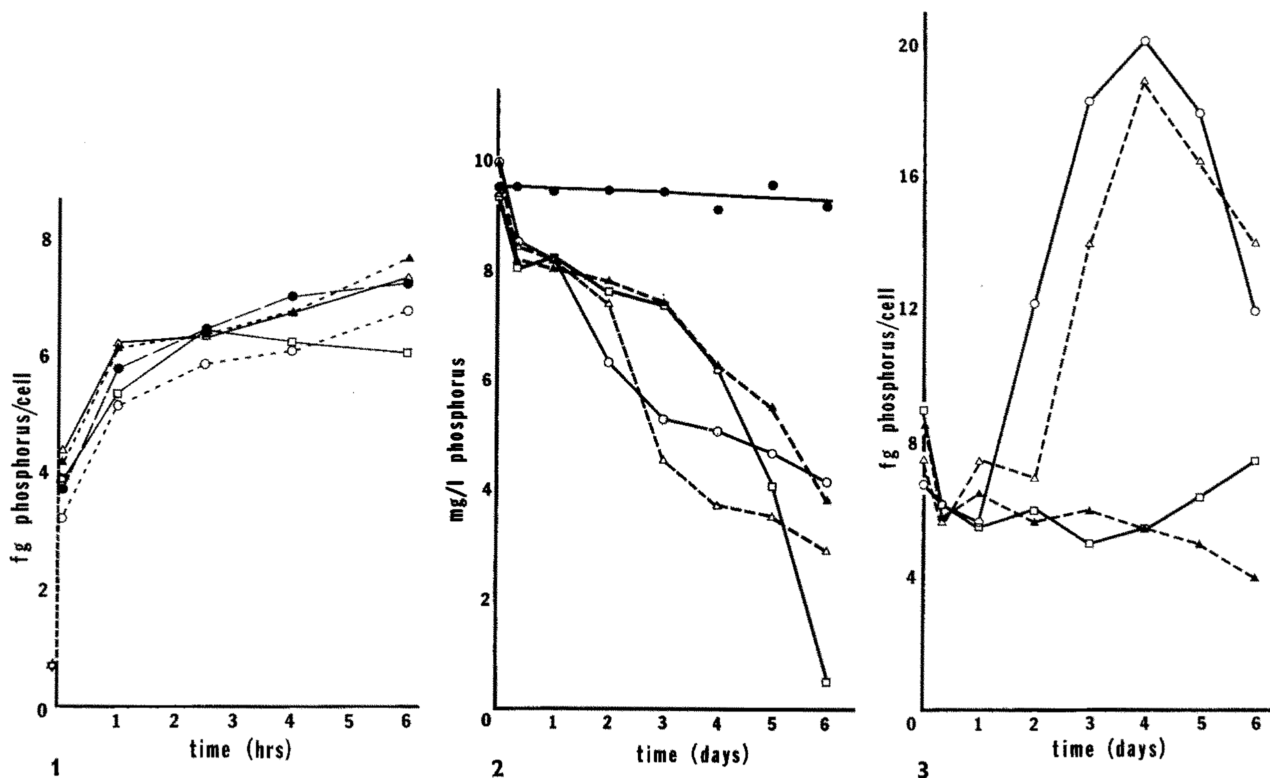

Fig. 1. Increases in cellular phosphorus by P-depleted cells undergoing rapid uptake when resuspended in medium containing 5 mg/l (0.16 mM) phosphorus as orthophosphate. Phosphorus concentration of inoculum cells previously maintained on zero exogenous phosphorus for 6 days (*); medium with restored phosphorus variously adjusted to pH 7.0 (○), pH 7.5 (●), pH 8.0 (△), pH 8.5 (▲), and pH 9.0 (□)

Fig. 2. Disappearance of 10 mg/l (0.32 mM) exogenous phosphorus in cultures of growing cells. Initial concentrations of exogenous sulfur were zero (○), 3.1 µM (△), 31 µM (□), 0.31 mM (▲), and 0.31 mM without cells (●)

Fig. 3. Changes in cellular phosphorus by cells growing on an initial 10 mg/l (0.32 mM) exogenous phosphorus. Initial concentrations of exogenous sulfur were zero (○), 3.1 µM (△), 31 µM (□), and 0.31 mM (▲)

added to correct for magnesium loss. Exogenous orthophosphate and cellular phosphorus were measured after the first 8 h of growth, then daily as described previously. Aliquot volumes necessary to provide readings within the range of precision decreased with time as cell numbers and/or cellular phosphorus increased. An air-bubbled, cell-free flask was provided to measure orthophosphate concentration change due to long-standing. Illumination was 2600 lux in 16-h periods interrupted by 8-h periods of darkness. Cells were removed after 47 and 120 h of growth and fixed for transmission electron microscopy.

Electron Microscopy. Suspensions of cells were mixed with a few drops of 2% osmium tetraoxide and spun down for 15 min with a clinical centrifuge at full speed. The cells were resuspended in 1% OsO_4 in pH 8.0 Michaelis buffer and stored at 4° C for 1 h, and a further 105 min at room temperature. After fixation the cells were resuspended in 1% aqueous uranyl acetate in buffer for 15 min, dehydrated in a graded ethyl alcohol-propylene oxide series, and embedded in Epon 812 essentially according to Luft (1961). Thin sections were cut on a Dupont diamond knife with an LKB Ultrotome III, post-stained with methanolic uranyl acetate (Stempak and Ward, 1964) and lead citrate (Venable and Coggeshall, 1965), and examined under an Hitachi HU-11E transmission electron microscope at 75 kv. Thick sections (≤250 um) were examined at 100 kv.

Results

The rapid increase in cellular phosphorus in cells previously starved for this element is shown in Fig. 1 over a 100-fold range in hydrogen ion activity. When the phosphorus-depleted cells were exposed to 0.16 mM exogenous phosphorus, their cellular levels of phosphorus increased 5–6 times their previous values in the time required to draw and centrifuge the first samples. Such rapid uptake was largely complete within 1 h, and the rates slowed substantially but continued thereafter in most cases. Optimum uptake was achieved in the pH 7.5–8.5 range, with the maximum somewhere near pH 8.5. Beyond that pH there was a decline in uptake after the initial 2–3 h, and final (6-h) cellular accumulation at pH 9.0 was 20% less than that at pH 8.5. In a second experiment (results not shown) cells deprived of exogenous phosphorus for 6 days were resuspended in medium containing 0.06 mM phosphorus, and exhibited an immediate increase in cellular phosphorus to

ca. four times that of the starved level. The amount of orthophosphate taken up very rapidly thus appears to be in part a function of the exogenous concentration of that anion.

Daily levels of exogenous phosphorus in cultures of growing cells are shown in Fig. 2. In the case of 1 mg/l (31 μM) sulfur initial concentration the loss of phosphorus from the growth medium was almost complete by day 6. Rates of loss increased after day 1 in cultures with 0.1 mg/l (3.1 μM) and zero sulfur initial concentrations, but these rates declined after day 3. Figure 3 represents the cellular phosphorus levels over the 6-day period. In the cases of cells grown on 3.1 μM and zero sulfur initial concentrations the increases were precipitous and reached maxima of three times those of the original cellular levels of phosphorus. Marked decreases in cellular phosphorus were manifested by both populations after the 4th day. The phosphorus level of cells grown on 10 mg/l (0.31 mM) sulfur initial concentration decreased slowly and steadily over the 6-day period. In descending order of initial exogenous sulfur concentration, final cell densities were: 950 $\times 10^6$, 925×10^6, 375×10^6, and 287.5×10^6 cells/ml.

After 47 h of growth only the cells provided with no exogenous sulfur exhibited any substantial cytoplasmic deterioration, including among other effects a decrease in the number of thylakoids and an enlargement of polyphosphate granules. This culture was already becoming chlorotic at that time. The cells grown on 3.1 μM sulfur initial concentration appeared only slightly affected in regard to the size and number of polyphosphate bodies, and those grown on ten and one hundred times more exogenous sulfur were quite normal in appearance. Figures 4 – 8 reveal that all sulfur concentrations tested but the highest led to increased phosphate uptake and deposition by 120 h. At this time the cells cultured on the two lowest concentrations of sulfur (zero and 3.1 μM) were chlorotic. The mean diameter of polyphosphate bodies in healthy cells is very near 200 nm. Since these inclusions tend increasingly to be lost from thin sections as they increase in size (Jensen, 1968), the number of spaces seen in fields of sectioned cells significantly increases (Fig. 4). The intact polyphosphate bodies of sulfur-starved cells are better visualized in thick sections (Fig. 8), and their mean diameter can be seen to lie in the 400 – 500 nm range after 120 h.

Energy dispersive x-ray microanalysis of thick sections of air-dried cells cultured on 0.31 mM sulfur as sulfate and zero sulfur has indicated the prevalent elements present in polyphosphate bodies of *Synechococcus* sp. are phosphorus and calcium, similar to results obtained with *Plectonema boryanum* (Sicko-Goad et al., 1975). This work will be reported elsewhere.

Earlier workers have suggested the association of nucleic acid fibrils with the polyphosphate (Voelz et al., 1966; Jensen and Sicko, 1974). Such an association is particularly well shown in Fig. 9. Within most of the polyhedral bodies seen in thin sections are minibodies which have been reported to be polyphosphate by Jensen and Sicko (1974). Of particular interest is the doubling or tripling in diameter (from 8 – 10 to 23 nm) of the minibodies as the effects of sulfur starvation become manifest.

Discussion

In the long-term study the concurrent loss of orthophosphate from the growth medium and the decline in cellular phosphorus levels in the first 8 h were effects of rapid cell division. As sulfur became progressively limiting in three of the four cultures, it was reflected by increases in both cellular phosphorus levels and exogenous phosphorus loss. After sudden and rapid increases in cellular phosphorus by the two most sulfur-limited populations, the sharp declines thereafter resulted from slow increases in cell number with accompanying decreases in phosphorus uptake.

That exogenous phosphorus loss is not only a function of cell number, but also of sulfur deficiency is best revealed by the culture provided with 31 μM sulfur. This concentration was not limiting until about day 4, when the cell number of the population had well surpassed those of the two previously sulfur-deficient populations (initial concentrations zero and 3.1 μM sulfur). Thus the large number of now-sulfur-depleted cells rapidly removed most of the remaining exogenous phosphorus. The same degree of exogenous orthophosphate loss did not occur with cells cultured on ten times greater exogenous sulfur (0.31 mM), although cell numbers of the two populations were very nearly the same.

In microorganisms a reciprocity of uptake/assimilation often exists between the major anions orthophosphate and sulfate. Levels of the different sulfur fractions increased in phosphorus-deficient cells of the green alga *Scenedesmus* (Kylin, 1964 a, b), and in cells of the cyanobacterium *Microcystis aeruginosa* an absence of one anion led to a measurable increase in the incorporation of the other (Volodin, 1970). The discovery that sulfur deficiency in cells of *Enterobacter aerogenes* increases polyphosphate content was made by Smith et al. (1954). The phenomenon was further investigated by Harold (1963) and Harold and Sylvan (1963) in *E. aerogenes*, and by Pine (1963) in *Escherichia coli*. From the substantial increases in size and number of polyphosphate granules revealed in the present study, it is concluded that cells of the photo-

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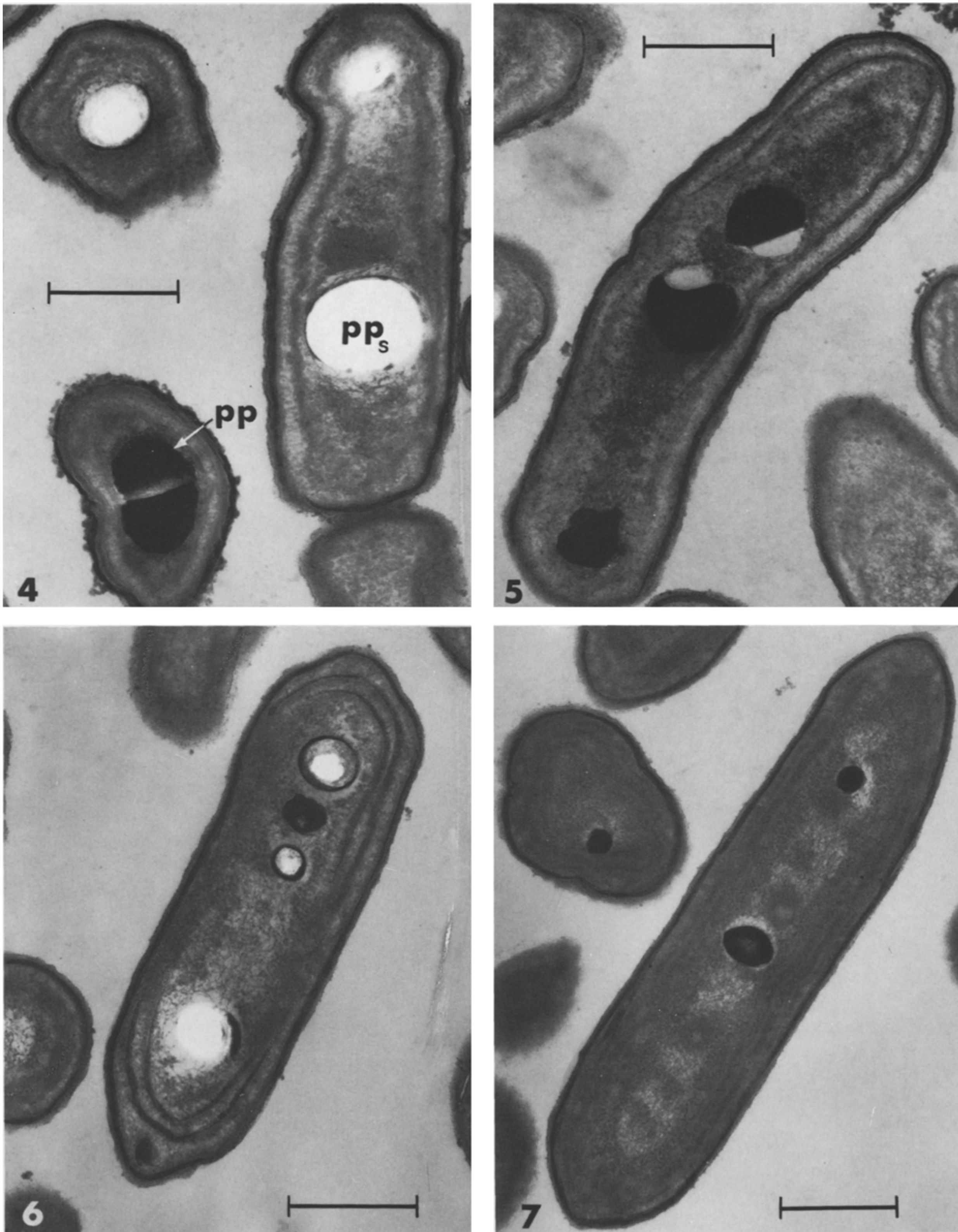
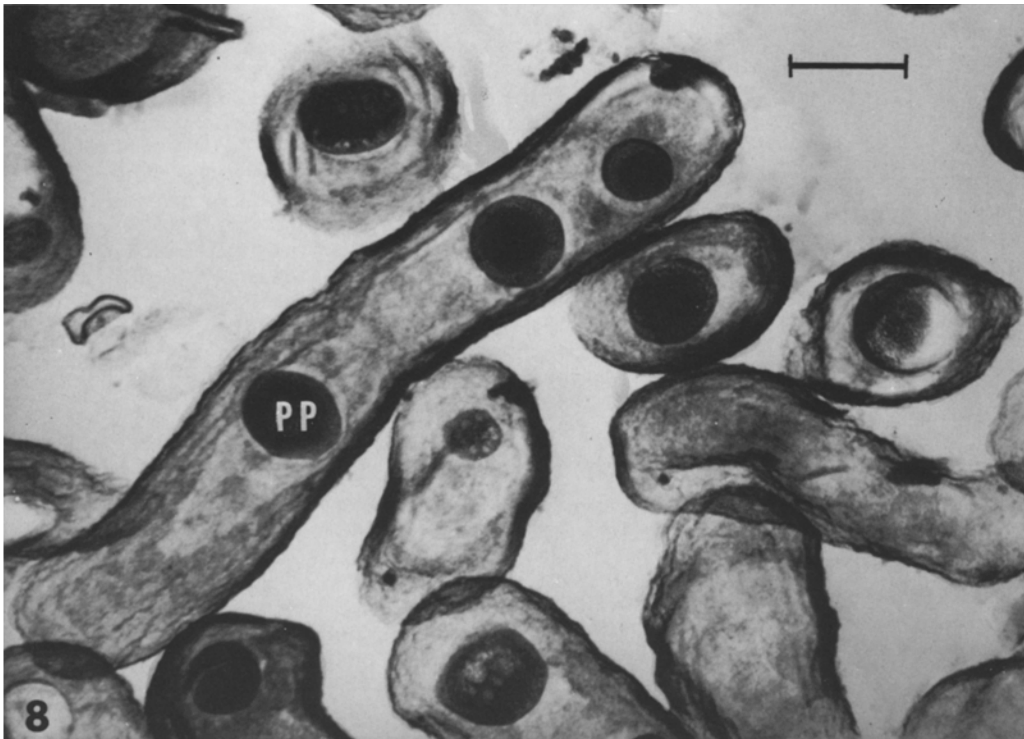


Figs. 4–7. Thin sections of cells grown for 120 h in medium containing 0.32 mM phosphorus as orthophosphate, but varying amounts of exogenous sulfur as sulfate, indicate that the sulfur has become limiting in all concentrations but the highest. Polyphosphate bodies (*pp*) or their remnant spaces (pp_s) are prevalent. All marker bars are equivalent to 500 nm. **Fig. 4.** Zero exogenous sulfur. **Fig. 5.** 3.1 μM sulfur. **Fig. 6.** 31 μM sulfur. **Fig. 7.** 0.31 mM sulfur



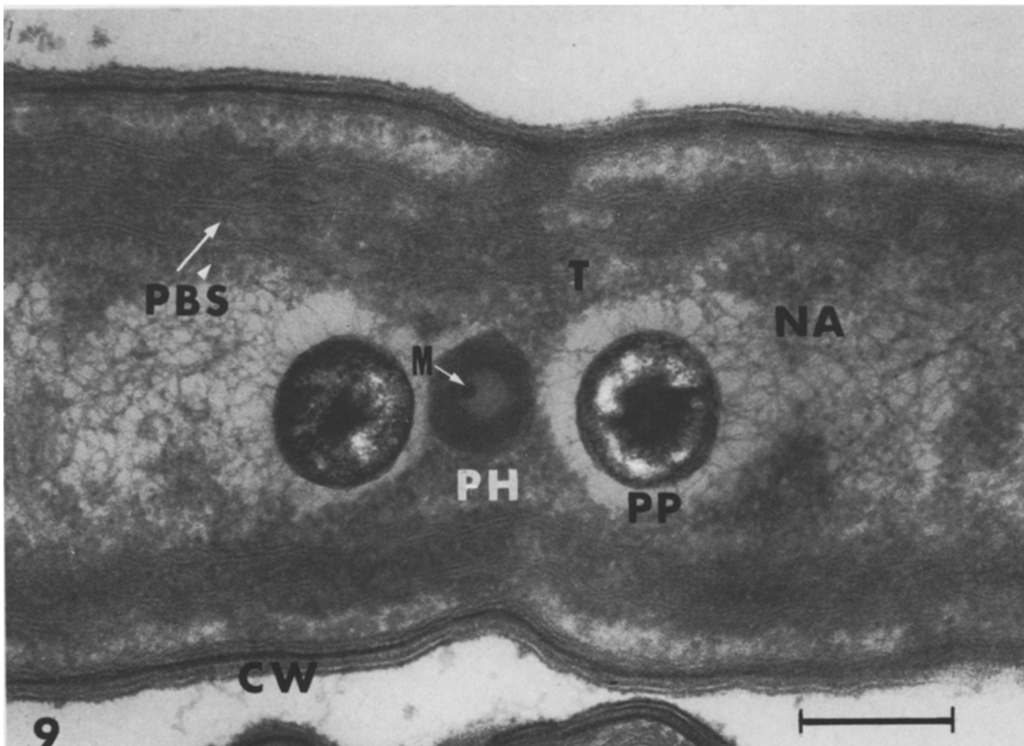


Fig. 8. Thick sections (>200 nm) of cells grown for 120 h in medium containing zero exogenous sulfur and 0.32 mM phosphorus display well preserved polyphosphate bodies (*PP*) of increased size and number. The marker bar is equivalent to 500 nm

Fig. 9. Nucleic acid fibrils (*NA*) are often observed to be continuous with polyphosphate bodies in thin sections of cells grown at all sulfur levels. Note also the polyphosphate minibody (*M*) within the polyhedral body (*PH*), the substructure of the cell wall (*CW*), and phycobilisomes (*PBS*) attached to the thylakoids (*T*). The marker bar is equivalent to 200 nm

autotrophic *Synechococcus* sp. respond to a deficiency of sulfur in a manner very similar to that of cells of the heterotrophic bacteria previously mentioned. Additionally, Vaillancourt et al. (1978) have used sulfur starvation followed by phosphorus starvation to test for polyphosphate-deficient mutants of this species.

The reciprocity of uptakes between orthophosphate and sulfate is likely due to a common transporter (or transporters) of these anions. Normal phosphate uptake in cells of *Synechococcus* sp. is energy-dependent and carrier-mediated, with a pH optimum between 7.6 and 8.5 (Falkner et al., 1974; Simonis et al., 1974; Ullrich-Eberius and Yingchol, 1974). Results of the present investigation indicate a similar pH range for rapid phosphate uptake, between pH 7.5 and 8.5, with the optimum nearer the more alkaline end. This finding, however, does not rule out the possibility that normal and rapid phosphate uptakes may be accomplished by different carrier molecules. Sulfate uptake has also been found to be energy-dependent and carrier-mediated in cells of this species, with pH optima of 7.5–8.0 and 8.0–8.5 indicated by Utkilen et al. (1976) and Jeanjean and Broda (1977), respectively. The former authors have found that sulfate uptake is competitively inhibited by sulfite and thiosulfate. Crompton et al. (1975) have demonstrated that the dicarboxylate carrier of the rate liver mitochondrion transports phosphate and sulfate, as well as sulfite and thiosulfate. In such a system the binding of orthophosphate and sulfate would be interdependent, the one reducing affinity for the other by the carrier (Crompton et al., 1975), and explaining at least in part why a deficiency of one anion would markedly increase uptake of the other.

Published evidence has not unequivocally determined whether adenosine-5′-phosphosulfate (APS) (Tsang and Schiff, 1975) or adenosine-3′-phosphate-5′-phosphosulfate (PAPS) (Schmidt, 1977), or both, serve for further sulfur reduction in species of *Synechococcus*. However sulfur or phosphorus deficiency would eventually diminish formation of APS and the next acceptor in the sequence of sulfur reduction. In cells of *Chlorella* this acceptor is very likely glutathione (Schmidt, 1972; Tsang and Schiff, 1978), which may ultimately confirm the suggestion by Harold and Sylvan (1963) for the involvement of oxidized glutathione in the control of condensed phosphate deposition.

Harold (1963) predicted that polyphosphate is likely the most prevalent phosphorus compound present when exogenous phosphate is available, and this suggestion was confirmed in rapid phosphate uptake studies with blue-green bacteria (Niemeyer and Richter, 1969; Sicko-Goad and Jensen, 1976). Although Batterton and Van Baalen (1968) found no evidence for reserve polyphosphate in cells of *Anacystis nidulans* after rapid uptake of orthophosphate, our findings and those of Niemeyer and Richter (1969) are to the contrary. Cells of the *A. nidulans* 625 strain of *Synechococcus* elaborate polyphosphate granules whose mean size and number increase with both the degree and duration of sulfur deficiency, which provides a measure of the extent of polymerization of the newly acquired phosphorus.

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Received April 28, 1978