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THE ULTRASTRUCTURAL EFFECTS OF EXOGENOUS SULFUR COMPOUNDS ON CONDENSED PHOSPHATE DEPOSITION IN SYNECHOCOCCUS SP. (ANACYSTIS NIDULANS)

City University of New York PH.D. 1979

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THE ULTRASTRUCTURAL EFFECTS OF EXOGENOUS SULFUR COMPOUNDS ON CONDENSED PHOSPHATE DEPOSITION IN *SYNECHOCOCCUS* SP. (*ANACYSTIS NIDULANS*)

by

NELSON H. LAWRY

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1979

Nelson H. Lawry

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ABSTRACT

The Ultrastructural Effects of Exogenous Sulfur Compounds on Condensed Phosphate Deposition in Synechococcus sp. (Anacystis nidulans)

by

Nelson H. Lawry

Advisor: Professor Thomas E. Jensen

As is the case in other cyanobacteria, cells of Synechococcus sp. (Anacystis nidulans) are capable of rapid orthophosphate uptake after a period of phosphorus deprivation. The maximum uptake occurs within the first hour after phosphate restoration. Cellular phosphorus accumulation, as determined by autoclaving cell aliquots with $K_2S_2O_8$, followed by Murphy-Riley spectrophotometric analysis, is a direct function of the exogenous orthophosphate concentration upon restoral. Optimum uptake is shown in the pH 7.5-8.5 range, with a dropoff at pH 9. Since the $H_2PO_4^{2-}$ anion is prevalent over this entire pH range, carrier-mediated rapid uptake is suggested. A ten-hour exposure to 10 µg/ml CAP (inhibitor then washed out) had no immediate effect on uptake or deposition, indicative of long-lived carrier proteins.

The ultrastructure of phosphorus-starved cells reveals no polyphosphate bodies, whereas that of cells five hours after phosphate replenishment shows such inclusions, moreover somewhat larger than those in normally cultured cells.

In utilizing a similar system it was found that sulfur depletion over a longer period (up to six days) also increases orthophosphate uptake and condensed phosphate deposition. Cells cultured with exogenous concentrations of zero, 3.1 µM, and 31 µM sulfate exhibit a sequential slowing of growth, increase in phosphorus accumulation, and increase in

the size (400-500 nm D) of polyphosphate bodies. Those grown on the control concentration of 0.31 mM sulfate show only a normal phosphorus level and regular-sized bodies (200 nm D). A normal pattern is also seen in cells provided with 0.31 mM thiosulfate or metabisulfite. In cases of extreme sulfur depletion additional polyphosphate bodies are formed, and also rings and caps of condensed phosphate adjacent to the plasma membrane.

The volume of a normal-sized cell of Synechococcus sp. is approximately 0.760 fl, and the cell mass about 800 fg. Total cellular phosphorus is usually in the 5-10 fg range, about 1% of total mass; total phosphorus increases up to 40 fg/cell under sulfur starvation, or about 5% of total cell mass.

Phosphorus deficiency causes a lesser degree of pigment loss than does sulfur deprivation, indicating that while the cells draw upon the phycobiliprotein as a sulfur reserve, a more plentiful but less self-limiting (to the photoautotrophic capacity) reservoir of phosphorus is available. Cells grown under sulfur deficiency/phosphorus sufficiency for six days, then shifted to sulfur sufficiency/phosphorus deficiency, soon recover their pigment levels and normal ultrastructural morphology, and survive well for three or four days after shift-over. Cells grown in complete medium, then transferred to phosphorus-deficient medium, suffer a substantial decline in both aspects within 24 hours.

Ultrastructurally the initially sulfur-deficient cells have massive polyphosphate bodies, which soon become porous and diminished in size irrespective of the presence of orthophosphate in the recovery medium, indicative of hydrolysis of the polymer. Such bodies in control cells are of normal size and quickly disappear if exogenous phosphate is omitted. It is concluded that polyphosphate serves as an effective

phosphorus reserve for short-term survival under limiting conditions.

Cells of this obligate photoautotroph also utilize L-cystine, L-djenkolic acid, DL-lanthionine, and glutathione as sole sulfur sources, but are unable to use L-cystathionine, DL-homocystine, L-methionine, L-cysteic acid, or taurine. The pattern is consistent with a unidirectional transsulfuration from L-cysteine to L-methionine, and suggests the presence of L-cystathionine-γ-synthase (E.C. 4.2.99.9) and L-cystathionine-β-lyase (E.C. 4.4.1.8). An identical pattern occurs in green plants and heterotrophic bacteria.

It is proposed that, in common with other prokaryote-like systems, the orthophosphate and sulfate anions are taken into the cyanobacterial cell by the same porter molecules. The absence of one anion would subject the system to input overload by the other. However, such an overload would be relieved or precluded by an alternate (e.g. organic) source of the missing anion.

To my folks,

Henry Madison Lawry and Laura Ella Chick Lawry

for their belief, trust, hope, encouragement

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-----finally to my friends, unnamed but not unloved, who made "da Bronx" an interesting, enjoyable, and memorable place during my years here.

The sand of the desert is sodden red, --
   Red with the wreck of a square that broke; --
The Gatling's jammed and the Colonel dead,
   And the regiment blind with dust and smoke.
The river of death has brimmed his banks,
   And England's far, and Honour a name,
But the voice of a schoolboy rallies the ranks:
   "Play up! play up! and play the game!"

Vitaï Lampada
Henry Newbolt

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INTRODUCTION

Six years ago Wolk (1973) noted the meager information available on sulfur metabolism in members of the cyanobacteria or blue-green algae. Six years prior to that Smith et al. (1967) had presented evidence to support the first convincing explanation for obligate photoautotrophy in a substantial number of these microorganisms, which specifically includes the frequently studied Anacystis nidulans (now considered to be a species of the genus Synechococcus).

Accordingly an investigation was undertaken on the utilization of various inorganic sulfur ions and shortchain organosulfur compounds by cells of this species. Of particular interest was the question whether any or all of the organosulfur compounds could serve individually as a sole sulfur source despite the absence of a general heterotrophic capability.

Schmidt (1977c), among other authors in recent years, has warned of the difficulties encountered in performing sulfur biochemistry, especially in regard to the incidence of isotopic exchange or side reactions when using labelled sulfur compounds. Since it has long been known that sulfur deficiency promotes a marked increase of condensed phosphate in cells of heterotrophic bacteria, it was of interest to determine if a similar deposition occurred upon sulfur depletion in cyanobacterial cells. Once this point was confirmed, such accumulation was used to monitor the cellular physiological state when a variety of potential sulfur sources was provided, and thus obviated the complications attendant to a direct study of sulfur chemistry. Since polyphosphate inclusion granules have a very characteristic electron image,

ultrastructural observation provided a dramatic and convincing method for corroborating changes in the extent of condensed phosphate deposition.

LITERATURE REVIEW

A. PHOSPHATE UPTAKE

General Considerations

Despite its relatively poor solubility in natural waters (Griffith et al., 1977), orthophosphate (PO_4^{3-}) is normally the most commonly available source of phosphorus to the microorganism. This anion is especially stable and does not undergo abiotic phosphorylation reactions (Griffith et al., 1977). Thus its activation in biological reactions necessarily requires an accompanying exergonic event. Because of general membrane impermeability to the anion, it is widely accepted that orthophosphate uptake is an energy-dependent, carrier-mediated process in the vast majority of living systems. Such a phosphate (trans)porter system was first suggested by Mitchell (1953, 1954), after the exchange hypothesis of Ussing (1947). In only a few of the microorganisms thus far investigated has orthophosphate transport been shown to be coupled to the hydrolysis of adenosine triphosphate (ATP).

The Process in Heterotrophic Bacteria

The first step in the active transport of inorganic phosphate may involve a membrane-located binding protein (BP). One such molecule has been found in cells of Escherichia coli by Medveczky and Rosenberg (1970). The binding is accomplished on a one:one molecular basis. Since phosphate starvation is not required for production of the binding protein, its synthesis can be assumed to be constitutive in this bacterium, and under normal conditions there exist 2×10^4 molecules of BP per cell (Medveczky and Rosenberg, 1970). Indirect evidence of its role is the demonstration that the BP must be added to reconstitute phosphate transport in spheroplasts of E. coli (Gerdes et al., 1977).

Uptake of orthophosphate exhibits a definite energy dependence in

cells of both Bacillus subtilis (Rosenberg et al., 1969) and E. coli (Medveczky and Rosenberg, 1971). Such transport is biphasic, with high- and low-affinity components under dual-gene control (Willsky et al., 1973). In E. coli cells orthophosphate binding protein is apparently associated only with the high-affinity component (Rosenberg et al., 1977). This component is regulated by the amount of available inorganic phosphate, and is repressed by orthophosphate in excess of one mM. In contrast the low-affinity component (orthophosphate affinity less by two orders of magnitude than that of the high-affinity component) is constitutive, capable of exchanging intra- and extracellular phosphate, and it continues to operate when the high-affinity component is repressed, i.e. when the intracellular phosphate "pool" is filled (Rosenberg et al., 1977). Whereas the low-affinity component is uncoupler-inhibited, the high-affinity component is not, although both are energy-dependent. The low-affinity component is driven by proton-motive force/pH gradient generated from aerobic or anaerobic respiratory electron transport, but the high-affinity component operates via phosphate bond energy (Konings and Rosenberg, 1978).

Phosphorus-deficient growth depletes the cellular phosphate pool, and upon restoration of inorganic phosphate both components act in a rapid refilling of the pool, probably in an additive manner (Rosenberg et al., 1977). Once filling is achieved, the high-affinity component is repressed and the uptake rate declines to that of non-phosphorus-deprived cells (Rosenberg et al., 1969). During filling phosphate flux is unidirectional, but thereafter exchange with exogenous inorganic phosphate occurs. Further or secondary uptake occurs via the low-affinity component to replace pool phosphate removed for esterification (Medveczky and Rosenberg, 1971).

The first evidence of arsenate competition for the common porter system of these two anions was obtained by Mitchell (1954) in cells of Micrococcus pyogenes. A similar competition occurs in cells of Streptococcus faecalis (Harold and Baarda, 1966), where a pH-expressed biphasic phosphate transport system obtains (optima at pH 5.5 and pH 8-9), but at the expense of ATP or a closely related variant (Harold and Spitz, 1975). Oxidative respiration is lacking in this organism. Arsenate is also transported in competition with orthophosphate in cells of Bacillus cereus (Rosenberg et al., 1969) and Escherichia coli (Medveczky and Rosenberg, 1970). In the latter bacterium only the high-affinity component is involved in this competitive transport.

In contrast to most of the other microorganisms investigated to date, cells of Micrococcus lysodeikticus exhibit a linear uptake of inorganic phosphate rather than a biphasic one, although two uptake peaks exist, at pH 6.5 and pH 8.0 (Friedberg, 1977). Transport regulation occurs by means of repression/derepression, and a rate increase is prevented by the addition of chloramphenicol (CAP), implying that de novo synthesis of the carrier must occur to increase phosphate uptake. Inorganic phosphate uptake is here again energy-dependent, as respiratory suppressors and uncouplers of oxidative phosphorylation inhibit the process (Friedberg, 1977). Friedberg (1977) has suggested the non-biphasic nature of uptake is due to an incomplete loading of the phosphate pool, in turn due to rapid polymerization of the cellular orthophosphate to polyphosphate (see Friedberg and Avigad, 1968).

The Process in Eukaryotic Autotrophic and Heterotrophic Microorganisms

In cells of the green alga Chlorella pyrenoidosa phosphate transport is also energy-requiring (Jeanjean, 1969), biphasic (Jeanjean et al., 1970), and rate-increase-mediated by de novo carrier synthesis (Jeanjean,

1973). Further information suggests that carrier synthesis is balanced by continual carrier destruction (Jeanjean and Ducet, 1974). As with the phosphate transport system in cells of Escherichia coli, a high-affinity component with a low saturation capacity, and a low-affinity, high-saturation capacity component operate in response to the concentrations of exogenous and endogenous inorganic phosphate (Jeanjean et al., 1970). Nyholm (1977) has pointed out that the former component is likely the primary element operating in algae under ecological conditions. With Chlorella too phosphate starvation affects the pattern of subsequent orthophosphate uptake (Jeanjean and Ducet, 1974; Jeanjean, 1975), and evidence indicates that transport of the anion is not an ATP-driven process, but rather depends upon respiratory and/or photosynthetic metabolism (Jeanjean, 1976). That the energy allowing phosphate uptake to function may be derived from more than one such source is indicated by the observations that in cells of Chlorella (Wintermanns, 1955), Ankistrodesmus (Simonis and Urbach, 1963), and Scenedesmus (Kylin, 1966), such transport is light-enhanced but not light-dependent.

A dual-component uptake system with differing affinities has been found to operate as well in the osmoorganotrophs Candida tropicalis (Blasco et al., 1976) and Neurospora crassa (Burns and Beever, 1977; Beever and Burns, 1977).

The Process in Cyanobacteria

Hutchinson (1973) has suggested that a special mechanism exists in blue-green bacteria for the efficient uptake of phosphate at very low concentrations in vivo, but he could not elaborate on precisely what it might be. Indeed, less specific information is available on phosphate transport in these obligately autotrophic, prokaryotic microorganisms. However it is very certain that the process is an energy-dependent,

carrier-mediated one in cells of Anacystis nidulans (Bornefeld et al., 1974; Falkner et al., 1974; Simonis et al., 1974). The optimum range for such uptake is quite narrowly defined in this organism: pH 7.6-8.2 (Falkner et al., 1974); pH 8.0 (Bornefeld et al., 1974; Simonis et al., 1974); or pH 7.0 with a more pronounced peak at pH 8.5, uptake falling off rapidly thereafter (Ullrich-Eberius and Yingchol, 1974). Studies on rapid orthophosphate uptake by cells of A. nidulans were carried out within this same range, at pH 8.1 (Batterton and Van Baalen, 1968).

The velocity of phosphate uptake is concentration-dependent on exogenous orthophosphate, with a K_m = 6.0 μM, and that uptake appears to be unidirectional in cells of A. nidulans (Falkner et al., 1974). Transport in blue-green bacteria is light-enhanced but not light-dependent (Talpasayi, 1962; Simonis et al., 1974), and DCMU inhibition suggests some degree of coupling to photosystem II or to non-cyclic photophosphorylation (Simonis et al., 1974). The light effect is even more pronounced in cells of A. nidulans starved for phosphorus (Bornefeld et al., 1974). However orthophosphate transport in cells of this species is also strongly sensitive to the uncoupler mCCCP (Falkner et al., 1974; Simonis et al., 1974), indicative of a contribution from oxidative phosphorylation and/or cyclic photophosphorylation to the energy required for inorganic phosphate uptake. All authors concluded that although ATP generation may support transport, ATP level is not a limiting factor in that process. A proton-motive/pH gradient mechanism may drive phosphate uptake in cells of Anacystis nidulans (Brinckmann and Simonis, 1978).

Pretreatment of cells of A. nidulans with CAP had no effect on orthophosphate transport, a reflection that in contrast to the condition obtaining in cells of Chlorella pyrenoidosa when pretreated with cycloheximide (CHI) (Jeanjean, 1973), phosphate porters in the cyanobacterium

are long-lived. Batterton and Van Baalen (1968) have indicated that in both normal and phosphate-starved cells of this species a great many phosphate binding sites are present, suggesting again the constitutive nature of binding proteins in prokaryotic organisms.

Organic Phosphates and Alkaline Phosphatases

Depletion of exogenous phosphorus not only induces an initially increased rate of inorganic phosphate uptake in a wide variety of microorganisms (Kuhl, 1962; Batterton and Van Baalen, 1968; Rosenberg et al., 1969; Jeanjean, 1969; Stewart and Alexander, 1971), but also a much increased level of alkaline phosphatase (Torriani, 1960; Bone, 1971; Healey, 1973; Ihlenfeldt and Gibson, 1975). Both processes are inhibited by orthophosphate, apparently via a repression of carrier synthesis in the first case (Jeanjean and Ducet, 1974), and of the enzyme itself in the second (Horiuchi et al., 1959; Torriani, 1960; Echols et al., 1961). The release of this enzyme into the extracellular milieu allows any of several organic phosphates to replace inorganic phosphate as the organism's source of phosphorus. The cleaved phosphate moiety is transported into the cell, while the remainder of the substrate molecule is excluded (Horiuchi et al., 1959)

Cytochemical staining techniques and electron microscopy have localized active phosphatases in the periplasmic space of such gram-negative bacteria as Escherichia coli (Malamy and Horecker, 1961; Done et al., 1965; Wetzel et al., 1970) and Pseudomonas aeruginosa (Cheng et al., 1970; Ingram et al., 1973; Bhatti et al., 1976), as well as in the cyanobacteria Plectonema boryanum (Doonan and Jensen, 1977) and Anabaena cylindrica (Doonan, 1978). Of interest is the observation that in the former blue-green bacterium only the outer walls are stained, but in the latter species the cross walls too exhibit localization of the enzyme.

Evidence has indicated that in cells of Anacystis nidulans alkaline phosphatase is peripherally located (Ihlenfeldt and Gibson, 1975), but its precise site has yet to be demonstrated.

B. SULFATE UPTAKE AND REDUCTION

General Considerations

Similar to orthophosphate, the sulfate anion (SO_4^{2-}) is a very stable and unreactive species in aqueous solution. In contrast to the frequently limiting amounts of inorganic phosphate in oligotrophic habitats, however, inorganic sulfate is rarely deficient, and in fact normally is present in moderate to high concentrations in both freshwater (as high as 0.1 mM) and marine (mean value 25 mM) environments (Schiff, 1962; Brock, 1970). Also unlike orthophosphate, sulfate sulfur must undergo a valency change, usually an 8-electron reduction, prior to its assimilation into organic molecules (Bandurski, 1965). Reductions are endergonic, but that of sulfate sulfur to the thiol level is substantially so, requiring some 180 kcal per mole (Gibbs and Schiff, 1960; Schiff and Hodson, 1973).

Sulfate is the universal sulfur source for virtually all autotrophs, and is used by some heterotrophic microorganisms as well (Bandurski, 1965). Within both classes the vast majority of microorganisms utilize the anion in assimilatory sulfate reduction to yield useful structural and/or metabolic organosulfur molecules. The sulfur is most often incorporated at the thiol level (-SH), oxidoreductive state -2. The enzymes involved in such reduction and assimilation are normally repressible by other sulfur species, both inorganic and organic. Among heterotrophs a very few, classically represented by the obligate anaerobes of the genus Desulfovibrio, exhibit dissimilatory sulfate reduction. The process

yields respiratory energy only for the microorganisms and most of the sulfate is not assimilated, but reduced to sulfide (S^{2-}) and excreted.

The Uptake Process in Heterotrophic Bacteria

The first step in cellular uptake of inorganic sulfate may be binding of the anion to the plasmalemma. A largely protein molecule tightly binding sulfate on a one:one molecular basis has been implicated in cells of Salmonella typhimurium (Pardee, 1966; Pardee and Prestidge, 1966), with approximately 1×10^4 binding proteins per cell (Pardee, 1968). The binding process is not energy-dependent, but is repressed by L-cysteine and inhibited by thiosulfate ($S_2O_3^{2-}$) (Pardee et al., 1966). Dreyfuss and Pardee (1965) suggested that binding represents an obligatory event in sulfate transport, and Pardee and Watanabe (1968), by the use of diazo-NDS and antibody inactivation of proteins, localized the BP on the plasma membrane adjacent to the cell wall in S. typhimurium. The gene coding for sulfate transport (cys A) in this organism does not code for the binder, although there is ultimately a single regulator of binder production, sulfate transport, and cysteine synthesis (Ohta et al., 1971). That the absence of BP is not directly correlated with a lack of sulfate transport (Pardee et al., 1966) may be the result of a two-component uptake system for sulfate similar to that for phosphate previously discussed.

Transport of inorganic sulfate across the membrane barrier is an energy-dependent, carrier-mediated process in such bacteria as E. coli and B. subtilis (Wheldrake and Pasternak, 1965; Springer and Huber, 1972), as well as in S. typhimurium (Dreyfuss, 1964; Dreyfuss and Pardee, 1965). Transport is effectively inhibited by endogenous reduced sulfur, particularly cysteine or its oxidized form, cystine, and methionine is also very inhibitory in some organisms. For example Dreyfuss (1964) determined

that glucose-energized sulfate uptake in cells of Salmonella typhimurium is inhibited by thiosulfate, sulfite, and cysteine. The two inorganic anions very likely inhibit competitively, as evidence exists that they are transported via the sulfate carrier; contrarily cysteine acts by negative feedback or endpoint repression, since it inhibits not only transport but also activation of sulfate and thiosulfate (Dreyfuss, 1964; Dreyfuss and Pardee, 1966; Kredich, 1971). Furthermore both the sulfate transport and activation processes repressed by cysteine are derepressed by sulfur starvation (Dreyfuss, 1964; Kredich, 1971). Transport of sulfate in cells of S. typhimurium exhibits characteristics reminiscent of a two-component uptake mechanism, and thus the rate of net sulfate influx depends upon the intracellular sulfate level. A low cellular level resulting from sulfur deprivation would induce a high rate of intake, while a high level would be consistent with a much reduced intake rate (Dreyfuss and Pardee, 1966; Schiff and Hodson, 1973).

The Uptake Process in Eukaryotic Microorganisms

Within both auto- and heterotrophic eukaryotes uptake of inorganic sulfate exhibits similar functioning. It is energy-linked in Chlorella cells (Wedding and Black, 1960) and biphasic in cells of C. pyrenoidosa, but there is no evidence yet of exchange by cellular and exogenous sulfate (Vallee and Jeanjean, 1968a). Uptake is increased in presulfur-starved cells, and in such cases in both C. pyrenoidosa and C. vulgaris inhibition by methionine and cysteine is maximal (Vallee and Jeanjean, 1968b; Passera and Ferrari, 1975).

Sulfate uptake is inhibited by thiosulfate in cells of Neurospora crassa (Ragland and Liverman, 1958), and as well by sulfite, methionine, cystine, and cysteine-HCl (and less so by cysteic acid and taurine) in cells of Penicillium chrysogenum (Segel and Johnson, 1961). Sulfate

influx far exceeds efflux ($K_{m_{efflux}}$ = 20 mM; $K_{m_{influx}}$ = 0.01 mM) in cells of N. crassa (Marzluf, 1974), and a two-component uptake based on high and low affinities has been demonstrated in cells of Saccharomyces cerevisiae. This osmotroph forms L-methionine as its first major organosulfur compound, and thus transport is regulated (via carrier synthesis) by exogenous methionine or *S*-adenosylmethionine (Breton and Surdin-Kerjan, 1971).

Assimilatory Sulfate Reduction

After uptake sulfate is reduced to the thiol level by one or two activation steps, and two reduction steps (Tsang and Schiff, 1976a). In heterotrophic microorganisms there are two activation events: (1) sulfate reacts with ATP via ATP sulfurylase (formally designated ATP-sulfate adenylyltransferase, E.C. 2.7.7.4) to yield adenosine 5′ -phosphosulfate (APS) and pyrophosphate (PP_i); (2) APS reacts with a second molecule of ATP via APS kinase (adenylyl 3′ -phosphotransferase, E.C. 2.7.1.25) to yield adenosine 3′ -phosphate 5′ -phosphosulfate (PAPS) and ADP (Hilz et al., 1959; Pasternak et al., 1965; Kline and Schoenhard, 1970; Kredich, 1971; Tsang and Schiff, 1975). The first reaction has an unfavorable equilibrium for product formation since the change in free energy is strongly positive, but it is "pulled" by product removal by the second reaction, whose enzyme has a very high substrate affinity, and the cleavage of PP_i by inorganic pyrophosphatase (E.C. 3.6.1.1). Both of these secondary reactions have a negative ΔF (Schiff and Hodson, 1973).

In autotrophic microorganisms carrying out assimilatory sulfate reduction the situation is somewhat less clear, for in this case APS not PAPS is the substrate for subsequent reduction (Schmidt, 1972, 1977a,b; Goldschmidt et al., 1975; Tsang and Schiff, 1975; Brunold and Schiff, 1976; Møller and Evans, 1976). These organisms must still contend with

the unfavorable equilibrium presented by the sulfate-ATP activation reaction, and perhaps the majority of APS is converted to PAPS. This compound could then serve as a reservoir not only for APS, but also in organisms capable of doing so, for esterification reactions. Present in both heterotrophs such as Escherichia coli and autotrophs such as Chlorella pyrenoidosa is the enzyme 3′5′-diphosphonucleoside 3′-phosphohydrolase (DPNPase), which dephosphorylates PAPS back to APS (Hodson and Schiff, 1971; Goldschmidt et al., 1975). Whether PAPS or APS serves as the sulfo-group donor in each type of organisms depends upon the specificity of the sulfotransferase present. In heterotrophs such as E. coli there is only PAPS-sulfotransferase (Tsang and Schiff, 1976a), whereas in C. pyrenoidosa and apparently in other photoautotrophs as well, only APS-sulfotransferase (Schmidt, 1972; Tsang and Schiff, 1975; Brunold and Schiff, 1976). Cells of the chemolithotroph Thiobacillus ferrooxidans normally oxidize reduced sulfur ions as an energy source, but when grown on ferrous iron or glucose, they can utilize sulfate rather than reduced sulfur ions. The sulfate is activated as APS, with no evidence for the involvement of PAPS (Tuovinen et al., 1975).

As a result of what Schiff and Hodson (1973) have termed "promiscuous reactions of coy intermediates," the major reduction pathway of sulfur after APS or PAPS formation has long been obscured, and still is uncertain in all details. Initial evidence suggested that in both Salmonella typhimurium (Dreyfuss and Monty, 1963a,b) and E. coli (Jones-Mortimer, 1968) sulfite and sulfide exist as free intermediates on the main reduction route, with free thiosulfate reducing by a feeder pathway. Many authorities, however, argued for a system of bound intermediates, because toxicity and lability of some inorganic sulfur ions render them unsuitable as free intermediates (Bandurski, 1965).

Later investigators have indicated that in fact the major sulfate reduction pathway in both autotrophic and heterotrophic microorganisms, as exemplified by E. coli and C. pyrenoidosa, respectively, proceeds via bound intermediates (Abrams and Schiff, 1973; Schmidt, 1973; Schmidt et al., 1974; Tsang and Schiff, 1976a). In each case the sulfonyl group is transferred from its respective nucleoside phosphosulfate to a carrier bound to thiosulfonate reductase (Schmidt et al., 1974). The bound sulfite group (car-S-SO_3^-) is then reduced to bound sulfide (car-S-S^-), probably by NADPH in E. coli (Tsang and Schiff, 1976a) and by ferredoxin or ferredoxin-NADPH reductase in C. pyrenoidosa (Schmidt, 1973; Schmidt et al., 1974). In both cases the bound thiol group is transferred to *O*-acetyl-L-serine via sulfhydrylase, requiring two electrons from an unknown donor, to form L-cysteine (Dreyfuss, 1964; Kredich and Tomkins 1966; Jones-Mortimer, 1968; Schmidt et al., 1974). The carrier in cells of E. coli has been indicated as thioredoxin or a closely related form (Tsang and Schiff, 1976a, 1978a), and that of C. pyrenoidosa and Rhodospirillum rubrum as a natural thiol, probably glutathione (Schmidt, 1972, 1977b; Tsang and Schiff, 1978b). The possibility arises that sulfonyl receptors are mandated by whether donated from APS or PAPS.

The presence of free intermediates -- sulfite, sulfide, thiosulfate, and others -- is very probably due to side reactions of the pathway, particularly in the presence of thiols, or to exogenous input (Tsang and Schiff, 1976a,b). It appears that especially in the case of APS-sulfotransferase from Chlorella cells, the enzyme is non-specific toward a range of thiol acceptors, yielding several free sulfur ions or organic thiosulfates as products (Schmidt et al., 1974; Tsang and Schiff, 1976b). The PAPS-sulfotransferase from E. coli cells is much more specific for the thioredoxin(-like) acceptor, and only a minor amount of side product

occurs in its absence (Tsang and Schiff, 1976a). In both organisms there are sulfite reductases existing outside the main reduction pathway that reduce sulfite to sulfide, which in turn reacts with *O*-acetyl-L-serine via OAS sulfhydrylase to form L-cysteine (Schmidt et al., 1974; Tsang and Schiff, 1976a). Additional evidence has prompted suggestion that the sulfite and thiosulfonate reductases are identical in cells of Escherichia coli (Tsang and Schiff, 1976a), although the enzymes are distinct entities in cells of Chlorella pyrenoidosa (Schmidt, 1973).

A great deal of evidence has accumulated that regulation of L-cysteine synthesis occurs by either endpoint inhibition of enzyme activity or endpoint repression of enzyme synthesis (Ellis et al., 1964; Dreyfuss, 1964; Dreyfuss and Pardee, 1966; Pasternak et al., 1965; Wheldrake and Pasternak, 1965; Jones-Mortimer, 1968; Kredich, 1971; Collins and Monty, 1975). Such repression of all enzymes along the sulfate activation-reduction pathway seems to be directly proportional to the intracellular concentration of cysteine (Wheldrake, 1967), and moreover is increased in the absence of thiols, suggesting a selective inhibition of the bound intermediate pathway, probably at thiosulfonate reductase (Schmidt, 1973; Schmidt et al., 1974).

In cells of E. coli and Salmonella typhimurium a positive control of cysteine synthesis is exerted by *O*-acetyl-L-serine, since the amino acid must be present for synthesis of most of the enzymes required for sulfate uptake, activation, and reduction (Kredich and Tomkins, 1966; Jones-Mortimer et al., 1968). However L-cysteine not only represses all enzymes in that pathway, but also suppresses serine transacetylase (serine → *O*-acetyl-L-serine) activity by feedback inhibition (Jones-Mortimer, 1968; Kredich, 1971), suggesting that again intracellular cysteine concentration is the ultimate regulator of L-cysteine synthesis. Ellis et

al. (1964) have postulated that L-cysteine is also the common repressor in suppression by sulfite, L-cystine, L-methionine, L-djenkolic acid, cystamine, and glutathione. Its formation from the latter four compounds is relatively slow and the repression of cysteine synthesis is incomplete, but is rapid from sulfite and L-cystine, and repression is complete if concentrations are sufficiently high. These two effective repressors of sulfate activation and reduction have no effect on sulfite reductase (Pasternak et al., 1965), lending additional weight to the suggestion of Ellis et al. (1964).

Dissimilatory Sulfate Reduction

In the dissimilatory-sulfate-reducing members of the genera Desulfovibrio and Desulfotomaculum inorganic sulfate is activated via ATP sulfurylase to APS (Peck, 1961), a reaction not repressed by either sulfite or cysteine (Wheldrake and Pasternak, 1965). APS is reduced to sulfite by an APS reductase, and sulfite to sulfide, both reductions relying on cytochrome c_3 with a very low E_o of -205 mv (Peck, 1959; 1961; Michaels et al., 1970). The ionic intermediates seem to be free, a possible evolutionary consequence of anoxygenic metabolism, although the adenylyl sulfate reductase reaction in cells of Desulfovibrio may involve a flavin-sulfite association (Michaels et al., 1970). Further details of this remarkable and unique sulfate metabolism, peripheral to the present study, are encountered in Trudinger (1969) and Roy and Trudinger (1970).

The Uptake, Activation, and Reduction Processes in Cyanobacteria

Uptake of inorganic sulfate is both energy-dependent and carrier-mediated in cells of Anacystis nidulans, with a maximum at pH 8 (Utkilen et al., 1976) or between pH 8 and 8.5 (Jeanjean and Broda, 1977), falling off thereafter in both cases. Some suggestion of binding has been obtained, along with evidence that sulfate uptake in this species is

inhibited by mCCCP, DCCD, chromate, and selenate, but not by DCMU (Jeanjean and Broda, 1977). Transport is also competitively inhibited by sulfite and thiosulfate (Utkilen et al., 1976). Increased sulfate uptake after sulfur starvation is partly reduced by 50 µg/ml chloramphenicol (Jeanjean and Broda, 1977), suggesting there is some de novo synthesis of the carrier.

The assimilatory sulfate reduction pathway in members of the blue-green bacteria is presently less certain than among the heterotrophic prokaryotes and autotrophic eukaryotes studied. After determining that both APS and PAPS can be degraded hydrolytically by cellular extracts of Anabaena cylindrica likely containing 3′- and 5′-nucleotidases, Sawhney and Nicholas (1976a,b) demonstrated that both APS and PAPS are elaborated from inorganic sulfate in cells of this species. Of note is the fact that while the ATP-sulfurylase reaction is not inhibited by 10 mM cysteine, methionine, or glutathione (Sawhney and Nicholas, 1976b), it is progressively inhibited by increasing amounts of inorganic phosphate (Sawhney and Nicholas, 1977).

Tsang and Schiff (1975) have reported an overwhelming predominance of APS-sulfotransferase activity vice PAPS-sulfotransferase activity in cellular extracts of a marine Synechococcus species and Oscillatoria wonnonichinii, and have suggested again that activity from PAPS is due to dephosphorylation by a DPNPase rather than a PAPS-sulfotransferase. On the other hand Schmidt (1977a) has predicted a species-specific pattern. Cells of a Plectonema strain exhibit a reduction specific for APS, while those of Spirulina platensis and strains of Synechococcus and Synechocystis show a reduction preferential for PAPS (Schmidt, 1977a). Furthermore Schmidt and Christen (1978) have isolated a PAPS-sulfotransferase from Synechococcus 6301 cells with optimum activity at pH 8, and a

dependence on a thioredoxin-like factor for activity.

Wildtype cells of Leuconostoc mesenteroides lack all enzymes for the activation and reduction of inorganic sulfate (Wheldrake and Pasternak, 1965), restricting members of this cyanobacterial-like species to obligate heterotrophy.

Cells of the thermophilic Synechococcus lividus strain Y52 are capable of both dark and photoreduction of sulfate and thiosulfate to hydrogen sulfide (Sheridan and Castenholz, 1968). Either sulfur anion can serve as a terminal electron acceptor for dark anaerobic oxidation, apparently analagous to the dissimilatory reduction of Desulfovibrio spp. Utkilen (1976) has indicated that thiosulfate can serve as an electron donor for photosynthesis by cells of Anacystis nidulans. However, Sheridan (1973) determined by more detailed investigations using S. lividus Y52 that thiosulfate acts as an electron acceptor for a photoreductant (probably water) participating in photosystem II. Thus it appears that sulfate and thiosulfate serve as terminal electron acceptors, in the dark replacing oxygen, and in the light replacing carbon dioxide (Sheridan, 1973).

C. INTERACTIONS BETWEEN PHOSPHATE AND SULFATE UPTAKE AND ASSIMILATION

The uptakes of inorganic sulfate and phosphate can be noted to possess a number of similarities. The passage of each element through the membrance barrier is an energy-linked, porter-borne process; each has been found in at least one organism to be associated with an initial binding protein, and subsequently with transport components of different affinities; and each by its deprivation induces the cell to respond by a rapid uptake of that element. The transports of both phosphate and sulfate exhibit biphasic or multiphasic kinetics in a variety of plants and tissues

(Nissen, 1974). The effects of these two inorganic species on each other's uptake and assimilation vary and require consideration.

Entry into Whole Cells (Including Cyanobacteria)

The increase in intracellular sulfur compounds -- i.e. inorganic sulfate, soluble reduced sulfur, and lipid sulfur -- as a result of phosphorus deficiency has been demonstrated in cells of Scenedesmus sp. (Kylin, 1964a). Kylin (1964b) suggested the following nonmutually exclusive possibilities in explanation: (1) a phosphorus deficiency may cause a change in membrane permeability to sulfate; (2) both anions may compete for the same adsorption and/or carrier sites; (3) a phosphorus deficiency should decrease the phosphorylation rate in redox reactions, and thereby may decrease the phosphate transport capacity; and (4) a phosphorus deficiency may decrease phosphate inhibition of pyrophosphatase, promoting APS formation and in turn sulfate uptake.

In a similar fashion the incorporation of sulfate sulfur and phosphate phosphorus into cellular material in the coccoid blue-green bacterium Microcystis aeruginosa increases markedly if the other anion is absent from the growth medium (Volodin, 1970). Closely coupled with one of Kylin's (1964b) possibilities above, is the finding that inorganic phosphate directly inhibits the activity of ATP-sulfurylase in cellular extracts from the filamentous cyanobacterium Anabaena cylindrica (Sawhney and Nicholas, 1977). The result of such inhibition would be a decrease in sulfate uptake and reduction.

Entry into and Functions within Chloroplasts

Uptake of sulfate into spinach chloroplasts requires ATP, and is rate-limited at either or both of the steps catalyzed by ATP-sulfurylase or ATP-sulfotransferase. On the contrary sulfite is directly bound in relation to the number of available sulfhydryl groups, forming car-S-SO_3^-.

Sulfite uptake into chloroplasts neither requires ATP nor is controlled by a rate-limiting step (Ziegler and Hampp, 1977). As indicated by exchange reactions with inorganic phosphate, phosphoglyceric acid, and dihydroxyacetone phosphate, both inorganic phosphate and sulfate are at least partly transported by the phosphate translocator of the chloroplast inner envelope (Hampp and Ziegler, 1977). As opposed to the electron transport function of the thylakoids, the inner envelope membrane seems primarily to carry on transport activity (Flügge and Heldt, 1976). In the study by Hampp and Ziegler (1977) the transport of sulfur anions occurred at a much slower rate than that of phosphate, and was enhanced by the presence of inorganic phosphate or phosphoglyceric acid.

Sulfate is a strong inhibitor of photophosphorylation in isolated chloroplasts by competition with phosphate (Asada et al., 1968; Baldry et al., 1968; Hall and Telfer, 1969), in which manner it acts as in inhibitor of energy transfer (Pick and Avron, 1973). Sulfate has also been shown to uncouple electron transport irreversibly from photophosphorylation by an interaction with the coupling factor (CF_1) (Ryrie and Jagendorf, 1971). Grebanier and Jagendorf (1977) have obtained evidence that sulfate and phosphate do not compete for the phosphorylation binding site, but they have suggested there may be a second phosphate binding site which allows the inhibition by an interaction between the two sites.

Entry into Mitochondria

Sulfate, sulfite, and thiosulfate are transported at least in part into the rat liver mitochondrion by the dicarboxylate carrier, as indicated by an exchange with inorganic phosphate and certain dicarboxylic acids (Chappell, 1968; Crompton et al., 1974a,b). Further, the phosphate and dicarboxylate anions occupy different binding sites on the transporter (Palmieri et al., 1971, 1974). Crompton et al. (1975) have determined

that sulfate, phosphate, and malonate influxes are mutually inhibitory, and have obtained additional data that suggest sulfate and malonate bind to a different site from that of phosphate. Thus while sulfate competes directly with malonate for a common binding site, there is also an interdependence with phosphate in that binding of one anion to its site decreases the affinity of the other anion for its carrier site, perhaps by an allosteric hindrance.

Both phosphate and dicarboxylate transporters have been identified in spinach chloroplasts (Heldt and Rapley, 1970; Fliege et al., 1978; Lehner and Heldt, 1978) as well as in mitochondria, and the possibility arises that both the sulfate and phosphate anions are generally translocated by more than one carrier type.

D. THE METABOLISM OF ORGANIC SULFUR COMPOUNDS

The Utilization by Heterotrophic Bacteria

In a lengthy investigation on biosynthesis by cells of Escherichia coli, Roberts et al. (1955) discovered that not only would sulfate, sulfite, thiosulfate, and sulfide serve as sole sulfur sources, but also L-cystine, DL-lanthionine, *S*-methylcystine, glutathione, and at a slower growth rate, cysteic acid and taurine (all at 10 µg S/ml). Homocyst(e)ine and methionine were found to be much less satisfactory, and L-djenkolic acid, cystathionine, allocystathionine, and glutathionine were ascertained not to serve at all. L-cysteine demonstrated an inhibitory effect on growth in this study. Almost all of the compounds serving as sulfur sources inhibited the uptake of inorganic sulfate.

Cellular sulfur in E. coli exists almost entirely as methionine and cyst(e)ine, with the former nearly all incorporated into protein, and the latter divided approximately equally into protein and glutathione.

Glutathione serves as a readily available internal sulfur reservoir in most gram-negative bacteria, accounting for a large proportion of the thiol groups (Roberts et al., 1955; Young and Maw, 1958; Fahey et al., 1978).

In the study by Roberts et al. (1955) L-cystine uptake was not inhibited by some of the sulfur compounds suppressing sulfate uptake, but both methionine and homocyst(e)ine reduced cystine transport by 50%. On the other hand methionine uptake was not inhibited by any sulfur compound tested. A shorter generation time followed from including both inorganic sulfate and methionine in the growth medium, and the glutathione and protein sulfur fractions which resulted were about equal. When sulfate alone served as the sulfur source, an overabundance of glutathione was formed, some of which was excreted and some (< 30%) was used for further synthesis. Little sulfur-containing protein was synthesized from exogenous glutathione.

Methionine and homocyst(e)ine support the methionine requirement of E. coli cells, but neither leads to the formation of cysteine or glutathione, and no sulfur is transferred from methionine to cysteine. The carbon skeleton for de novo synthesis of cysteine was determined to originate ultimately from serine (Roberts et al., 1955). The conversion of L-serine, by acetylation to *o*-acetyl-L-serine (OAS), and sulfhydrylation of this intermediate to L-cysteine, was confirmed in both cells of E. coli and Salmonella typhimurium (Kredich and Tomkins, 1966) as described previously.

In most bacteria L-cysteine can be converted to a number of organosulfur compounds, and in many cases it reacts with other amino acids from converging metabolic pathways (Smith, 1971). Most commonly however it is incorporated into protein or glutathione via peptide synthetases,

or converted to methionine via a nonreversible transsulfuration pathway. Synthesis of the tripeptide glutathione is nonribosomal and involves two peptide bonds: between L-cysteine and glutamic acid, catalyzed by γ-glutamylcysteine synthetase, to form γ-glutamyl cysteine; and between this compound and glycine, catalyzed by glutathione synthetase, to yield glutathione (γ-glutamylcysteinylglycine).

The major route of methionine synthesis has been established from several investigations using both E. coli and S. typhimurium. Aspartic acid is the ultimate source for L-homoserine, which reacts with succinyl Co A via homoserine-*O*-transsuccinylase to yield *O*-succinyl homoserine (OSH) and reduced coenzyme A (Rowbury, 1964; Rowbury and Woods, 1964b). L-cysteine reacts with OSH via cystathionine-γ-synthetase in a γ-replacement reaction to form L-cystathionine and succinic acid (Kaplan and Flavin, 1964, 1966). L-cystathionine undergoes a β-elimination reaction via cystathionine-β-lyase (β-cystathionase) to yield L-homocysteine, pyruvic acid, and ammonia (Wijesundera and Woods, 1962; Delavier-Klutchko and Flavin, 1965b). L-homocysteine is converted to L-methionine by a methylation reaction involving an L-homocysteine methylase. The methyl donor is either of two folic acid derivatives, N^5-methyltetrahydropteroyl glutamates, and two pathways exist for the methylation, one cobalamin-dependent and the other not (Guest and Woods, 1962; Guest et al., 1964; Woods et al., 1964; Morningstar and Kisliuk, 1965; Cauthen et al., 1966).

The formation of L-homocysteine methylase, cystathionine-β-lyase, γ-cystathionine synthetase, and homoserine-*O*-transsuccinylase are all repressed by L-methionine (Harold, 1962; Rowbury, 1964; Rowbury and Woods, 1961, 1964a,b, 1966). In addition the activity of the last-named enzyme is inhibited by L-methionine (Rowbury, 1964). As opposed to the positive control (induction) of cysteine synthesis, production of methio-

nine is under negative control (repression) in these enteric heterotrophic bacteria.

Transport of exogenous amino acids across the plasma membrane is both energy- and carrier-dependent. Each permease is specific for a single amino acid or a group of amino acids, and may be stereospecific as well (Oxender, 1972; Ayling and Bridgeland, 1972). Cells of E. coli can utilize D-methionine, probably by oxidative deamination/transamination to L-methionine (Cooper, 1966). Although uptake of L-methionine is only slightly inhibited by D-methionine (but strongly inhibited by L-ethionine), uptake of D-methionine is substantially inhibited by L-methionine (Piperno and Oxender, 1968; Kadner, 1977). Transport of L-methionine by E. coli cells occurs via dual components, the first having an affinity 200 times greater than the second for the amino acid (Kadner, 1974, 1977; Kadner and Watson, 1974). The high affinity component appears also to be the D-methionine carrier, whereas the low affinity component transports only L-methionine (Kadner and Watson, 1974; Kadner, 1977). No binding protein has been demonstrated with either methionine transport component, and both components are energized by ATP or a related derivative (Kadner and Winkler, 1975).

The loss of most methionine transport activity has little effect on the size of the methionine pool (Kadner and Watson, 1974), because L-methionine can be produced by transsulfuration from L-cysteine. Conversely however, methionine transport activity is partially controlled by the intracellular methionine pool (in addition to repression) (Kadner, 1975). Although well known in fungi, this method, termed transinhibition by Cuppoletti and Segel (1974) is rather uncommon in bacteria.

Although cells of E. coli have not been shown to possess a binding protein for methionine, they do synthesize a protein which binds L-cystine

in a competitive manner with diaminopimelic acid. Cystine transport takes place by a dual component system displaying different affinities (Berger and Heppel, 1975). Within cells of Salmonella typhimurium a similar two-component pattern occurs for L-methionine uptake (Ayling and Bridgeland, 1972), but a tri-component system may obtain for L-cystine transport (Baptist and Kredich, 1977). Control of the latter process is exerted by sulfur limitation, and the presence of *O*-acetyl-L-serine and the cysB gene product, suggesting that the L-cystine transport system is part of the cysteine regulon (Kredich, 1971).

The (Trans)sulfuration Pathway in Green Plants

L-methionine synthesis in green plants proceeds primarily by a unidirectional transsulfuration involving L-cystathionine as an intermediate (Giovanelli and Mudd, 1971; Datko et al., 1974a,b), and to a lesser extent by direct sulfuration from sulfide (Giovanelli and Mudd, 1967; Datko et al., 1977). Similar to that in bacteria, transsulfuration involves a γ-replacement and β-elimination, i.e. from L-cysteine to L-homocysteine (Giovanelli and Mudd, 1971). In both transsulfuration and direct sulfuration the preferred α-aminobutyryl donor is *O*-phosphohomoserine, although *O*-acetylhomoserine is utilized slowly (Datko et al., 1974b, 1977).

Conversely prokaryotes such as Escherichia coli, Salmonella typhimurium, and Bacillus subtilis cannot use *O*-phosphohomoserine for either transsulfuration or direct sulfuration (Datko et al., 1974b, 1977). They instead perform both processes using *O*-succinyl-L-homoserine or *O*-acetyl-L-homoserine (Rowbury, 1964; Rowbury and Woods, 1964b; Kaplan and Flavin, 1966; Wiebers and Garner, 1967; Flavin and Slaughter, 1967; Flavin, 1975). Datko et al. (1977) have suggested that the predominance of the transsulfuration route over that of direct sulfuration in green plants may be due to the relative availabilities of L-cysteine and sulfide.

The Transsulfuration Pathway in Animals

L-methionine is an essential ingredient in the diet of animals ranging from protozoons such as Tetrahymena (Kidder and Dewey, 1945; Dang and Cook, 1977) to mammals (Young and Maw, 1958). As in the enteric bacteria and green plants, transsulfuration involves L-cystathionine as an intermediate and is irreversible, but in the opposite direction. L-methionine reacts with ATP via methionine adenosyltransferase to form *S*-adenosylmethionine, which produces *S*-adenosylhomocysteine under catalysis by methyl transferase. This organosulfur compound is hydrolyzed via adenosylhomocysteinase to form free L-homocysteine, which reacts with L-serine via cystathionine-γ-synthetase to yield L-cystathionine (Selim and Greenberg, 1959). Cleavage takes place by cystathionine-γ-lyase to form L-cysteine, 2-oxobutyrate, and ammonia (Matsuo and Greenberg, 1958a).

The (Trans)sulfuration Pathways in Fungi

Most fungi appear to carry out both β- and γ-replacements, and γ- and β-eliminations, and thus can transsulfurate in either direction (Flavin, 1962; Flavin and Slaughter, 1964; Delavier-Klutchko and Flavin, 1965b). However product formation is substantially in the direction of L-homocysteine (and hence L-methionine). In cells of Neurospora crassa and Aspergillus nidulans methionine is synthesized by a γ-replacement involving *O*-acetylhomoserine and L-cysteine, to form L-cystathionine and acetate, and a β-elimination to yield L-homocysteine, pyruvate, and ammonia (Nagai and Flavin, 1966; Kerr and Flavin, 1969, 1970; Paszewski and Grabski, 1974, 1975).

Cells of Saccharomyces cerevisiae carry out a direct sulfhydration of *O*-acetylhomoserine via OAH sulfhydrylase, forming L-homocysteine and acetate, as the main pathway of L-methionine synthesis (Cherest et al., 1969; Savin and Flavin, 1972; Masselot and Surdin-Kerjan, 1977). This

pathway is also present in cells of N. crassa and Asp. nidulans (Wiebers and Garner, 1967a,b; Kerr and Flavin, 1970; Paszewski and Grabski, 1973), but Kerr (1971) has suggested that in these fungi it is a secondary route appreciably utilized only when exogenous sulfur is limiting.

The Utilization of Djenkolic Acid by Heterotrophic Bacteria

The use of L-djenkolic acid as a sulfur source by bacteria is anomalous. Roberts et al. (1955) found it neither to support growth nor to inhibit sulfate uptake in cells of Escherichia coli, but Ellis et al. (1964) reported that it represses sulfate activation in this species, and postulated that djenkolic acid acts via cleavage to L-cystine.

Cells of Salmonella typhimurium were determined to use djenkolic acid as a sole source of sulfur, but the generation time was twice that of cells grown on L-cysteine (Dreyfuss and Monty, 1963a). Furthermore repression of the sulfate reduction pathway by L-cysteine was reported to be derepressed by djenkolic acid (Dreyfuss and Monty, 1963b; Kredich and Tomkins, 1966). However Pardee (1968) found growth of S. typhimurium to be poor with djenkolic acid as the sulfur source, and moreover subsequent sulfate uptake was greater the longer the cells remained on the thioacetal. Kredich (1971) has proposed that the derepression noted by himself and others is triggered not by djenkolic acid, but by the concomitant sulfur starvation.

It may well be the amino acid is a species-specific sulfur source, for cells of Brucella suis exhibited near-normal growth with L-djenkolic acid as the only exogenous sulfur compound present (Rode et al., 1951).

The Catalytic Activities of γ- and β-Cystathionases

Both cystathionine-β-lyase (E.C. 4.4.1.8) and cystathionine-γ-lyase (E.C. 4.4.1.1) catalyze the cleavage of a variety of organic disulfides, thioethers, and other organosulfur compounds. Greater study has been

made on enzyme E.C. 4.4.1.1, a multifunctional pyridoxal-phosphate-dependent protein (Binkley, 1955; Matsuo and Greenberg, 1958b) whose activities have resulted in extensive nomenclatural synonymy (Florkin and Stotz, 1973). Names and catalyses include: (a) cystathionine-γ-lyase, L-cystathionine cysteine lyase (deaminating), or γ-cystathionase, from the γ-elimination of L-cystathionine to L-cysteine, 2-oxobutyrate, and ammonia (Carroll et al., 1949; Matsuo and Greenberg, 1958a); (b) L-homoserine dehydratase or L-homoserine hydrolyase (deaminating), from the cleavage of L-homoserine to 2-oxobutyrate, ammonia, and water (Binkley and Olson, 1950; Matsuo and Greenberg, 1958a); (c) L-cystine desulfhydrase, from the cleavage of L-cystine to thiocysteine, pyruvate, and ammonia (Cavallini et al., 1960; Flavin, 1962); and (d) L-cysteine desulfhydrase or L-cysteine hydrogen sulfide lyase (deaminating), from the cleavage of L-cysteine to hydrogen sulfide, pyruvate, and ammonia (Binkley, 1950; Binkley and Okeson, 1950; Kumagai et al., 1975).

Cystathionine-γ-lyase also catalyzes the cleavage of several other thioethers, disulfides, and thioacetals, among them allo-cystathionine, L-lanthionine, L-homocystine, and L-djenkolic acid (Binkley, 1950; Flavin, 1962; Greenberg et al., 1964). Binkley (1950) proposed that the cysteine moiety is the specific point of attack, and supported this thesis by noting when allo-cystathionine is cleaved to L-homocysteine, the product is not susceptible to further attack. Flavin (1962) extracted a cystathionase from cells of Neurospora crassa which catalyzed the cleavage of L-cystine to pyruvate and the alkyl hydrogen disulfide, thiocysteine. The latter compound is a labile one which decomposes spontaneously to L-cysteine and hydrogen sulfide. In an analagous manner L-djenkolic acid was predicted to cleave catalytically to pyruvate and *S*-thiomethylcysteine, the latter then decomposing spontaneously to L-

cysteine, formaldehyde, and hydrogen sulfide (Greenberg et al., 1964).

Enzyme E.C. 4.4.1.8, known variously as cystathionine-β-lyase, β-cystathionase, and cystathionine-L-homocysteine lyase (deaminating), also requires pyridoxal phosphate as a prosthetic group (Flavin and Slaughter, 1964; Florkin and Stotz, 1973). Substrates which are subject to catalytic cleavage vary widely depending upon the source of the enzyme. L-cystathionine is universally cleaved by a β-elimination to L-homocysteine, pyruvate, and ammonia (Delavier-Klutchko and Flavin, 1965a, b). L-djenkolic acid is also efficiently cleaved by all such β-cystathionases, presumably to the same products as derive from the activity of rat liver γ-cystathionase, *S*-thiomethylcysteine, pyruvate, and ammonia (Greenberg et al., 1964; Delavier-Klutchko and Flavin, 1965a; Giovanelli and Mudd, 1971).

However, whereas the β-cystathionase extracted from cells of Neurospora crassa rapidly catalyzes the cleavage of L-lanthionine and L-cystine (Flavin and Slaughter, 1964), the enzyme from Escherichia coli cells and that from spinach tissue do so slowly (Delavier-Klutchko and Flavin, 1965a; Giovanelli and Mudd, 1971). The amino acid *S*-methyl-L-cysteine (but not L-cysteine) is a good substrate for the bacterial enzyme, but not for the enzyme from spinach. Other good substrates for the cystathionine-β-lyase from E. coli cells are allo-cystathionine and D-cystine; additional poor substrates include *O*-succinyl-L-homoserine, *O*-phosphohomoserine, DL-homoserine, DL-methionine, and L-homocystine (Delavier-Klutchko and Flavin, 1965a).

Amino Acid and Organosulfur Utilization by Cyanobacteria

Substantial evidence exists that a variety of L-amino acids is taken up by many blue-green bacteria. Asparagine serves as a nitrogen source for several marine cyanobacteria, including the red-pigmented Phormidium

persicinum (Pintner and Provasoli, 1958; Van Baalen, 1962), and arginine and glycine do likewise for both nitrogen-fixing and non-fixing species (Wyatt et al., 1971). Glycine and glutamine support the heterotrophic growth of cultures of Chlorogloea fritschii (Fay and Fogg, 1962). Threonine (Maclean et al., 1965) and leucine (Singer and Doolittle, 1975) are transported and utilized by auxotrophs of Anacystis nidulans, as are phenylalanine and tryptophan by auxotrophs of Synechococcus cedrorum (Kaney and Jhabvala, 1975) and Agmenellum quadruplicatum (Ingram et al., 1972), respectively.

Several amino acids including L-methionine are readily taken up by cells of Phormidium luridum and Fremyella diplosiphon (Crespi et al., 1970). Methionine serves as a precursor of organic molecules in cells of Anabaena variabilis (Botham and Pennock, 1971), but as a sole sulfur source it allows only indifferent growth by cultures of this species and Anacystis nidulans (Prakash and Kumar, 1971). The derivative L-methionine-DL-sulfoximine is transported into cells of A. cylindrica and A. variabilis, where it binds and inactivates glutamine synthetase, the enzyme catalyzing the assimilation of newly fixed nitrogen (Stewart and Rowell, 1975; Ownby, 1977).

Cells of Anabaena flos-aquae use *O*-succinylhomoserine or *O*-acetylhomoserine (but not *O*-phosphohomoserine) as the aminobutyryl donor in the transsulfuration pathway from L-cysteine to L-homocysteine (and in turn to L-methionine) (Datko et al., 1974b). In addition extracts from cells of Agmenellum quadruplicatum exhibit no *O*-phosphohomoserine sulfhydrylase activity, ruling out direct sulfhydration with OPH as the α-aminobutyryl donor (Datko et al., 1977). A methionine-requiring mutant of A. nidulans does not grow on sulfate alone, or with L-cystathionine or L-homocysteine substituted for the L-methionine. No evidence exists

that homoserine-*O*-transsuccinylase is controlled via repression/derepression by L-methionine, and this inability has been offered by Delaney et al. (1973) as further support that transcriptional control is generally lacking in cells of the cyanobacteria (Hood and Carr, 1971, 1972; Carr, 1973).

The methionine analog, ethionine, appears to be particularly detrimental to blue-green bacteria. DL-ethionine is about 100 times more inhibitory to the growth of cultures of Synechococcus cedrorum and Anabaena cylindrica than to the growth of cultures of eukaryotic phytoflagellates such as Euglena gracilis and Ochromonas danica (Aaronson and Ardois, 1971). Mutants of Plectonema boryanum and Nostoc sp. MAC resistant to L-ethionine have been isolated, but no ethionine incorporation occurs in cells of the former, and uptake and (or) assimilation of L-methionine also are (is) diminished (Hentschel et al., 1978).

Acquired sulfur is not only assimilated into amino acids and protein, but also into other cellular material, including lipid. Benson (1963) determined that a significant amount of chloroplast sulfur was found as plant sulfolipid (α-sulfoquinovosyl diglyceride). This compound has been reported in cells of Anacystis nidulans and Anabaena variabilis (Nichols et al., 1965; Hirayama, 1967). The route of synthesis is still unknown, but the suggestion has been made that PAPS and/or sulfite serves as the direct precursor (Benson, 1971; Goodwin, 1971).

E. FORMATION OF CONDENSED PHOSPHATE

General Considerations

Griffith et al. (1977) have asserted that it is not the stable orthophosphate, but the thermodynamically unstable condensed phosphate, which is vital to life in aquatic ecosystems. Long-chain polyphosphate can

serve as the precursor for trimeta- and orthophosphate, and the phosphorus turnover rate is very rapid by microorganisms in aquatic ecosystems. Given the small amount of phosphorus necessary to sustain abundant life, Griffith et al. (1977) feel that prevention of the eventual eutrophication of man-inhabited lakes is almost impossible.

In chemical structure condensed phosphates are polymers of orthophosphate with phosphoanhydride linkages that are thermodynamically equivalent to those of ATP (Meyerhof et al., 1953; Yoshida, 1955). The most commonly occurring condensed phosphate of biological origin is polyphosphate, an unbranched linear polymer of general formula $M_{n+2}P_nO_{3n+1}$. Polyphosphate is alkali-stable and acid-labile, hydrolyzable to orthophosphate in hot acid. The cyclic condensed phosphates ($M_nP_nO_{3n}$) of common biological occurrence are primarily tri- and tetrametaphosphates, and to a lesser extent penta- and hexametaphosphates, all of which convert to the linear polyphosphates in strong alkali. No other form of condensed phosphate is found as a common biological product. The structural formulae for some condensed phosphates are shown in Figure 1, after Thilo (1959); that author also reviewed the condensed phosphates (Thilo, 1962).

Although it was once believed there were two polyphosphate fractions in yeast (and other) cells, one form soluble and the other form insoluble in cold trichloroacetic acid (TCA) (Wiame, 1949), Katchman and Van Wazer (1954) and Langen and Liss (1958) suggested differently. Using differential extraction Langen et al. (1962) showed that under normal conditions long-chain polyphosphate is formed initially, and then is broken down into progressively shorter molecules, finally into orthophosphate. During the rapid uptake of "overcompensation" too, high molecular weight polyphosphate is formed first, then reduced to low molecular weight poly-

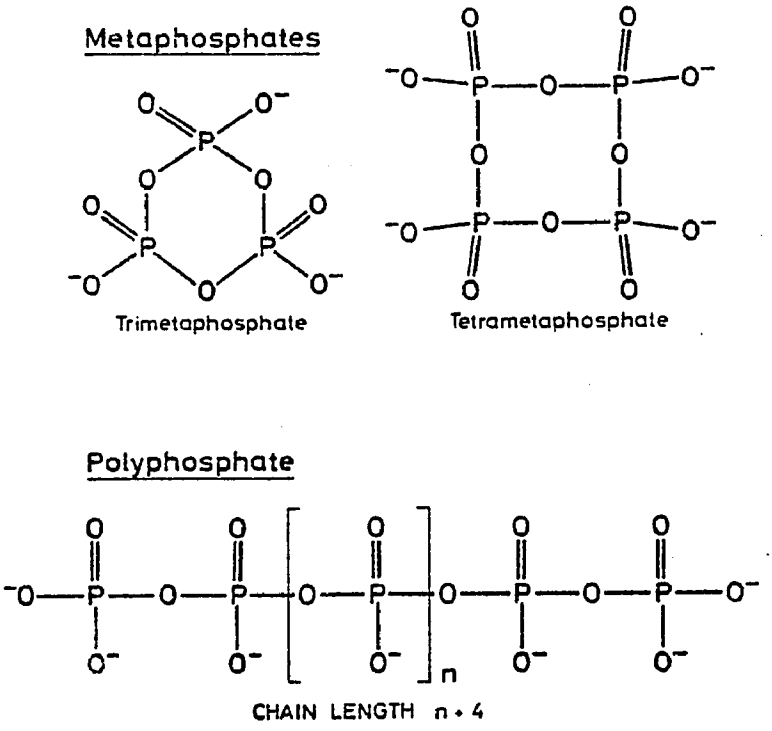


Figure 1. The structures of the prevalent biologically produced condensed phosphates

phosphate (Liss and Langen, 1962).

Metaphosphates are cold-TCA-soluble, but most of the polyphosphate is not. Chain lengths extend to about 300 in yeast cells (Liss and Langen, 1960), and up to 500 in cells of Enterobacter (Aerobacter) aerogenes (Harold, 1963b). Pure polyphosphate is acid-soluble regardless of chain length (Harold, 1966), but this substance is a very strong polyanion and bears negative charges even at extremely acid pH (Van Wazer, 1958). Thus polyphosphate is not only strongly basophilic and most retains basic dyes at pH 1 (nucleic acids and short-chain polyphosphate destain at pH 3.5) (Fuhs, 1969), but also it possesses a high adsorption affinity for positively charged macromolecules (Wiame, 1949). High molecular weight polyphosphate will coprecipitate with protein (Katchman and Van Wazer, 1954), bind with cell wall polygalactosamine (Harold, 1962a), etc., and so remain cold-TCA-insoluble.

The polyanionic nature of the polymer is probably the reason that both inorganic cations, most commonly magnesium and calcium (Konig and Winkler, 1948; Widra, 1959; Friedberg and Avigad, 1968), and RNA (Konig and Winkler, 1948; Winder and Denneny, 1956; Widra, 1959; Dirheimer et al., 1963; Ebel et al., 1963; Stahl and Ebel, 1963) have been found tightly complexed with the polyphosphate. Although most authors have felt the RNA complexes were extraction artifacts due to ionic bonding, nucleic acid strands have been observed in cells fixed for electron microscopy to be embedded within polyphosphate bodies (Voelz et al., 1966; Jensen and Sicko, 1974). Furthermore Smith et al. (1954) reported that while polyphosphate bodies took up as much as 20% of the volume of nutrient-deficient cells of E. aerogenes, "meta"phosphate accounted for less than 1% of the cellular dry weight. Therefore whether the sizable organic component, including RNA, lipid, and protein, reported in poly-

phosphate granules of various bacteria by Widra (1959) and Friedberg and Avigad (1968) is fact or artifact is still an open question. In contrast similar granules isolated from members of the ciliate protozoon, Tetrahymena pyriformis, were shown to consist entirely of calcium magnesium pyrophosphate (Rosenberg, 1966).

Volutin

Meyer (1904) first observed metachromatically staining inclusion bodies in cells of Spirillum volutans, which he named volutin granules (they were otherwise referred to as Babes-Ernst granules). Although Liebermann had chemically identified "meta"phosphate in yeast cells in 1888, it was not until the mid-1930s that researchers began to link volutin with condensed phosphate. Macfarlane (1936) rediscovered meta/polyphosphate in yeast, and Jeener and Brachet (1944) found that when inorganic phosphate was added to such cells starved for phosphorus, a massive amount of basophilic material -- which they incorrectly assumed to be RNA -- rapidly accumulated in the cells. Wiame (1946a,b) and Schmidt et al. (1946) identified this material as "meta"phosphate, and soon thereafter Wiame (1947a,b) demonstrated that in yeast cells the basophilic substance, the metachromatic granules, and polyphosphate were one and the same. Konig and Winkler (1948) and Ebel (1949) did likewise using bacterial cells. By the use of TCA extraction Hughes et al. (1963) showed that the bulk of the polyphosphate in cells of Chlorobium thiosulfatophilum was localized in granules which were both metachromatic and electron-dense.

Polyphosphate Formation and Degradation

The reversible transfer of the terminal phosphate group between polyphosphate and adenosine triphosphate, catalyzed by the enzyme polyphosphate kinase (Yoshida and Yamataka, 1953; Kornberg et al., 1956; Kornberg,

1957; Muhammed, 1961; Levinson et al., 1975), has been shown to be the solitary route of long-chain polyphosphate synthesis. However it appears that while ATP is required to polymerize orthophosphate into polyphosphate, in most cases ATP is not produced upon polyphosphate degradation to orthophosphate (Harold, 1962b). Intact, energy-generation-blocked cells of Neurospora crassa do not degrade polyphosphate to form ATP (Harold, 1962b). Moreover in cells of Corynebacterium xerosis and Enterobacter aerogenes polyphosphate degradation is hydrolytic, the cleavage of terminal orthophosphate groups catalyzed by one or more polyphosphatases, dissipating the bond energy rather than retaining it (Muhammed et al., 1959; Harold and Harold, 1965).

Occurrence of Polyphosphate in Traditional Bacteria

Since the earliest attempts at electron microscopy preceded the chemical identification of volutin, an effort was made to link electron scattering bodies observed in myco- (Lembke and Ruska, 1940), coryne- (Morton and Anderson, 1941), and other bacteria (Knaysi and Mudd, 1943), with the classical metachromatic granules, and with the work then in progress on the nature of volutin in yeast cells. Wiame (1946a) noted the great similarity of metachromatic bodies in cells of yeast and Corynebacterium diphtheriae, and Konig and Winkler (1948) confirmed them as the same granules which were highly electron-scattering. Macrary (1951) identified these inclusions as polyphosphate bodies. Sall et al. (1956) further investigated polyphosphate accumulation and disappearance in cells of C. diphtheriae, and thereafter (Sall et al., 1958) they correlated variation in amount of polyphosphate with the stages of the cell division cycle.

Similar studies were done to determine the nature of the granules in the mycobacteria (Knaysi et al., 1950; Winder and Denneny, 1954, 1955,

1956, 1957; Mudd et al., 1956; Spitznagel and Sharp, 1959; Drews, 1960a, b).

Much of the knowledge of bacterial polyphosphate metabolism was derived from studies with Enterobacter (Aerobacter) aerogenes. Smith et al. (1954) and Wilkinson and Duguid (1960) examined volutin production in cells of this species, and noted certain effects which would later receive more attention. (a) Little if any polyphosphate was formed under normal conditions, but it accumulated when growth decreased or was limited by some nutrient imbalance, e.g. a nitrogen or sulfur deficiency (but not a carbon, phosphorus, magnesium, or potassium deficiency). (b) Polyphosphate was very rapidly formed when exogenous orthophosphate was made available to phosphorus-starved cells. (c) Large polyphosphate granules diminished in size as normal growth was restored after a period of inhibition. (d) Although the granules of nutrient-deficient cells occupied up to 20% of the cell volume, total "meta"phosphate constituted less than 1% of the cellular dry weight.

Widra and Wilburn (1959) observed the volutin granules of E. aerogenes by electron microscopy. Such inclusions were lacking in mutants blocked for polyphosphate synthesis (Harold and Harold, 1963).

Harold (1963a) determined that under certain conditions polyphosphate accounted for most of the total phosphorus in cells of E. aerogenes. Further investigation revealed that nucleic acid synthesis inhibited that of polyphosphate, and moreover stimulated polyphosphate degradation by inorganic polyphosphatase (Harold, 1963a; Harold and Harold, 1965). If growth and nucleic acid synthesis were hindered by nutrient exhaustion (e.g. sulfur), polyphosphate degradation was inhibited in consequence, and the polymer accumulated. The accumulation was halted upon resumption of normal growth, and phosphate was quantitatively transferred from poly-

phosphate to the nucleic acid. Little or no phosphate derived from RNA during the polyphosphate accumulation in cells of E. aerogenes, rather the source was exogenous orthophosphate (Harold, 1963a). These results differ from those obtained with cells of Neurospora crassa, which indicated that RNA degradation was an important source of polyphosphate phosphorus (Harold, 1960).

Harold (1964) demonstrated that the rapid phosphate uptake and polyphosphate deposition in phosphorus-depleted cells -- which he termed the "polyphosphate overplus" phenomenon -- was correlated with a much elevated level of polyphosphate kinase. During exponential growth such cells contained low levels of polyphosphate kinase, alkaline phosphatase, and polyphosphatase, but when the cells depleted the available phosphorus, all three enzyme levels markedly rose, at least the first two by derepression (Harold, 1964). Subsequent exposure to exogenous orthophosphate led to an immediate and rapid synthesis of polyphosphate, which was independent of nucleic acid synthesis (Harold, 1964).

When grown in TRIS-buffered medium containing a high level of orthophosphate (75 mM), cells of E. aerogenes were particularly sensitive to sulfur deprivation in triggering polyphosphate synthesis. On this basis sulfate, sulfite, sulfide, cyst(e)ine, and oxidized or reduced glutathione (all 0.7 mM sulfur) served as sole sulfur sources, whereas methionine, homocyst(e)ine, cyastathionine, cysteic acid, cysteamine (all 0.7 mM sulfur), lipoic acid, thiamin, biotin, pantothene (all 0.3 mM sulfur), and coenzyme A (0.15 mM sulfur) did not (Harold and Sylvan, 1963). A competition for metabolic pathways, or a certain level of a cellular organosulfur compound was suggested as suppressing polyphosphate deposition. Preference was given to the latter alternative because suppression was non-stoichiometric. The level of such a controlling substance would

be decreased by sulfur starvation, allowing polyphosphate to be deposited. Evidence was presented that oxidized glutathione was the controlling compound in wild-type cells. Cystine-requiring mutants grown in medium containing orthophosphate but lacking a sulfur source did not deplete their glutathione pool or accumulate polyphosphate (Harold and Sylvan, 1963).

Investigations with other bacteria have either confirmed the above pattern, or provided evidence of variation. Szulmajster and Gardiner (1960) demonstrated that cells of a Clostridium species elaborated polyphosphate under anaerobic conditions when creatinine served as both the nitrogen source and electron donor. Pine (1963) showed an increase in "meta"phosphate was linked to the exhaustion of cellular sulfur stores in Escherichia coli. Studies using Chlorobium thiosulfatophilum revealed that polyphosphate was present in all stages of growth, approximately 67% of total cellular phosphorus as polyphosphate in normally growing cells, and an even greater proportion in older ones. Such high amounts of polyphosphate may have been the result of little or no polyphosphatase normally present in the cells (Hughes et al., 1963). Further work provided evidence that polyphosphate was degraded in cells of this species by phosphorylating ADP to ATP, which in turn was hydrolyzed via ATPase to release inorganic phosphate and regenerate ADP (Cole and Hughes, 1965). Under phosphorus-limiting conditions polyphosphate formation was suppressed, growth continued normally, and large amounts of sulfur were released into the medium (Hughes et al., 1963).

Voelz et al. (1966) studied polyphosphate overplus in cells of Myxococcus xanthus, and reported variation in granule formation under varying conditions. In some cases deposition of polyphosphate was by embedment in nucleic acid fibrils. The authors predicted that exponential and

stationary phase cellular organization differ. Friedberg and Avigad (1968) found that 50% of the total phosphorus was contained within polyphosphate bodies of cells of Micrococcus lysodeikticus, although the weight of these inclusions was 10% of cellular dry weight. The authors reported both significant organic contribution to the polyphosphate fraction, especially protein and lipid, and marked differences in shape, pattern, and organization of the bodies from those of other microorganisms. Terry and Hooper (1970) discovered that sulfur-, nitrogen-, or phosphorus-depleted cells of Nitrosomonas europaea accumulated polyphosphate at the expense of the nucleic acids (differing from the pattern in cells of Enterobacter aerogenes), but upon restoration of the deficient nutrient, phosphate was transferred from polyphosphate to nucleic acid.

The polyphosphate kinase from cells of Arthrobacter atrocyaneus was purified and characterized by Levinson et al. (1975). Isolated polyphosphate bodies from cells of Desulfovibrio gigas were shown to consist of short-chain magnesium polyphosphate (perhaps tripolyphosphate) and organic carbon, but no sulfur or nitrogen was detected (Jones and Chambers, 1975). Conditions for the accumulation of guanosine polyphosphate nucleotides in cells of Brevibacterium ammoniagenes have been outlined by Sato and Furuya (1977).

Occurrence of Polyphosphate in Cyanobacteria

One of the first electron microscopic studies of blue-green bacteria, and the first tentative identification of polyphosphate granules in the division as well, was made by Bringmann (1950). He noted electron-dense and metachromatic inclusions in cells of Lyngbya aerugineo-coerulea and L. amphivaginata, and suggested they were composed of inorganic "meta"-phosphate, DNA, and RNA. The presence of polyphosphate was confirmed in a variety of blue-green bacteria, including the genera Phormidium and

Oscillatoria (Ebel, 1952; Drews and Niklowitz, 1956, 1957; Keck and Stich, 1957; Ebel et al., 1958b).

Correll and Tolbert (1962) found a consistent molar ratio of 7 P : 1 RNA in polyphosphate from actively growing cultures of both Anabaena variabilis and the eukaryotic alga Chlorella pyrenoidosa. Approximately 50% of the total phosphorus was in the form of polyphosphate in cells of the former species, of which 30% was reported to be complexed with RNA. Talpasayi (1963), using both the toluidine blue stain of Keck and Stich (1957) and the lead nitrate-ammonium sulfide method of Ebel et al. (1958a), identified polyphosphate bodies in several species of cyanobacteria, including Anabaena cylindrica, Chlorogloea fritschii, and Mastigocladus laminosus. Such inclusions are few and small in younger filaments and spores, large and numerous in older filaments, and absent from terminal cells, mature spores, and heterocysts. The presence of RNA in polyphosphate bodies was predicted from the pattern obtained with fluorescence microscopy.

Jensen (1968) demonstrated that lead-sulfide-stained granules, electron-dense bodies, and electron-transparent spaces resulting from TCA-extraction in cells of Nostoc pruniforme all corresponded to the presence of polyphosphate. He reported on the pattern of formation of polyphosphate bodies in cells of Plectonema boryanum (Jensen, 1969), which is quite similar to that later found in free and lichenized cells of a Nostoc sp. (Boissiere, 1976). Fuhs (1969) determined that polyphosphate bodies in cells of Oscillatoria borneti and Anabaena cylindrica exhibit refractive indices very similar to that of synthetic potassium polyphosphate.

Batterton and Van Baalen (1968) found no polyphosphate after rapid orthophosphate uptake by cells of Anacystis nidulans. However, Niemeyer

and Richter (1969), using pulse-labeled ^{32}P-orthophosphate in phosphorus-starved cells of the same species, showed that most of the rapidly acquired phosphate is incorporated into condensed phosphate, very largely moderate- to long-chain polyphosphate, and trimetaphosphate. Bornefeld et al. (1974), utilizing Anacystis nidulans, and Sicko-Goad and Jensen (1976), using Plectonema boryanum, reported that the greatest increase in phosphorus is in the TCA-insoluble polyphosphate fraction upon restoration of that element to starved cells. Other studies on the phosphate metabolism in cells of P. boryanum have included the ultrastructural sequence of polyphosphate overplus (Jensen and Sicko, 1974), the effects of fixation and post-staining on fine structure (Jensen et al., 1977), x-ray energy dispersive analysis of polyphosphate bodies (Sicko-Goad et al., 1975; Kessel, 1977), and the effects of pH and cations on rapid phosphate uptake (Sicko-Goad et al., 1978).

Kuhl (1962) observed that many algae take up phosphorus far in excess of their needs, then store it (as condensed phosphates) as an internal reservoir. Under conditions of phosphorus deficiency cells of the eukaryotic alga Chlorella make immediate use of polyphosphate, indicating the constitutive nature of the degradative enzyme(s) (Baker and Schmidt, 1964). Eight different species of blue-green bacteria utilized condensed phosphates during growth, and in some cases excreted excess condensed phosphates into the aquatic environment (Davis and Wilcomb, 1968). Assimilation of detergent phosphorus during a 24-hour period by cyanobacterial cells was sufficient to support growth for an additional seven days in medium with no exogenous phosphorus (Stewart and Alexander, 1971).

Sulfur deficiency has been shown to induce the uptake of substantial amounts of orthophosphate, and the elaboration of very large polyphosphate bodies in cells of Synechococcus sp. (Anacystis nidulans) (Lawry

and Jensen, 1979). Successive sulfur- and phosphorus-free growth medium has been used to isolate mutants of this microorganism with an inability to synthesize polyphosphate (Vaillancourt et al., 1978).

F. ULTRASTRUCTURE OF MEMBERS OF THE GENUS SYNECHOCOCCUS AND LIKE GENERA

Electron microscopic investigations of cells of Anacystis nidulans and species of the genus Synechococcus revealed the fundamental cellular architecture of these bacilliform cyanobacteria to be a Feulgen-positive central area, suggested to be in large part nucleic acid, surrounded by a peripheral area containing a few (four or five) well ordered and concentric photosynthetic lamellae or thylakoids (Drews et al., 1961; Ris and Singh, 1961; Menke, 1961, 1966; Echlin, 1964; Hall and Claus, 1965; Lang, 1968; Gantt and Conti, 1969). The definitively descriptive work on cells of Synechococcus lividus by Edwards et al. (1968) was followed up by a freeze-fracture examination corroborating the features described (Holt and Edwards, 1972).

More specialized studies on the fine structure of species of Synechococcus (including Anacystis nidulans) and Agmenellum have been concerned with the cell wall (Drews and Meyer, 1964; Allen, 1968b; Golecki, 1977; Gleason and Ooka, 1978), the photosynthetic membranes (Allen, 1968a), the phycobilisomes (Edwards and Gantt, 1971; Wildman and Bowen, 1974; Cosner, 1978), cell division/separation (Allen, 1968b; Allen and Stanier, 1968; Kunisawa and Cohen-Bazire, 1970; Ingram and Aldrich, 1974; Sturgeon et al., 1975), the possible appearance of microtubules (Bailey-Watts et al., 1968), and AS-1M cyanophage infection (Sherman et al., 1976).

Carbon dioxide deprivation in cells of S. lividus has been shown to lead to complete losses of the tetrapyrrole pigments and thylakoids, and subsequently to cytoplasmic disorder, as seen at the electron microscope

level. Restoration of CO_2 permits a rapid synthesis of pigments and thylakoids, and thereafter leads to a return in cytoplasmic integrity and growth by the population (Miller and Holt, 1977).

MATERIALS AND METHODS

General

"Anacystis" nidulans UTEX 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 (Hughes et al., 1958; Allen, 1968c), buffered at pH 8.3 with 1 g/l glycylglycine (see Appendix 1). Cultures were grown at 40°C under 2600 lux cool white fluorescent illumination supplemented by a 25w incadescent bulb, on a 16-hour light/8-hour dark cycle. Both stock and experimental flasks were aerated through cotton-plugged Pasteur pipettes. Despite proper conditions growth was slow, the generation time varying between 8 and 24 hours.

Exogenous orthophosphate concentrations were determined colormetrically by the Murphy-Riley phosphomolybdic acid reduction technique (Murphy and Riley, 1962; Hayashi, 1976) (see Appendix 2). Solutions were allowed to develop in 2.25 cm D optically matched tubes, and measured directly at 882 nm on a Bausch and Lomb Spectronic 20 spectrophotometer. 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 method, and expressing the amount determined on a per-cell basis. Replicate cell counts were obtained using a Petroff-Hauser chamber.

Short-Term Uptake

Cells one week old were washed three times in phosphorus-free medium under the conditions described. On day six the cells were again washed three times in phosphorus-free medium, and resuspended at a density of

ca. 94 x 10^6 cells/ml in medium containing 0.16 mM (5 mg/l) 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 to promote orthophosphate uptake. The first samples (zero hour) for phosphorus analysis were drawn as quickly as possible after all flasks were inoculated, and thereafter at intervals over a period of six hours.

In a second experiment cells were similarly prepared, but an aliquot was removed from the phosphorus-starved culture and resuspended for a period of ten hours in medium containing 10 µg/ml chloramphenicol (CAP) but no phosphorus. This period was timed to coincide largely with the dark phase, because aqueous solutions of CAP can become toxic in some cases under illumination (Hoxmark and Nordby, 1977). All portions were washed three times in phosphorus- and sulfur-free medium, the untreated cells were resuspended at a density of ca. 90 x 10^6 cells/ml in medium variously containing 0.31 mM, 31 µM, 3.1 µM and zero (10, 1.0, 0.1, and zero mg/l) sulfur as sulfate. The CAP-treated cells were resuspended at a density of 75 x 10^6 cells/ml in medium containing 0.31 mM (10 mg/l) sulfur as sulfate. All cultures contained 0.064 mM (2 mg/l) phosphorus as orthophosphate. Zero-hour samples were removed as quickly as possible after inoculation, and thereafter samples were taken for phosphorus determination at intervals over a period of five hours.

In all experiments deletion of K_2HPO_4 was compensated by the addition of appropriate quantities of KNO_3; of $MgSO_4$ by appropriate amounts of $Mg(NO_3)_2$.

Long-Term Uptake

A. Inorganic Sulfur Sources

Cells one week old were washed four times in sulfur-free medium and

resuspended at a density of 23-26 x 10^6 cells/ml in cultures containing 0.32 mM (10 mg/l) phosphorus as orthophosphate, but no sulfur source. Preliminary experiments had determined that cells can endure 24-30 hours of sulfur starvation with little appreciable change in morphology, pigment content, or total phosphorus level. Following this initial period changes due to sulfur starvation rapidly become manifest. After 24 hours of growth sterile-filtered $MgSO_4$, $K_2S_2O_3$ and $K_2S_2O_5$ were each added to separate flasks at a concentration of 0.31 mM sulfur, and aeration of all flasks was halted.

Since both the thiosulfate and metabisulfite anions are subject to slow oxidation to sulfate in aerated alkaline medium containing catalytic amounts of Zn^{2+} and Cu^{3+} (Postgate, 1963), it was necessary to utilize pre-starved growing cells in need of immediately usable sulfur. A cell-free culture containing 0.31 mM sulfur and 0.32 mM phosphorus was maintained during the experimental period of three days. Daily phosphorus measurements were made and cells were fixed for TEM examination 24 hours after the exogenous sulfur was added (48 hours after inoculation of the cells).

B. Sulfur Starvation

Cells were prepared as in Part A and resuspended at a density of 28-29 x 10^6 cells/ml in cultures containing 0.32 mM, and zero sulfur as sulfate. Exogenous orthophosphate and cellular phosphorus were measured after the first eight hours of growth, then daily for six days. Aliquot volumes necessary to provide readings within the range of precision decreased with time as cell numbers and/or cellular phosphorus increased. An aerated cell-free flask was provided to measure orthophosphate concentration as a function of longstanding. Cells were removed after 47 and

120 hours of growth and fixed for TEM examination.

C. Recovery from Sulfur Starvation

Cells were prepared as in Part A and resuspended at a density of ca. 250 x 10^6 cells/ml in cultures containing either 0.31 mM or zero sulfate, and 0.35 mM (11 mg/l) phosphorus as orthophosphate. The cultures were maintained under normal growth conditions for six days. Exogenous and cellular phosphorus were monitored every other day. At the end of 5½ days the cells cultured with no exogenous sulfur were divided into two equal volumes, the first maintained as before, the other with 10 µg/ml CAP.

After an additional twelve hours all cells were washed three times in sulfur- and phosphorus-free medium, and each aliquot was divided into a pair of flasks containing medium with 0.31 mM sulfate. One flask of each pair contained 0.225 mM (7 mg/l) phosphorus, and the other flask zero phosphorus. Initial counts varied between 15 and 30 x 10^6 cells/ml. Phosphorus was monitored daily for six days. Changes in pigment content were recorded with a Beckman Model 25 spectrophotometer using the whole cell methods of Jones and Myers (1965) and Miller and Holt (1977), with a reading at 708 nm as a correction for light scattering. Cells were removed and fixed for TEM examination after 46, 98, and 144 hours of growth.

D. Organosulfur Sources

Cells were prepared as in Part A and grown in sulfur-free medium for 34 hours, washed thrice again, and resuspended in cultures containing one of several sulfur amino acids at a concentration of 0.31 mM sulfur, and 0.32 mM phosphorus as orthophosphate. Phosphorus was monitored daily for six days, and at the end of this period cells were fixed for TEM observa-

tion. Aliquots were transferred to fresh cultures containing the same organosulfur compound, at a concentration equal to or greater than that present previously, depending upon the response to the compound as a sulfur source during the first six-day period. Again the period of growth was six days, and cells were removed and prepared for ultrastructural examination. The organosulfur compounds used and final concentrations (of sulfur) provided were: L-methionine, DL-homocystine, L-djenkolic acid, L-cysteic acid, and taurine, 0.93 mM sulfur; L-cystine, DL-lanthionine, and reduced glutathione, 0.62 mM sulfur. Control cultures were maintained on 0.31 mM and zero sulfate during both six-day growth periods. All chemicals listed except L-methionine were obtained from Nutritional Biochemical Corporation (ICN Pharmaceuticals, Inc.), Cleveland, Ohio; L-methionine was procured from General Biochemicals (North American Mogul Products Company), Chagrin Falls, Ohio. In another experiment cells were prepared as in the first half of Part D, inoculated at a final density of 50-56 x 10^6 cells/ml into cultures containing 0.31, 1.0, and 2.31 mM (10, 32, and 74 mg/l) sulfur as L-cystathionine (from Calbiochem, LaJolla, California), and grown for seven days. Control populations, phosphorus monitoring, and TEM protocol were as previously described, except that cells were fixed for observation at 47 hours too.

All organosulfur compounds but L-cystathionine were tested for authenticity by dissolving 1-2 mg/ml of each in 10% isopropanol, and running 3-5 µl by one-dimensional ascending chromatography on Whatman Number One paper, with a solvent of 60 n-butanol : 15 glacial acetic acid : 25 water, v/v/v (Smith, 1960). Spots were visualized with a 0.2% solution of ninhydrin in acetone.

Transmission Electron Microscopy

Suspensions of cells were mixed with a few drops of 2% osmium tetraoxide and spun down for fifteen minutes with a clinical centrifuge at full speed. The cells were resuspended in 1% OsO_4 in pH 8.0 Michaelis buffer (see Appendix 3) and stored at $4^{o}C$ for one hour, then a further 100 minutes at room temperature. After fixation the cells were resuspended in 1% aqueous uranyl acetate in buffer for fifteen minutes, dehydrated in a graded ethyl-alcohol 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 War, 1964), and lead citrate (Venable and Coggeshall, 1965), and examined with an Hitachi HU-11E transmission electron microscope at 75 kv. Thick sections ($\geq$ 250 µm) were examined at 100 kv. Electron sensitized negatives were shot on Kodak 4489 Electron Microscope Film (8.3 x 10.2 cm).

Energy Dispersive X-ray Microanalysis

Cells from control, sulfur-deficient, and recovery cultures were removed at periodic intervals, washed once, and air-dried on nylon grids. They were examined with a JEOL-U3 scanning electron microscope on STEM mode, coupled to a Princeton Gamma Tech energy dispersive x-ray spectrophotometer. Both polyphosphate-containing and general cytoplasmic areas of cells were analyzed at an accelerating voltage of 25 kv, a rate varying between 950 and 4500 c.p.s., and a duration of 100 seconds.

RESULTS AND OBSERVATIONS

Short-Term Uptake

Phosphorus determinations were consistent and reproducible. For example in the two short-term studies (varying pH and exogenous sulfate levels) stock culture cells prior to phosphorus starvation contained 6.94 and 6.90 fg P/cell. After three days in phosphorus-free medium such cells contained 1.04 and 1.06 fg P/cell, and after six days 0.72 and 0.69 fg P/cell, respectively.

The rapid increase of phosphorus in cells previously starved for this element is shown over a hundred-fold range in hydrogen ion activity (Figure 2). When the phosphorus-deficient cells were exposed to 0.16 mM exogenous phosphorus, their cellular phosphorus levels increased 5-6 times their previous value (0.72 fg P/cell) in the time required to draw and centrifuge the first samples. Such rapid intake was largely complete within one hour, and the rates slowed substantially but uptake 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 two to three hours, and final (six-hour) cellular accumulation at pH 9.0 was 20% less than that at pH 8.5.

In the second experiment (Figure 3) similarly deprived cells showed a proportionate increase in phosphorus when exposed to 0.064 mM exogenous phosphate. Their internal phosphorus levels increased 3.5-4 times their previous value (0.69 fg P/cell) in the drawing and centrifugation period and achieved most of the remaining increase during the first hour. The range of values through five hours of uptake was narrow, with differences among populations of cells exposed to varying levels of exogenous sulfate manifested during the final time interval only. At the fifth

Figure 2. Increases in cellular phosphorus by P-depleted cells undergoing rapid uptake when resuspended in medium containing 0.16 mM phosphorus as orthophosphate. Phosphorus level of inoculum cells previously maintained on zero exogenous phosphorus for six days (✡); medium with restored phosphorus variously adjusted to pH 7.0 (○), pH 7.5 (●), pH 8.0 (△), pH 8.5 (▲), and pH 9.0 (□).

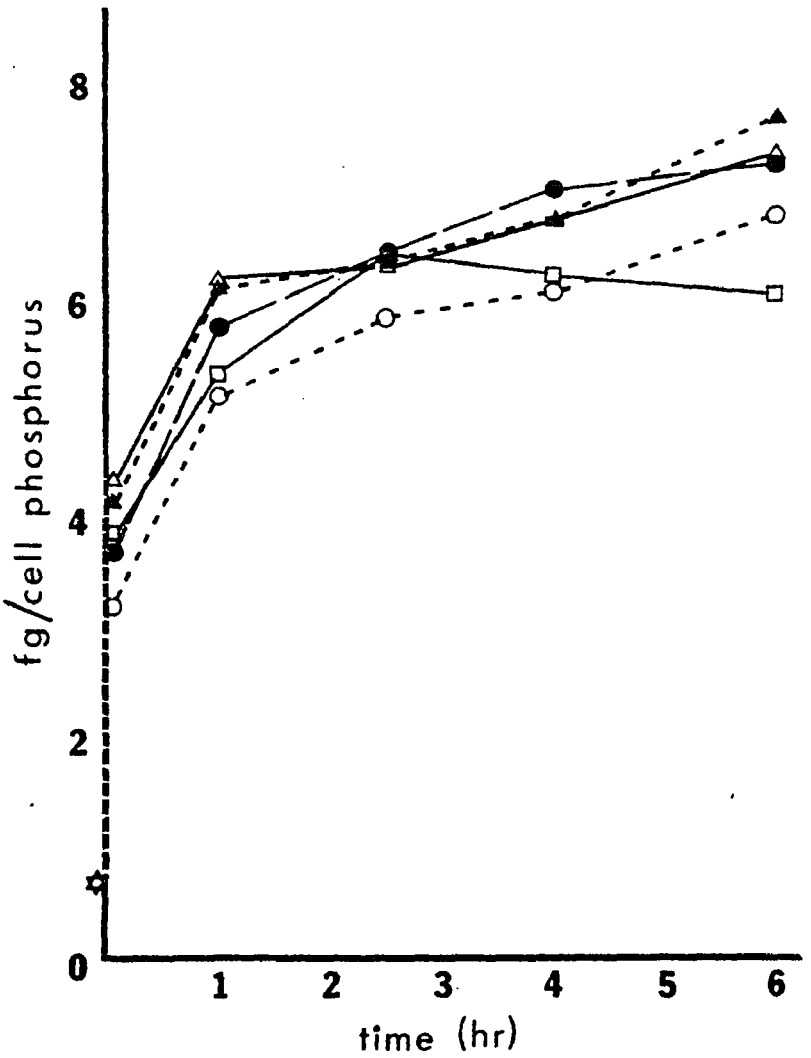
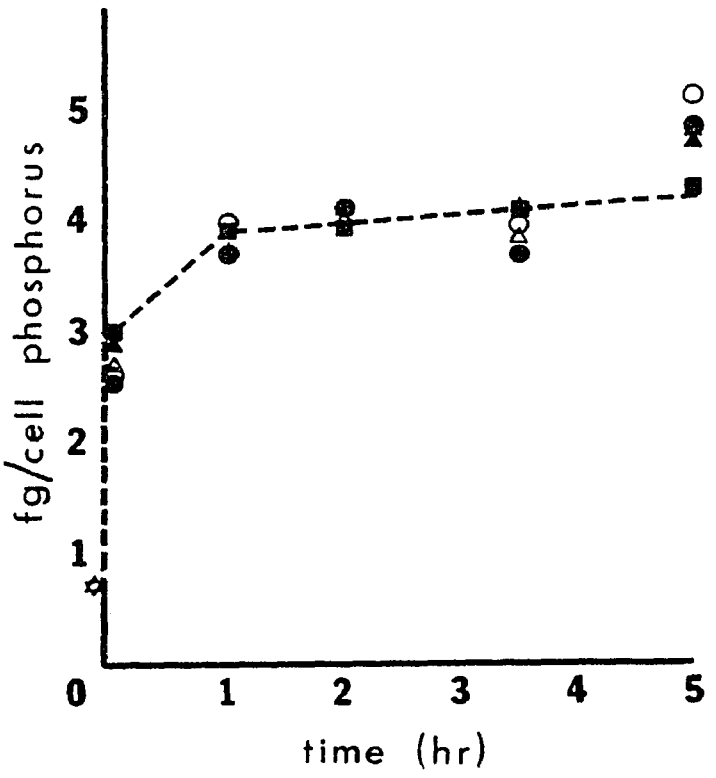

fg/cell phosphorus
0
2
4
6
8
1
2
3
4
5
6
time (hr)

Figure 3. Increases in cellular phosphorus by P-depleted cells undergoing rapid uptake when resuspended in medium containing 0.064 mM phosphorus as orthophosphate. Phosphorus level of inoculum cells previously maintained on zero exogenous phosphorus for six days (✡); medium with restored phosphorus various containing zero (○), 3.1 µM (△), 31 µM (●), and 0.31 mM (▲) sulfate. A second inoculum was pretreated in 10 µg/ml CAP overnight and resuspended in medium with 0.31 mM sulfate (■).


fg/cell phosphorus
0
1
2
3
4
5
time (hr)

hour phosphorus accumulation of cells in sulfur-free medium was more than 9% greater than that of cells in medium containing 0.31 mM sulfate, and cells in medium with 31 and 3.1 µM sulfate manifested intermediate levels. The CAP-treated cells also showed rapid uptake of phosphorus, but accumulation at the fifth hour was 9% less than non-CAP-treated cells in medium containing the same sulfate concentration (0.31 mM). Although four of the populations exhibited increases in cell number, the CAP-treated cells showed no increase whatsoever.

Cells deprived of exogenous phosphorus contained no visible polyphosphate bodies at the ultrastructural level (Figure 4). No differences can be discerned in the size and number of polyphosphate granules among the cells experiencing rapid phosphate uptake with varying levels of exogenous sulfate, including those pretreated with 1 µg/ml CAP (Figures 5-7). Cells starved for six days for phosphorus, then induced to rapid uptake of that element, did not lend themselves to electron microscopy, and in consequence such micrographs lack quality. However, it can be noted that after rapid phosphate uptake polyphosphate bodies were 25% larger than in normally growing cells (cf. Figures 11-14).

Long-Term Studies

A. Inorganic Sulfur Sources

Partial depletion of cellular sulfur, which was necessary to insure that the thiosulfate and metabisulfite anions were taken up and utilized immediately as such, and not after they had been allowed to oxidize the sulfate, gave rise to incongruities in the patterns of exogenous phosphorus disappearance and cellular phosphorus accumulation. Increase in cell number was normal (Figure 8), with day-by-day differences resulting from flask rotation within the growth chamber. However, the concentra-

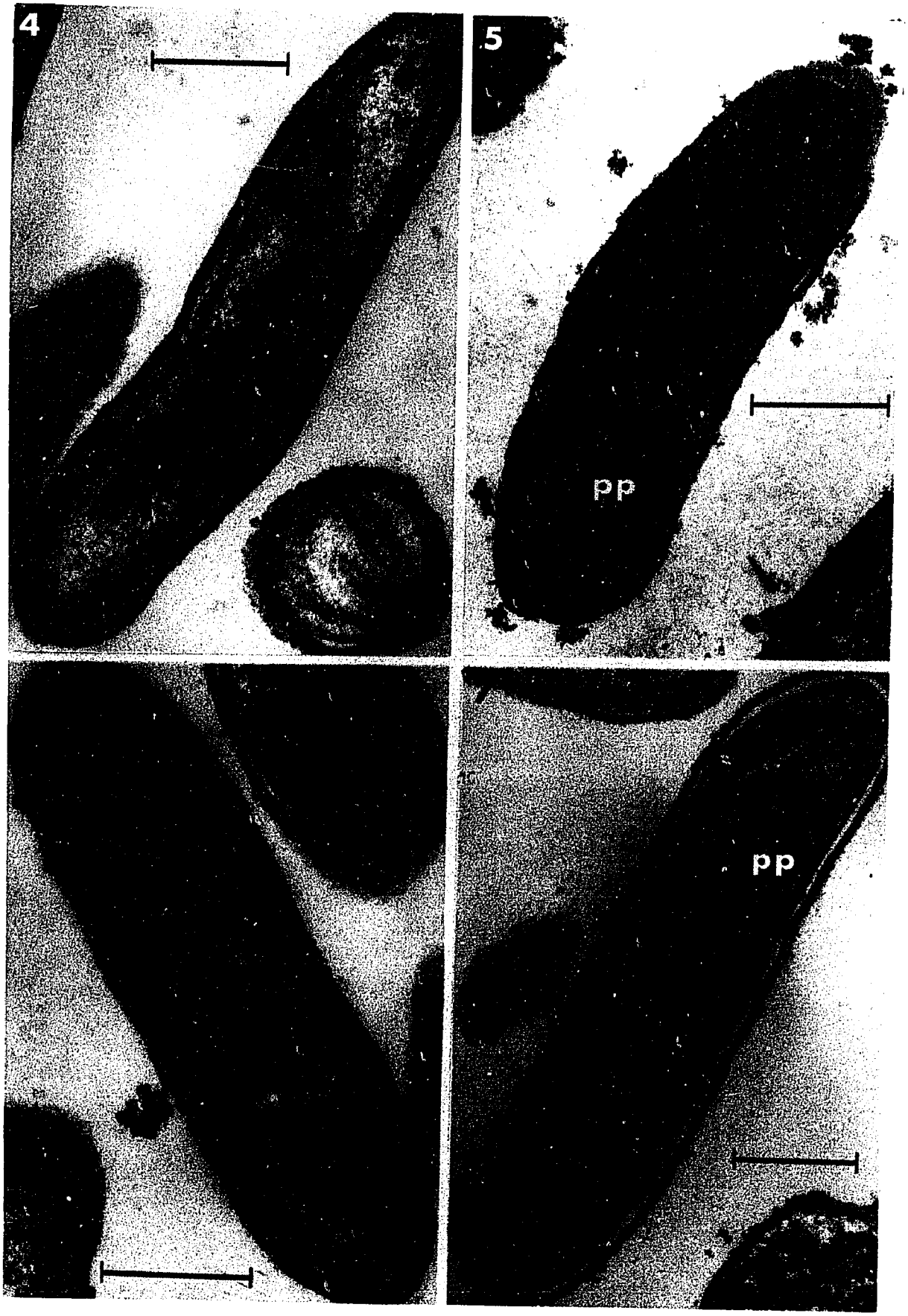
Figure 4. Thin sections of cells grown for six days in medium lacking phosphorus. The marker bar is equivalent to 500 nm.

Figures 5-7. Thin sections of cells grown for six days in medium lacking phosphorus, then resuspended for five hours in medium containing 0.064 mM phosphorus as orthophosphate, and varying concentrations of sulfate. Polyphosphate bodies (pp) are recognized as very electron-dense inclusions; all marker bars are equivalent to 500 nm.

Figure 5. Cells placed in medium lacking sulfur.

Figure 6. Cells placed in medium containing 0.31 mM sulfate.

Figure 7. Cells placed in medium containing 0.31 mM sulfate after having been pretreated overnight in 10 µg/ml CAP.


4
5
pp
pp

tions of exogenous phosphorus markedly decreased after 24 hours, the point at which the various sulfur anions were added to the cultures (Figure 9). Apparently the rapid uptake of sulfur was accompanied or very quickly followed by an uptake of orthophosphate. All cultures showed a concomitant increase in cellular phosphorus, then a decrease after a further 24 hours (Figure 10).

An accounting of total phosphorus per ml is shown in Table 1. Discrepancies appear after the addition of the sulfur anions to the sulfur-deficient cells, which are apparently the amounts of orthophosphate bound to the cell membranes and removed in sequential washings. An attempt to measure this lost phosphorus was made but the amount per wash was either below the limit of precision of the apparatus or not detectable at all.

Electron micrographs of cells fixed 24 hours after the addition of the sulfur anions show no differences in the cellular architecture and the pattern of polyphosphate bodies, whether metabisulfite (Figure 11), thiosulfate (Figures 12, 13), or sulfate (Figure 14) served as the sole sulfur source.

B. Sulfur Starvation

Growth curves of populations growing with various concentrations of exogenous sulfate are shown in Figure 15. In descending order of initial concentration, 0.31 mM, 31 µM, 3.1 µM, and zero sulfur, final (six-day) cell densities were 950, 925, 375, and 287.5 x 10^6 cells/ml.

Daily levels of exogenous phosphorus in the cultures are shown in Figure 16. Rates of loss increased after 24 hours in cultures with 3.1 µM and zero sulfur initial concentrations, but these rates declined after day three. In the case of 31 µM sulfur initial concentration the loss

Figure 8. Cell number per ml of cultures grown with various sulfur anions: 0.15 mM metabisulfite (□); 0.15 mM thiosulfate (△); 0.31 mM sulfate (○). The sulfur ions were added after 24 hours of growth.

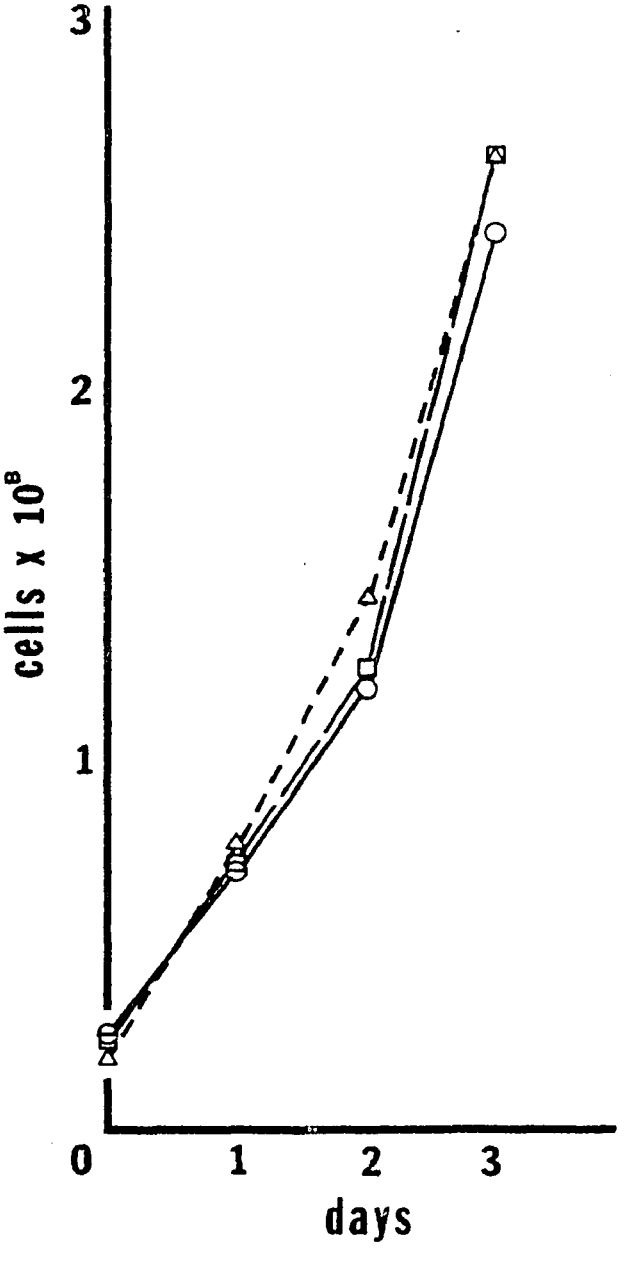

3
2
1
0
cells x 10⁸
1
2
3
days

Figure 9. Disappearance of 0.32 mM (10 mg/l) exogenous phosphorus in cultures containing (after 24 hours) different sulfur anions. Initial concentrations of those anions were: 0.15 mM metabisulfite (□); 0.15 mM thiosulfate (Δ); 0.31 mM sulfate (○); and 0.31 mM sulfate without cells (●). The arrowhead indicates the addition of the inorganic sulfur ions.

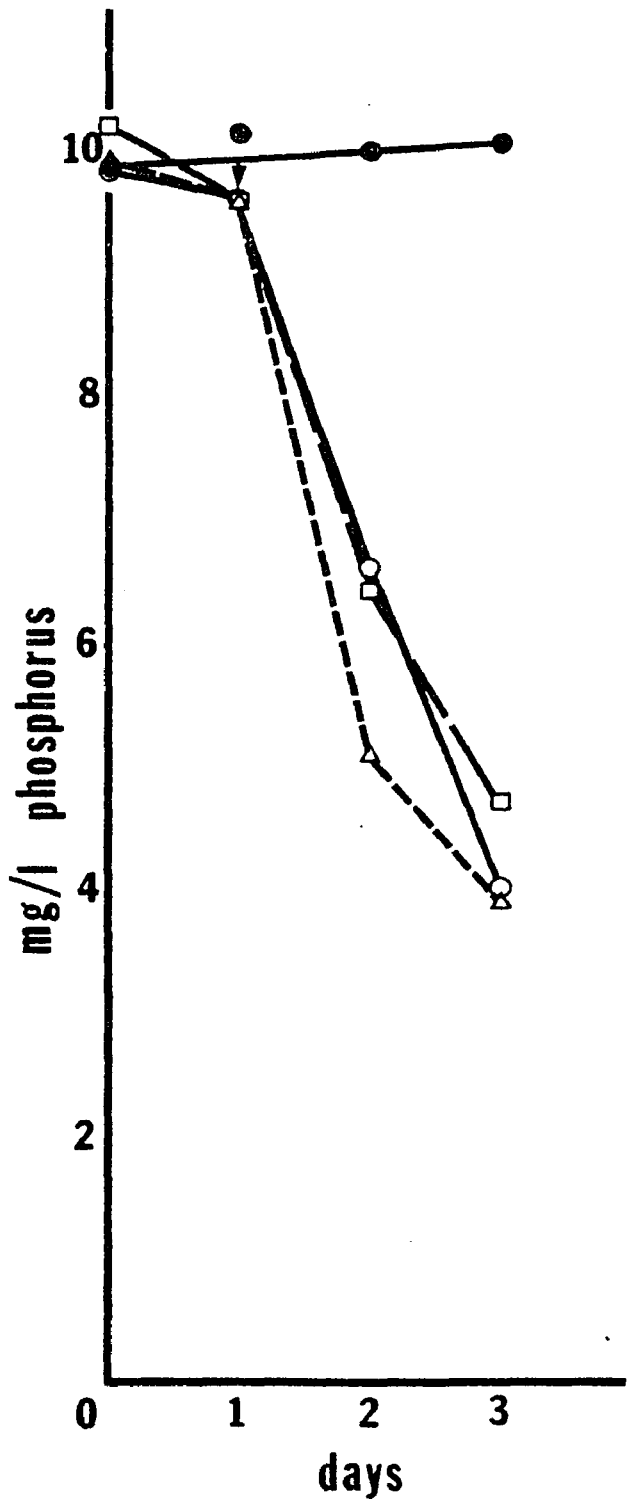
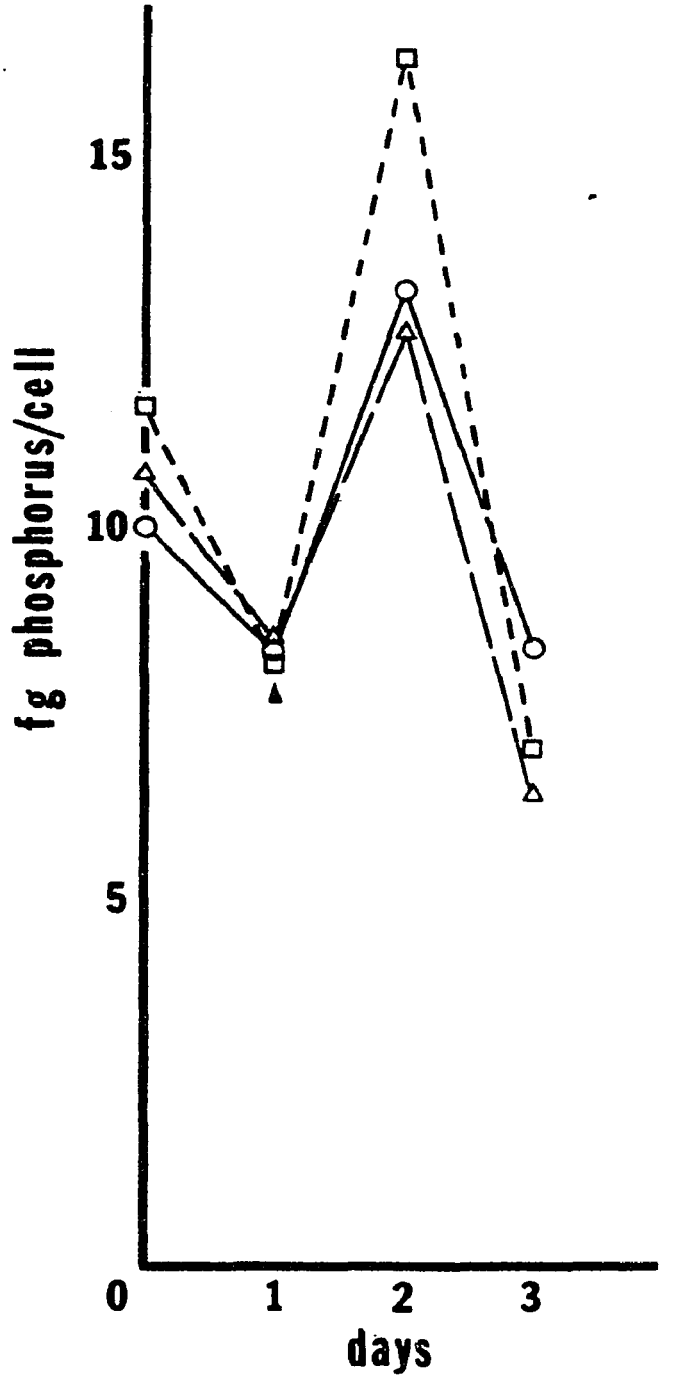

10
8
6
4
2
0
1
2
3
mg/l phosphorus
days

Figure 10. Changes in cellular phosphorus levels by cultures grown on an initial 0.32 mM exogenous phosphorus, and after 24 hours 0.31 mM exogenous sulfur supplied as different anions. Initial concentrations of the sulfur anions were: 0.15 mM metabisulfite (□); 0.15 mM thiosulfate (△); 0.31 mM sulfate (○). The arrowhead indicates the addition of the inorganic sulfur ions.


15
10
5
0
1
2
3
fg phosphorus/cell
days

sulfur anion provided		day zero	day one	day two	day three
SO_4^{2-}	exogenous P/ml	9.79	9.58	6.60	4.02
	cellular P/ml	0.26	0.58	1.28	1.98
	total P/ml	10.05	10.16	7.88	6.00
$S_2O_3^{2-}$	exogenous P/ml	9.89	9.55	5.08	3.90
	cellular P/ml	0.26	0.62	2.34	1.57
	total P/ml	10.15	10.17	7.42	5.47
$S_2O_5^{2-}$	exogenous P/ml	10.15	9.55	6.40	4.69
	cellular P/ml	0.25	0.60	1.48	1.68
	total P/ml	10.40	10.15	7.88	6.37
cell-free	exogenous P/ml	9.83	10.10	9.94	10.02
	cellular P/ml	--	--	--	--
	total P/ml	9.83	10.10	9.94	10.02

Table 1

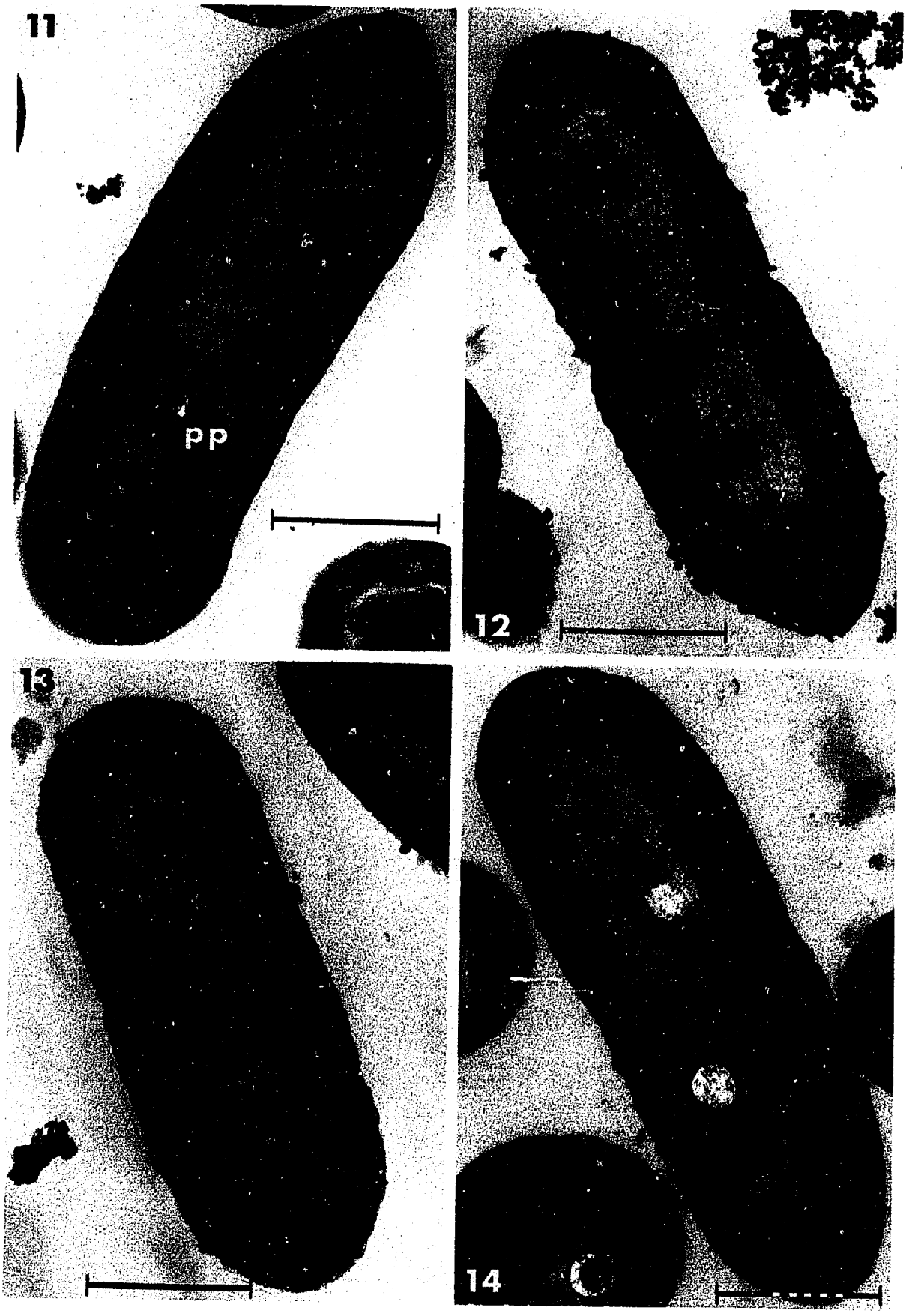
An accounting of total phosphorus per ml culture when various inorganic sulfur ions were provided

Figures 11-14. Thin sections of cells grown for 48 hours with 0.32 mM phosphorus as orthophosphate, but only the latter 24 hours with an inorganic sulfur compound in the medium. Polyphosphate bodies are shown as pp; all marker bars are equivalent to 500 nm.

Figure 11. Cells grown with 0.15 mM metabisulfite.

Figures 12, 13. Cells grown with 0.15 mM thiosulfate.

Figure 14. Cells grown with 0.31 mM sulfate.


11
pp
12
13
14

of phosphorus from the medium was almost complete by day six. Figure 17 represents the cellular phosphorus levels during the six-day period. For 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 fourth day. The phosphorus level of cells grown on 31 µM sulfur initial concentration slowly increased toward the end of the growth period, whereas that of cells grown on 0.31 mM sulfur initial concentration decreased slowly and steadily over the entire six days. Table 2 shows an accounting of total phosphorus per ml. Again significant losses of bound phosphate, increasing with time, by multiple washings are indicated.

Figures 18-21 show representative cells of the four populations after 47 hours of growth. Only the cells with no exogenous sulfur exhibited any substantial cytoplasmic deterioration, including other effects a decrease in the number of thylakoids and an enlargement of polyphosphate granules (Figure 18). The culture from which those cells were removed was already becoming chlorotic at this time. The cells grown on 3.1 µM sulfate initial concentration appear only slightly affected in regard to the size of polyphosphate bodies (Figure 19), and those grown on ten and one hundred times more exogenous sulfate are quite normal in appearance (Figures 20, 21). Figures 22-25 reveal that all sulfur concentrations tested but the highest led to increased phosphate uptake and deposition by 120 hours. 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 their sections as they

Figure 15. Cell number per ml of cultures grown with various concentrations of exogenous sulfate: zero (○); 3.1 µM (△); 31 µM (▲); 0.31 mM (●) sulfur.

cells x 10^8
0
2
4
6
8
10
days
0
1
2
3
4
5
6

Figure 16. Disappearance of 0.32 mM (10 mg/l) exogenous phosphorus in cultures of growing cells. Initial concentrations of exogenous sulfur (as sulfate) were zero (○); 3.1 µM (△); 31 µM (□); 0.31 mM (▲); and 0.31 mM without cells (●).

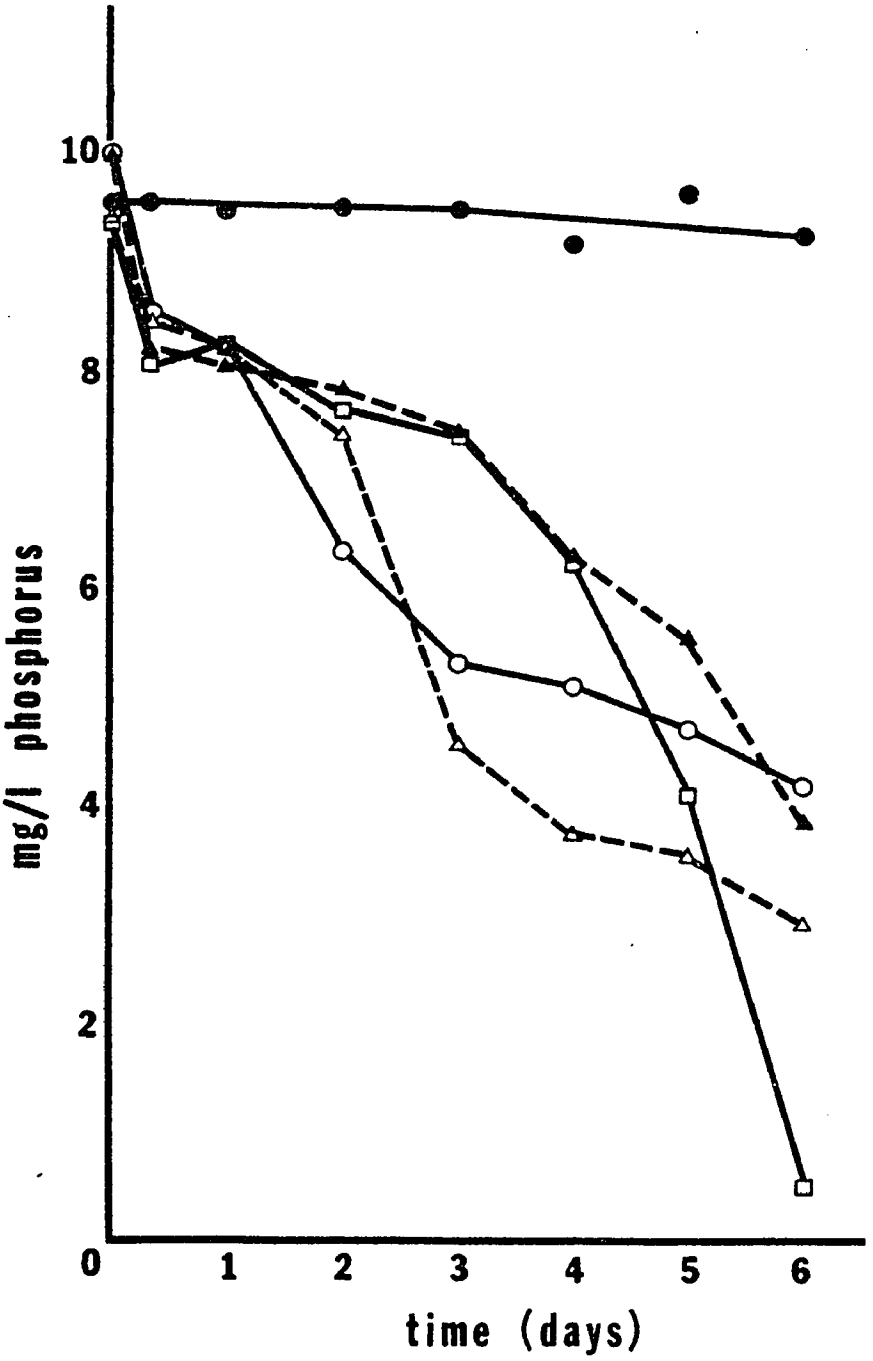
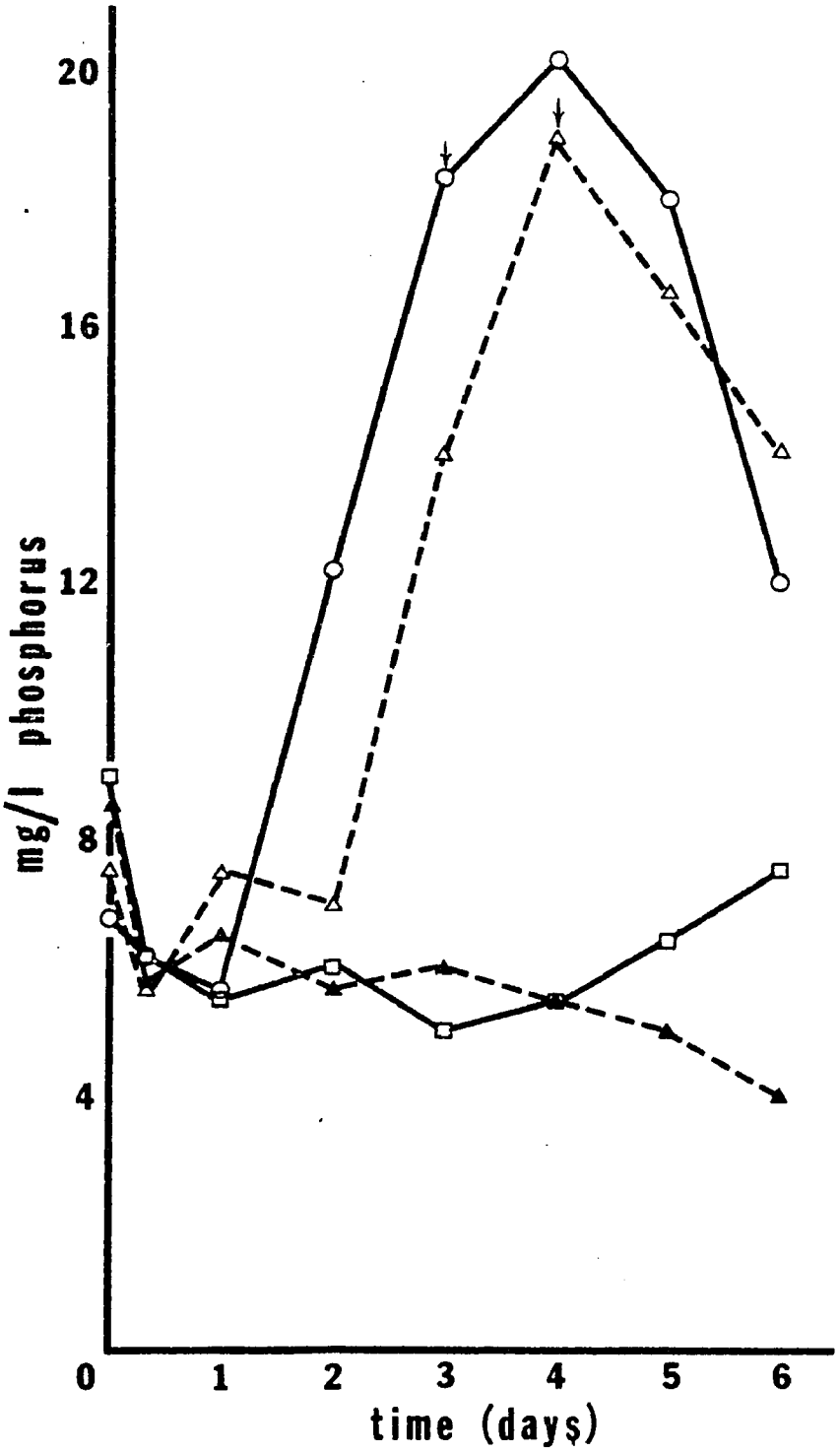

10
8
6
4
2
0
1
2
3
4
5
6
mg/l phosphorus
time (days)

Figure 17. Changes in cellular phosphorus by cultures grown on an initial 0.32 mM exogenous phosphorus (as orthophosphate). Initial concentrations of exogenous sulfur (as sulfate) were zero (○); 3.1 µM (△); 31 µM (□); and 0.31 mM (▲). The arrows indicate the days when those cultures became fully chlorotic.


20
16
12
8
4
0
mg/l phosphorus
1
2
3
4
5
6
time (days)

concentration of exogenous sulfate provided		day zero	+ 8 hrs.	day one	day two	day three	day four	day five	day six
zero sulfur	exogenous P/ml	9.96	8.52	8.19	6.35	5.31	5.07	4.66	4.15
	cellular P/ml	0.20	0.24	0.43	2.17	2.98	3.65	3.81	3.44
	total P/ml	10.16	8.76	8.62	8.52	8.29	8.72	8.47	7.59
0.31 mM SO_4^{2-}	exogenous P/ml	9.38	8.42	8.18	7.38	4.56	3.72	3.57	2.89
	cellular P/ml	0.21	0.26	0.39	1.02	3.59	5.19	5.60	5.26
	total P/ml	9.59	8.68	8.57	8.40	8.15	8.91	9.17	8.15
31 µM SO_4^{2-}	exogenous P/ml	9.36	8.04	8.24	7.64	7.40	6.24	4.09	0.47
	cellular P/ml	0.25	0.26	0.39	0.68	1.45	2.34	3.94	7.00
	total P/ml	9.61	8.30	8.63	8.32	8.85	8.58	8.03	7.47
3.1 µM SO_4^{2-}	exogenous P/ml	9.95	8.15	8.06	7.80	7.43	6.27	5.51	3.84
	cellular P/ml	0.25	0.28	0.42	0.74	1.36	2.30	3.10	3.71
	total P/ml	10.20	8.43	8.48	8.54	8.79	8.57	8.61	7.55
cell-free	exogenous P/ml	9.53	9.56	9.45	9.48	9.47	9.11	9.60	9.16
	cellular P/ml	--	--	--	--	--	--	--	--
	total P/ml	9.53	9.56	9.45	9.48	9.47	9.11	9.60	9.16

Table 2

An accounting of total phosphorus per ml culture when various concentrations of inorganic sulfate were provided

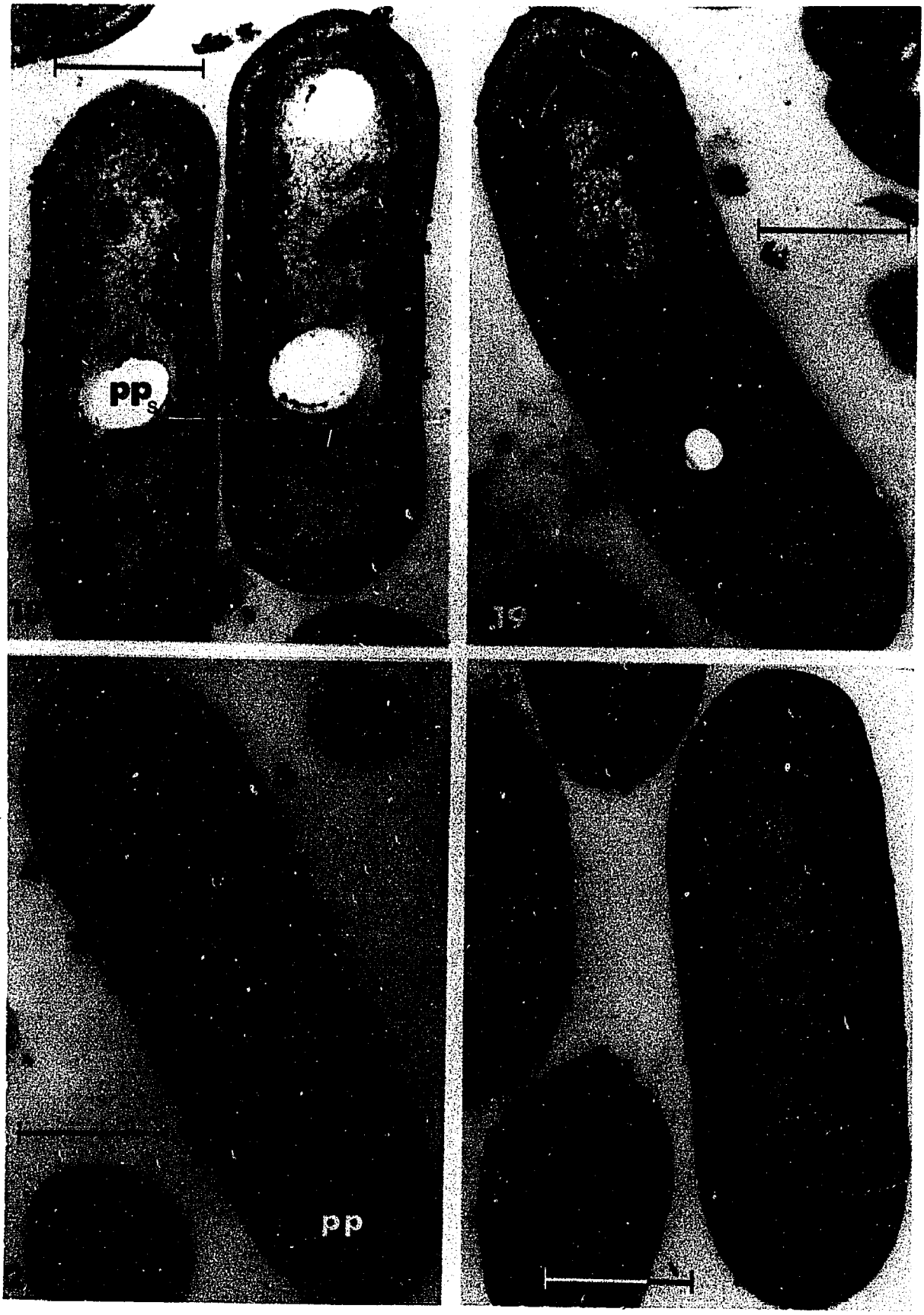
Figures 18-21. Thin sections of cells grown for 47 hours in medium containing 0.32 mM orthophosphate, but varying amounts of sulfate. Polyphosphate bodies (pp) are intensely electron-dense, and their remnant spaces (pp_s) are electron-transparent. All marker bars are equivalent to 500 nm.

Figure 18. Cells from medium lacking sulfur.

Figure 19. Cells from medium containing initially 3.1 µM sulfate.

Figure 20. Cells from medium containing initially 31 µM sulfate.

Figure 21. Cells from medium containing initially 0.31 mM sulfate.


PPs
pp

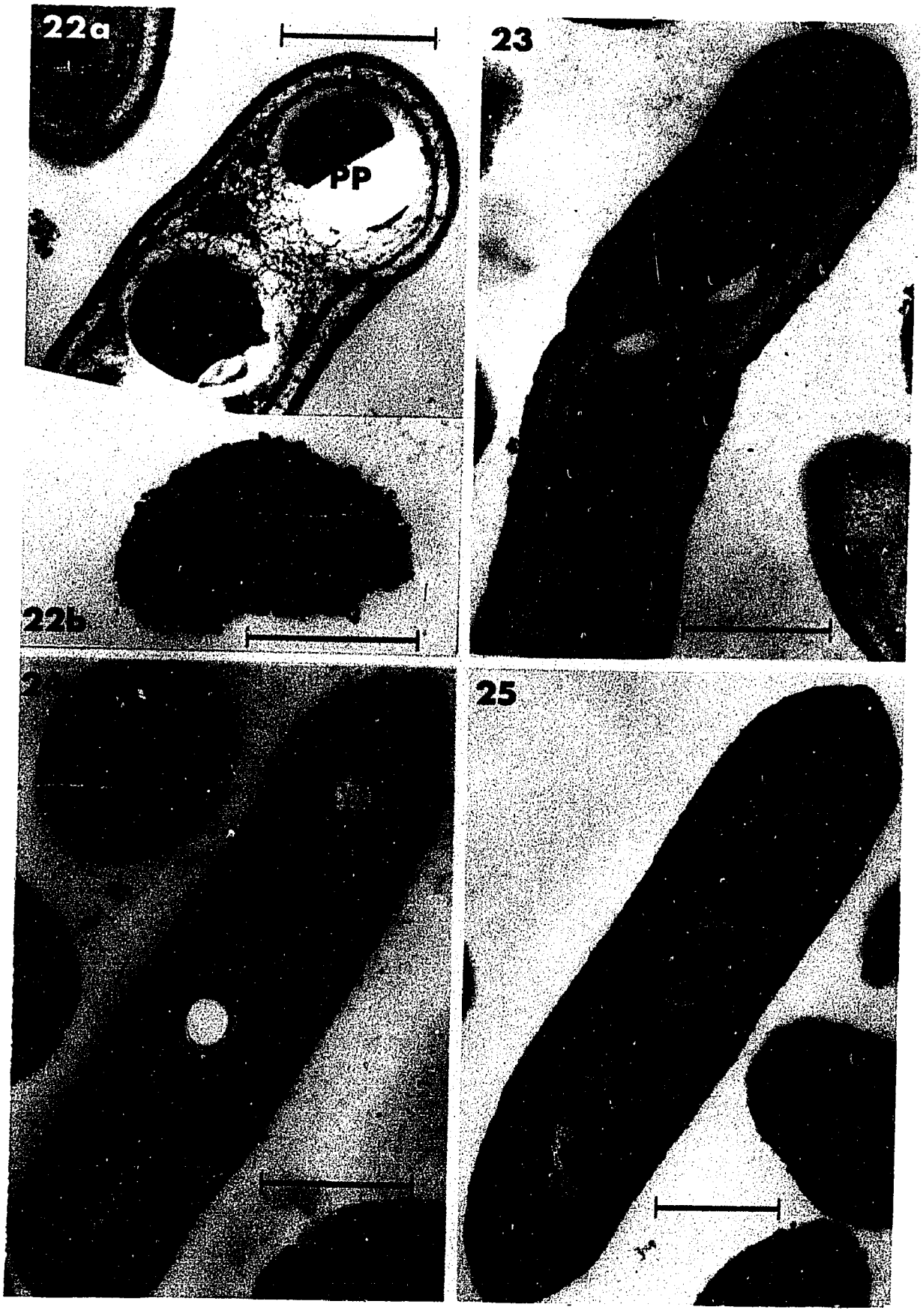
Figures 22-25. Thin sections of cells grown for 120 hours in medium containing 0.32 mM orthophosphate, but varying amounts of sulfate. Polyphosphate bodies are designated (pp); all marker bars are equivalent to 500 nm.

Figure 22 a,b. Cells from medium lacking sulfur.

Figure 23. Cells from medium containing initially 3.1 µM sulfate.

Figure 24. Cells from medium containing initially 31 µM sulfate.

Figure 25. Cells from medium containing initially 0.31 mM sulfate.


22a
PP
22b
23
25

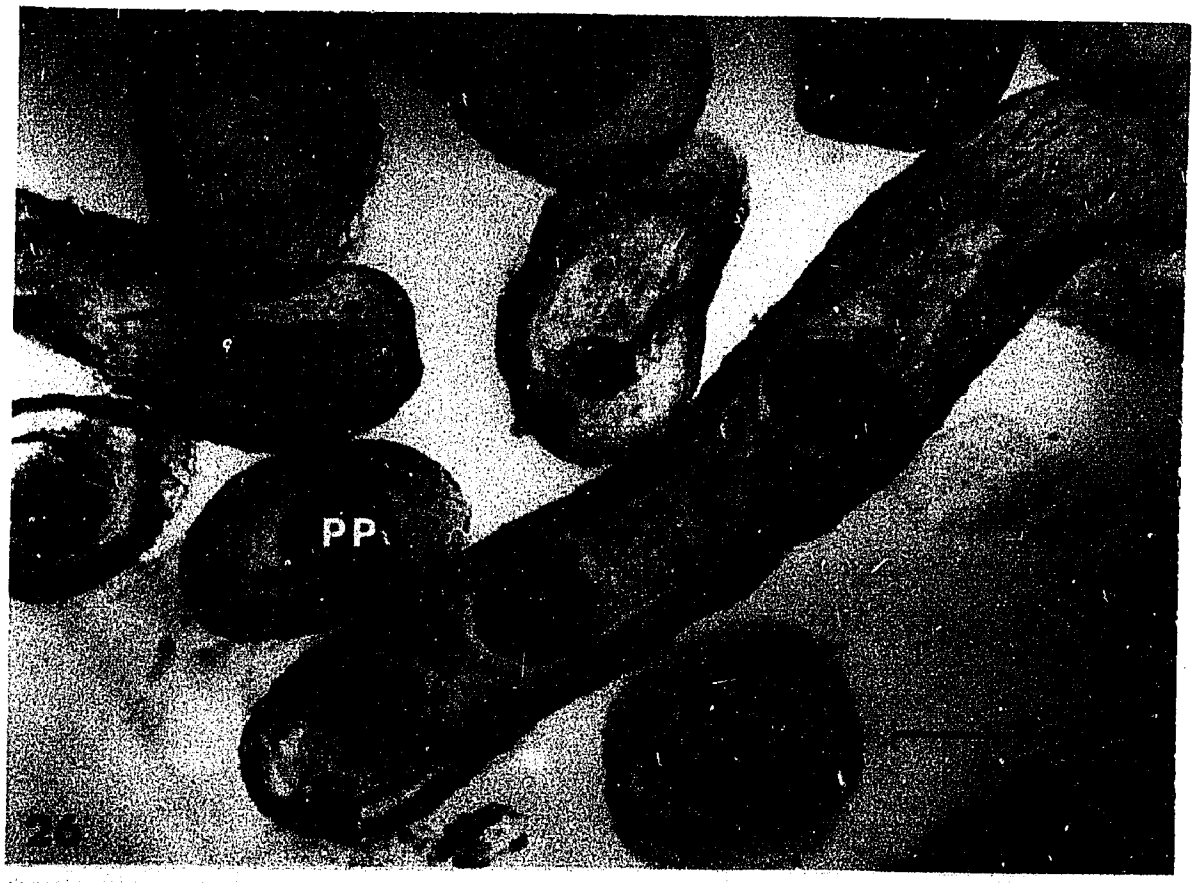
increase in size (Jensen, 1968), the number of spaces seen in fields of sectioned cells significantly increased (Figures 18, 22). The intact polyphosphate bodies of sulfur-starved cells are better visualized in thick sections (Figure 26), and their mean diameter can be seen to lie in the 400-500 nm range after 120 hours. After six days of starvation in this experiment, only the mean size of the polyphosphate bodies increased. Number of bodies per cell remained rather constant between two and four (normal cells exhibit two or three, and rarely four, polyphosphate granules per cell).

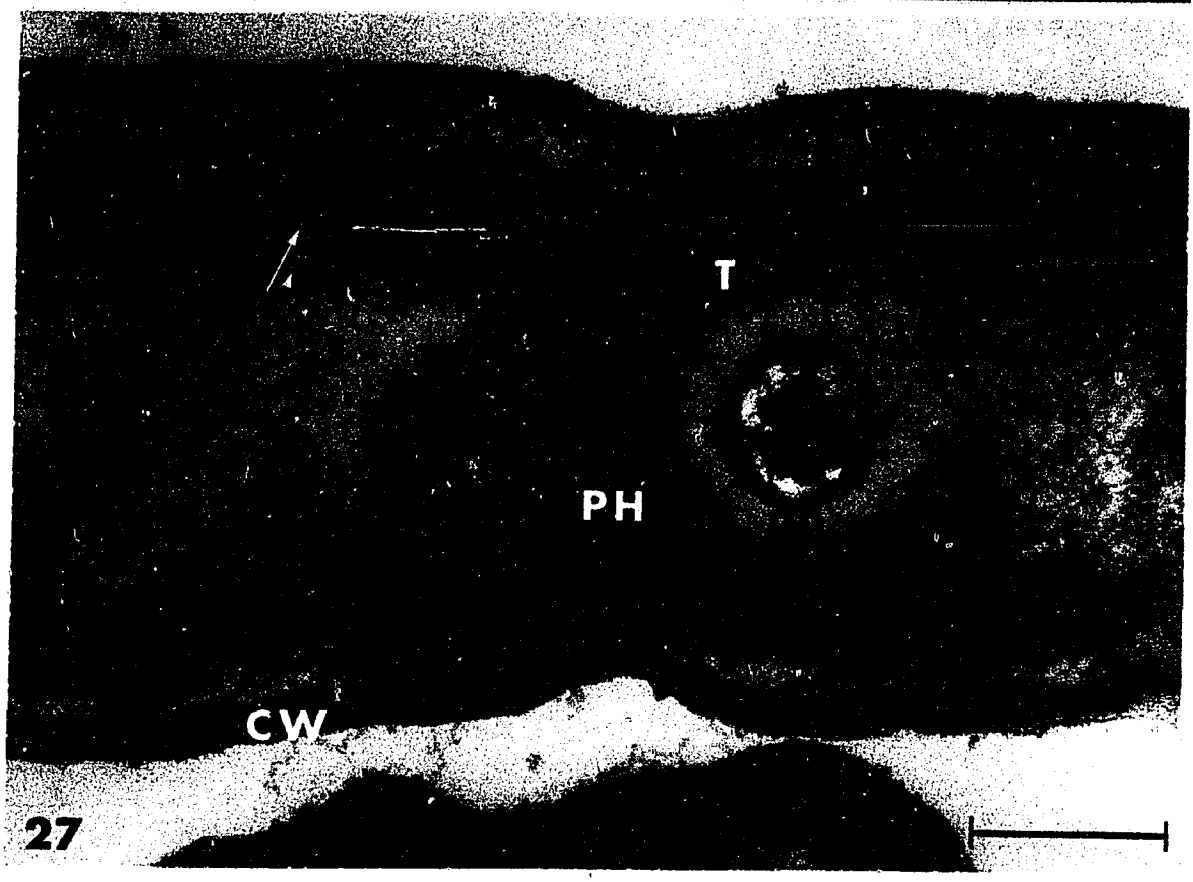
An association of nucleic acid fibrils with the polyphosphate bodies is seen in Figure 27. Within the polyhedral body is a minibody similar to those appearing in cells of Plectonema boryanum, and reported to be polyphosphate by Jensen and Sicko (1974). Of particular interest is the doubling or tripling in diameter (from 8-10 to 23 nm) of the minibodies as the effects of sulfur starvation become manifest.

In order to determine if sulfur deficiency generally causes an increase in polyphosphate in cyanobacteria, cells of Chlorogloea fritschii were similarly prepared and cultured variously with 0.31 mM, 31 μM, and zero sulfate in Allen's medium. After 72 hours cells were removed and fixed for TEM observation. Control cells exhibited many small polyphosphate bodies (Figures 28, 29) and in sulfur-deficient cells these inclusions did increase in size (Figures 30-33). Some of the electron transparent spaces, however, may have been once occupied by poly-β-hydroxybutyric acid granules rather than polyphosphate bodies. The most dramatic increase was that of the size of the cyanophycin or multi-L-arginyl-poly(L-aspartic acid) granules in cells grown in the absence of exogenous sulfate. This copolymer was produced to such an extent that the indi-

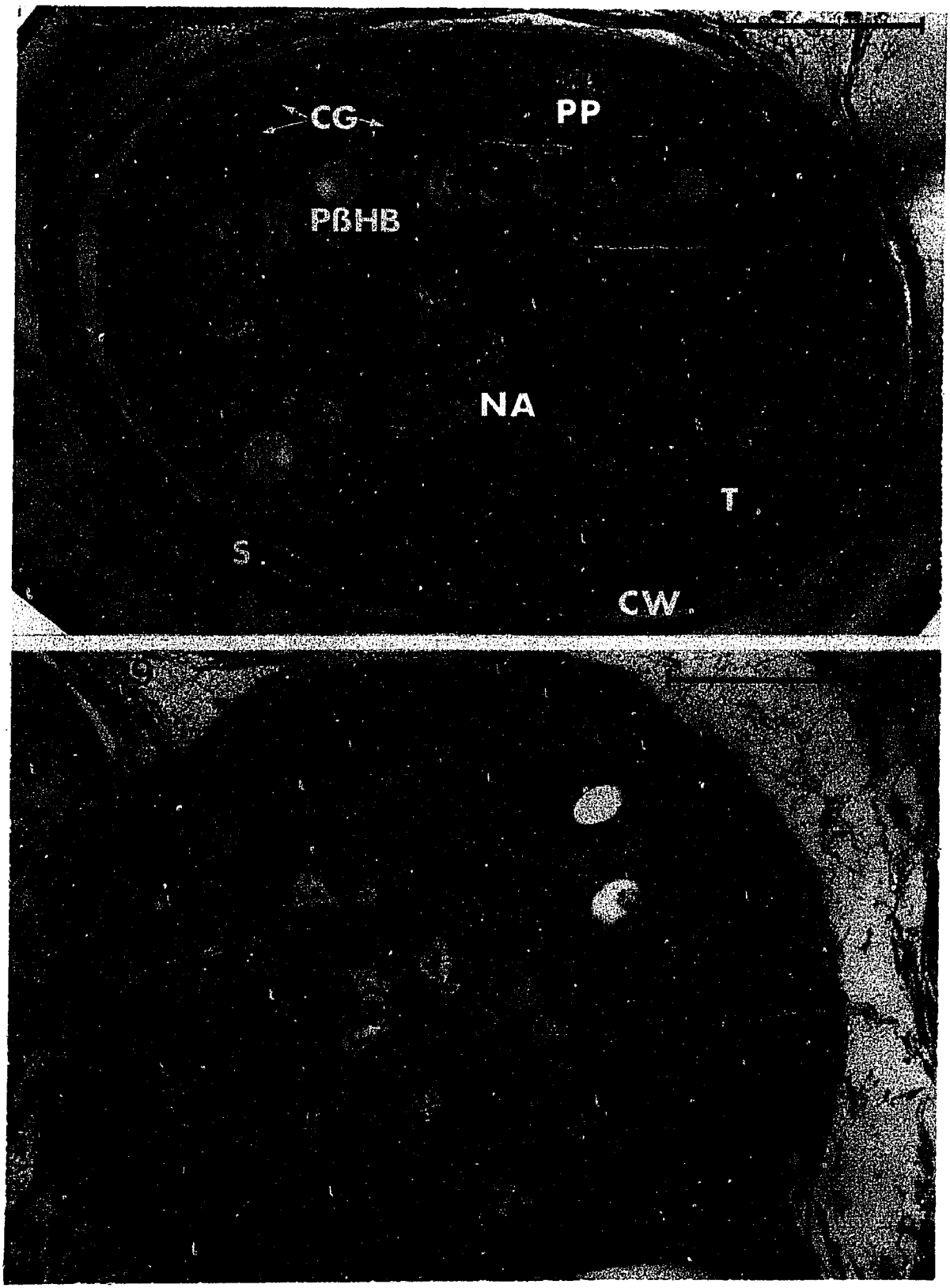
Figure 26. Thick sections (> 200 nm) of cells grown for 120 hours in medium initially containing 0.32 mM orthophosphate and zero sulfur display well preserved polyphosphate bodies (PP) of increased size. The marker bar is equivalent to 500 nm.

Figure 27. Nucleic acid fibrils (NA) are often observed to be continuous with polyphosphate bodies (PP) in thin sections of cells grown at all sulfate 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.

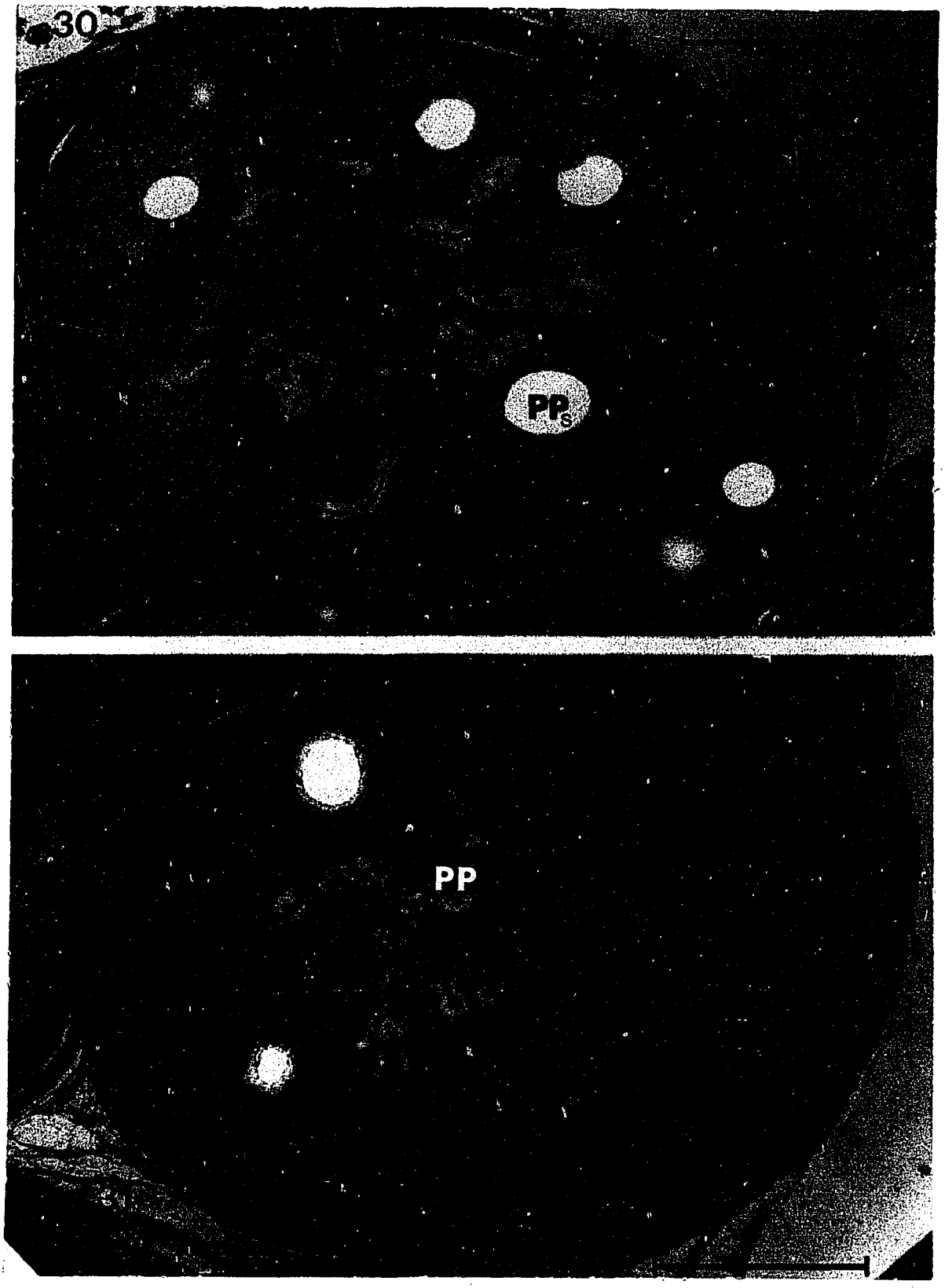

PP
26


T
PH
CW
27

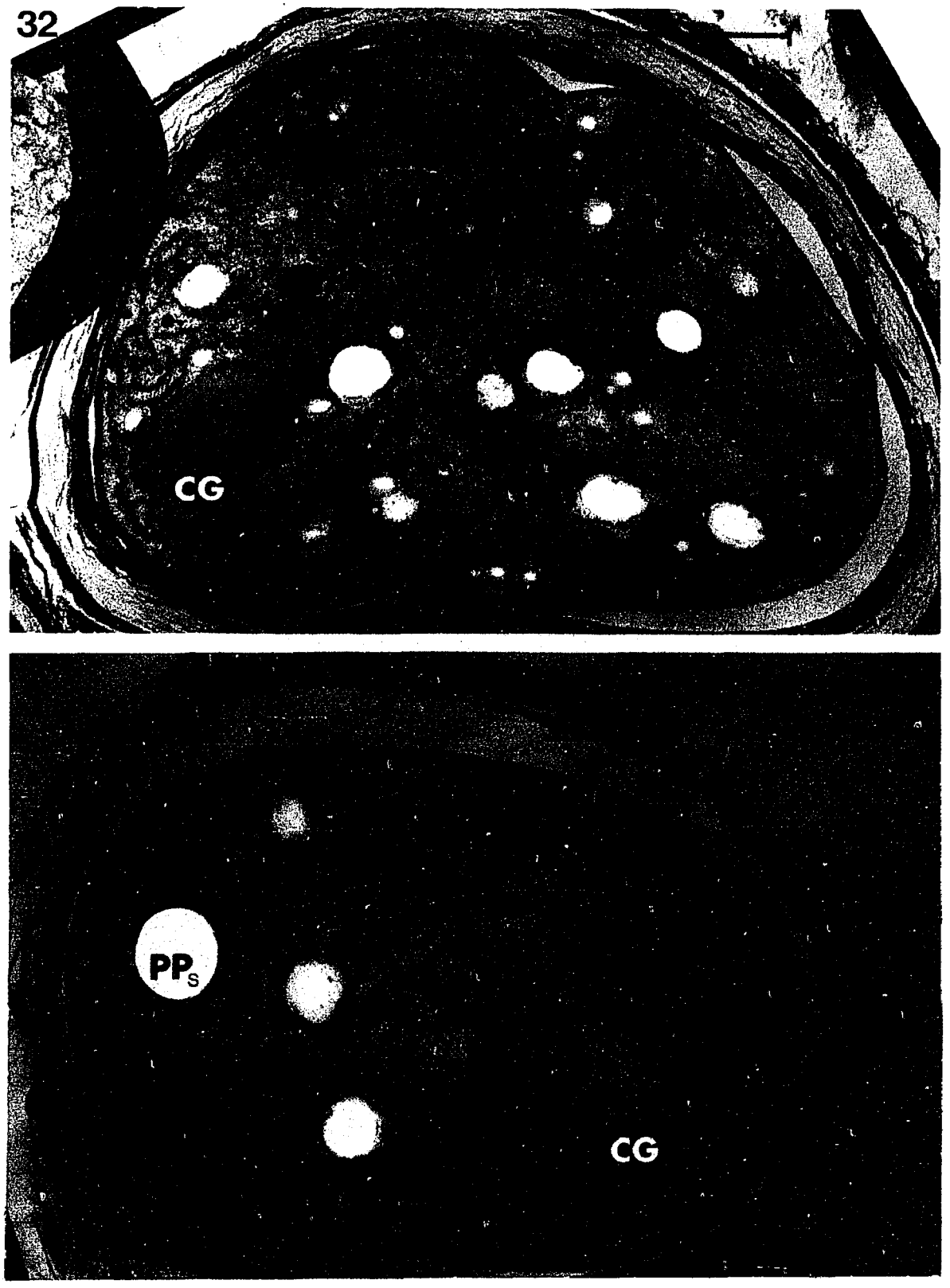
Figures 28, 29. Thin sections of cells of Chlorogloea fritschii grown for 72 hours in medium containing 0.31 mM sulfate and 0.32 mM orthophosphate. The surrounding sheath (S), cell wall (CW), thylakoids (T), nucleic acid fibrils (NA), polyphosphate bodies (PP), cyanophycin granules (CG), and poly-β-hydroxybutyric acid inclusions (PβHB) are evident. The marker bars are equivalent to 1 μm.


CG
PP
PβHB
NA
T
S
CW

Figures 30, 31. Thin sections of cells of Chlorogloea fritschii grown for 72 hours in medium containing 31 µM sulfate and 0.32 mM orthophosphate. Polyphosphate bodies (PP) and their remnant spaces (PP_s), and a polyhedral body or carboxysome (PH) are evident. The marker bars are equivalent to 1 µm.


30°
PPs
PP

Figures 32, 33. Thin sections of cells of Chlorogloea fritschii grown for 72 hours in medium containing zero sulfur and 0.32 mM orthophosphate. Polyphosphate bodies (PP) and their remnant spaces (PP_s), and much enlarged cyanophycin granules (CG) are evident. The marker bars are equivalent to 1 µm.


32
CG
PPs
CG

vidual granules apparently coalesced to form very few and very large bodies (Figures 32, 33).

C. Recovery from Sulfur Starvation

Growth of cells recovering from a six-day period of sulfur depletion was quite slow (Figure 34). The rates of cell division of the two starved cultures subsequently provided with both exogenous sulfur and phosphorus (one of these cultures was treated with 10 µg/ml CAP overnight) progressively increased with time. However, by day six their total cell numbers were little increased over those of cultures (a) not starved for sulfur but subsequently denied exogenous phosphorus, and (b) starved for sulfur, and subsequently provided with exogenous sulfur, but denied exogenous phosphorus. Control cells not starved and directly transferred to fresh, complete medium, exhibited about a 50% increase in cell number over the four previously mentioned cultures at the end of six days.

Recovery from nutrient deficiency was also manifested in changes in the photosynthetic pigment ratios (Figure 35). The ratios of chlorophyll a to carotenoids, and c-phycocyanin to carotenoids progressively increased for five days in all cultures containing exogenous phosphate, and in the CAP-treated culture lacking exogenous phosphate. The pigment ratios decreased after 24 hours in the non-sulfur-starved cells transferred to sulfur-sufficient, phosphorus-deficient medium, and after 96 hours in the sulfur-starved cells transferred to like medium.

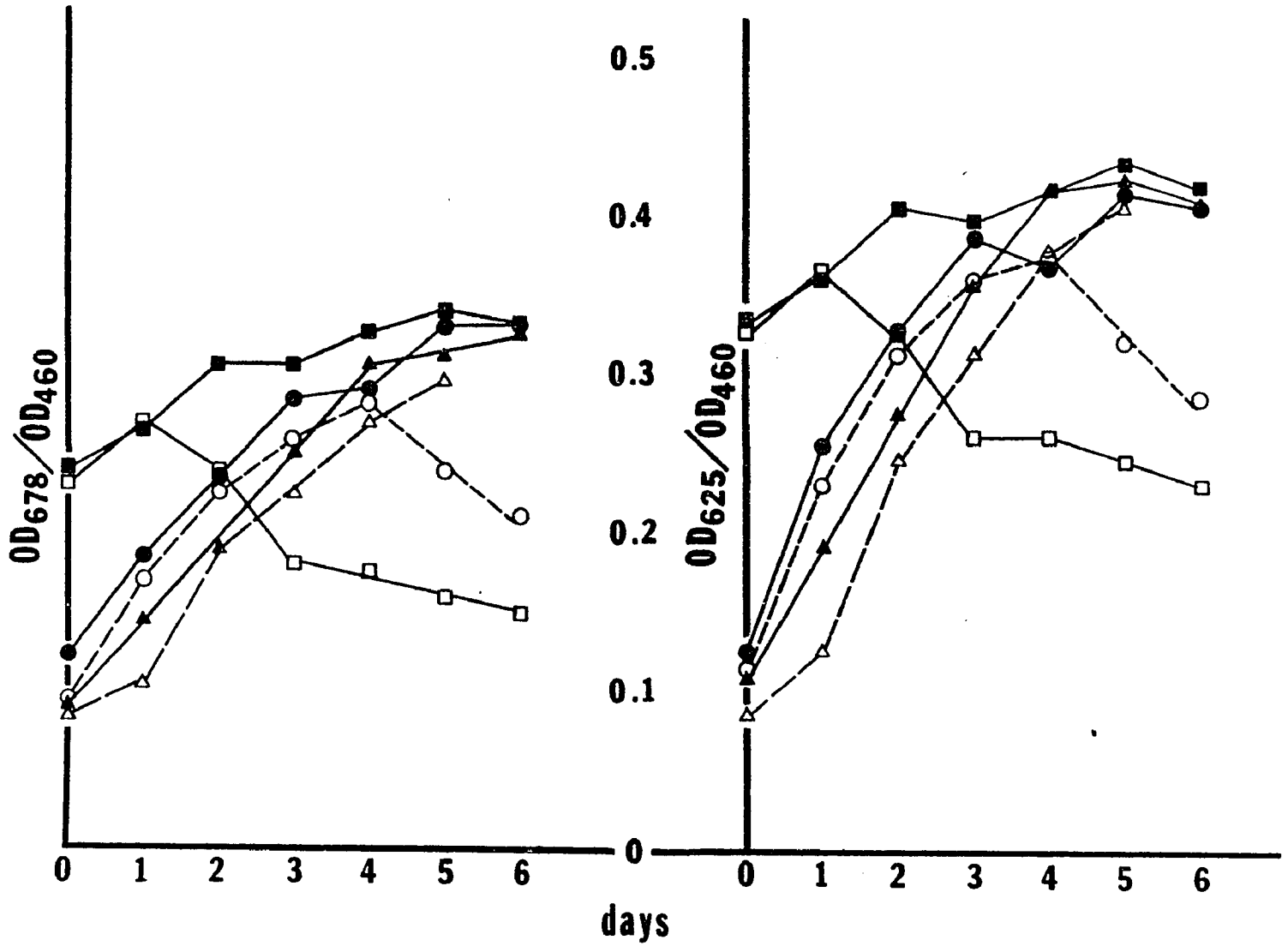
Actual amounts of chlorophyll a and c-phycocyanin are shown in Figures 36 and 37. In the following listing change in chlorophyll a is given first, followed by a change in c-phycocyanin within parentheses:

(a) in sulfur-starved cells restored to complete medium, increases of almost 200% (> 300%) during the first four days, then declines of over

Figure 34. Cell numbers per ml of populations recovering from sulfur depletion after transfer to medium containing 0.31 mM sulfate. S-starved cells were transferred either directly to medium containing 0.22 mM orthophosphate (○) and medium lacking it (●), or treated initially overnight with 10 µg/ml CAP, and transferred to medium containing 0.22 mM orthophosphate (△) and medium lacking it (▲). Cells grown originally in sulfur-sufficient medium were transferred to fresh medium containing 0.22 mM orthophosphate (□), and to medium lacking it (■).

cells x 10⁶
0
1
2
3
4
5
6
7
8
days
0
1
2
3
4
5
6

Figure 35. The changes in absorption ratios of whole cells measured at 678 nm and 460 nm, and 625 nm and 460 nm, after transfer from sulfur-deficient medium to that containing 0.31 mM sulfate. S-starved cells were transferred either directly to medium containing 0.22 mM orthophosphate (●) and medium lacking it (○), or treated overnight with 10 µg/ml CAP, and transferred to medium containing 0.22 mM orthophosphate (▲), and medium lacking it (△). Cells grown originally in sulfur-sufficient medium were transferred to fresh medium containing 0.22 mM orthophosphate (■), and to medium lacking it (□).


OD_{678}/OD_{460}
OD_{625}/OD_{460}
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6
0 1 2 3 4 5 6
days

20% (almost 25%) in the last two days;

(b) in sulfur-starved cells restored to exogenous sulfur but not exogenous phosphorus, increases of 200% (almost 290%) during the first three days, followed by declines of 33% (almost 35%) in the last three days;

(c) in sulfur-starved, CAP-treated cells restored to complete medium, increases of about 350% (> 400%) in the first five days, then declines of almost 20% (almost 25%) in the final 24 hours;

(d) in sulfur-starved, CAP-treated cells restored to exogenous sulfur but not exogenous phosphorus, increases of 300% (almost 500%) in five days;

(e) in non-sulfur-starved (control) cells transferred to fresh, complete medium, increases of 33% (almost 20%) in the first five days, then declines of 14% (14%) in the last 24 hours;

(f) in non-sulfur-starved cells transferred to medium lacking phosphorus, decreases of over 50% (almost 50%) in six days.

Disappearance of 0.22 mM orthophosphate by recovering cultures is shown in Figure 38. Control cells steadily depleted the exogenous phosphorus over the six-day period (ca. 40% loss from the medium). Cells recovering from sulfur starvation removed about 28% of the phosphate removed by the control cells, and cells recovering from sulfur starvation but pretreated with CAP appear to have removed no net orthophosphate from the medium. Cultures with no exogenous phosphorus either did not show a consistent concentration (very low) of phosphorus in the medium, or such was not detectable by the method utilized.

Decrease of cellular phosphorus by recovering cultures is shown in Figure 39. Marked decreases in internal phosphorus occurred in the first

Figures 36, 37. The changes in chlorophyll a and c-phycocyanin levels of whole cells after transfer from sulfur-deficient to sulfur-sufficient (0.31 mM sulfate) medium. S-starved cells were transferred either directly to medium containing 0.22 mM orthophosphate (●) and medium lacking it (○), or treated overnight with 10 µg/ml CAP, and transferred to medium containing 0.22 mM orthophosphate (▲) and medium lacking it (△). Cells grown originally in sulfur-sufficient medium were transferred to fresh medium containing 0.22 mM orthophosphate (■), and to medium lacking it (□).

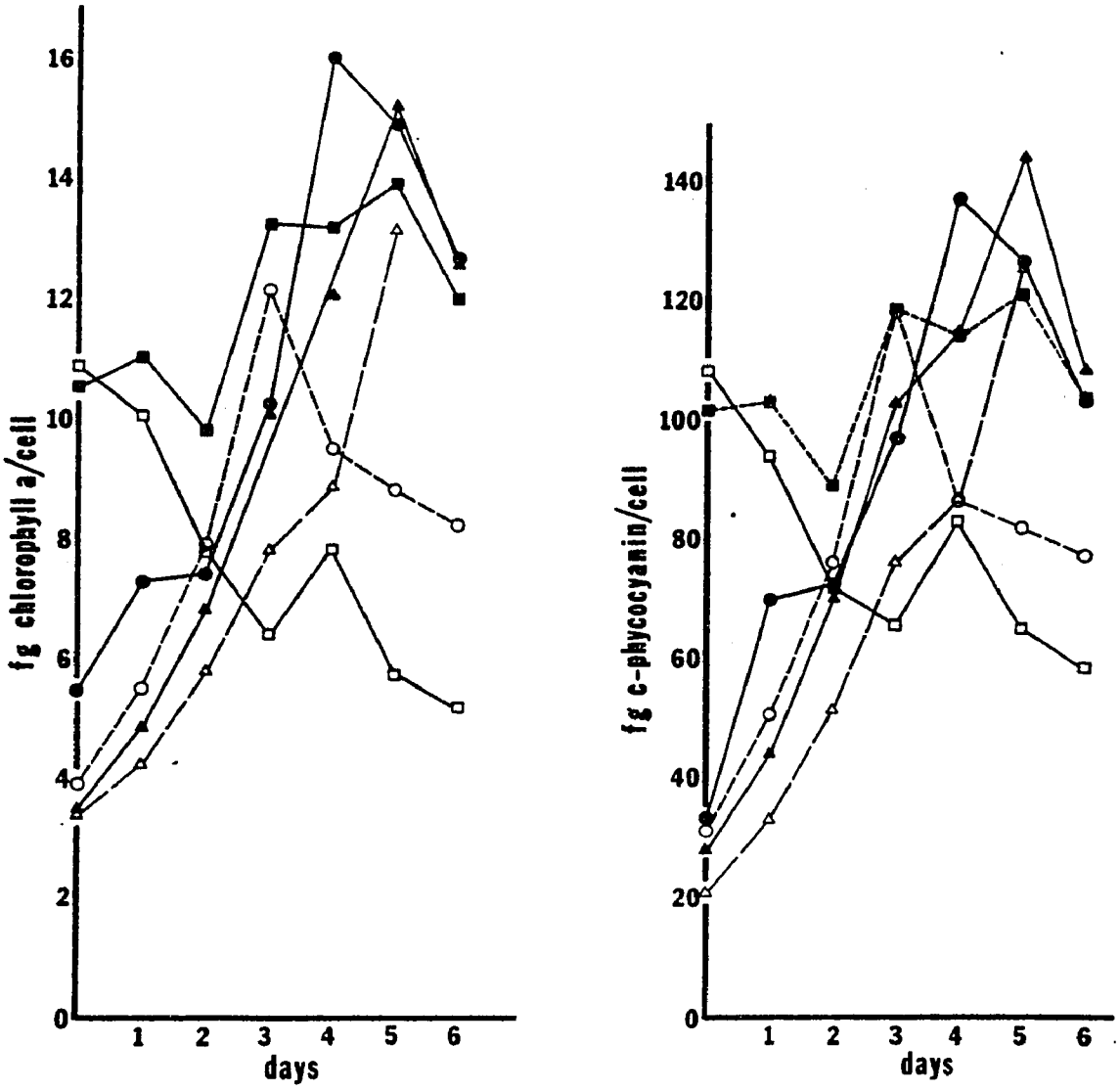
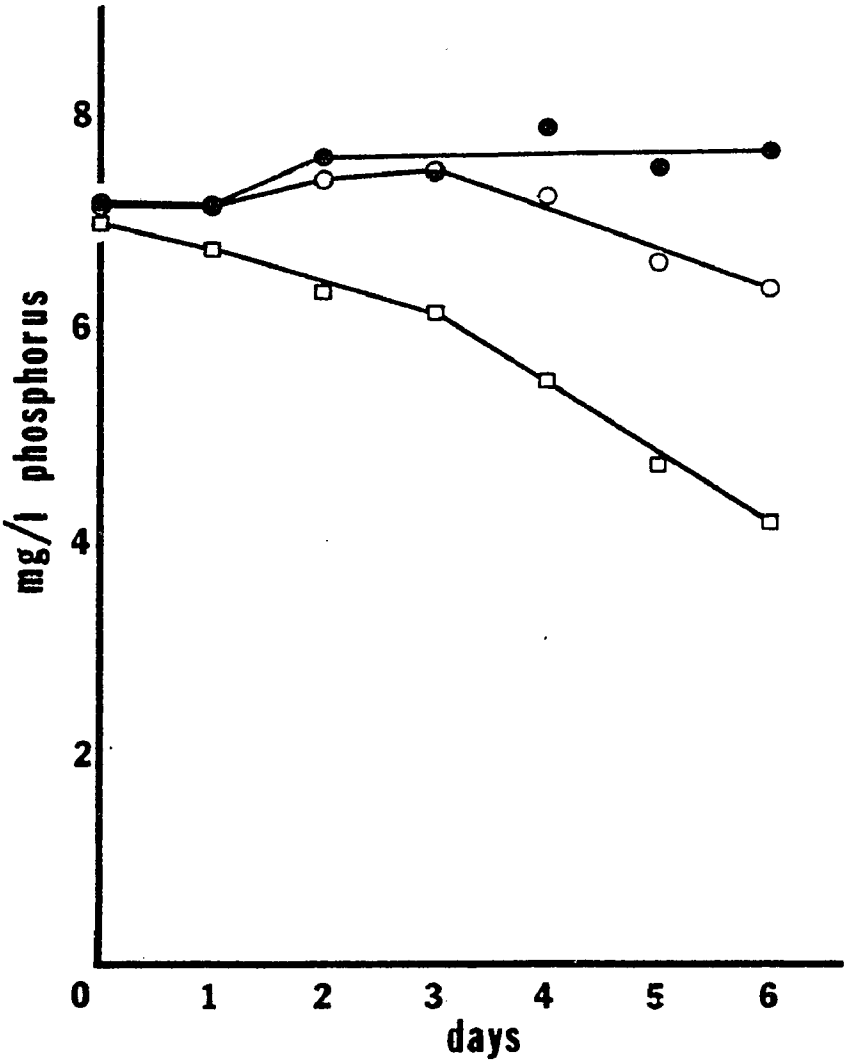

fg chlorophyll a/cell
0
2
4
6
8
10
12
14
16
1
2
3
4
5
6
days
fg c-phycocyanin/cell
0
20
40
60
80
100
120
140
1
2
3
4
5
6
days

Figure 38. Disappearance of 0.22 mM (7 mg/l) exogenous orthophosphate in cultures of sulfur-starved cells transferred to medium containing 0.31 mM sulfate. S-starved cells were transferred either directly (○) or treated initially overnight with 10 µg/ml CAP (●). Cells grown originally in sulfur-sufficient medium were transferred to fresh medium (□).


8
6
4
2
0
1
2
3
4
5
6
mg/l phosphorus
days

two or three days after the restoration of exogenous sulfur. At the end of six days cells provided with exogenous phosphate had a mean phosphorus level in the 5-6 fg range, and those not so provided had a mean phosphorus level about 1 fg (values not exceeding 2 fg P/cell).

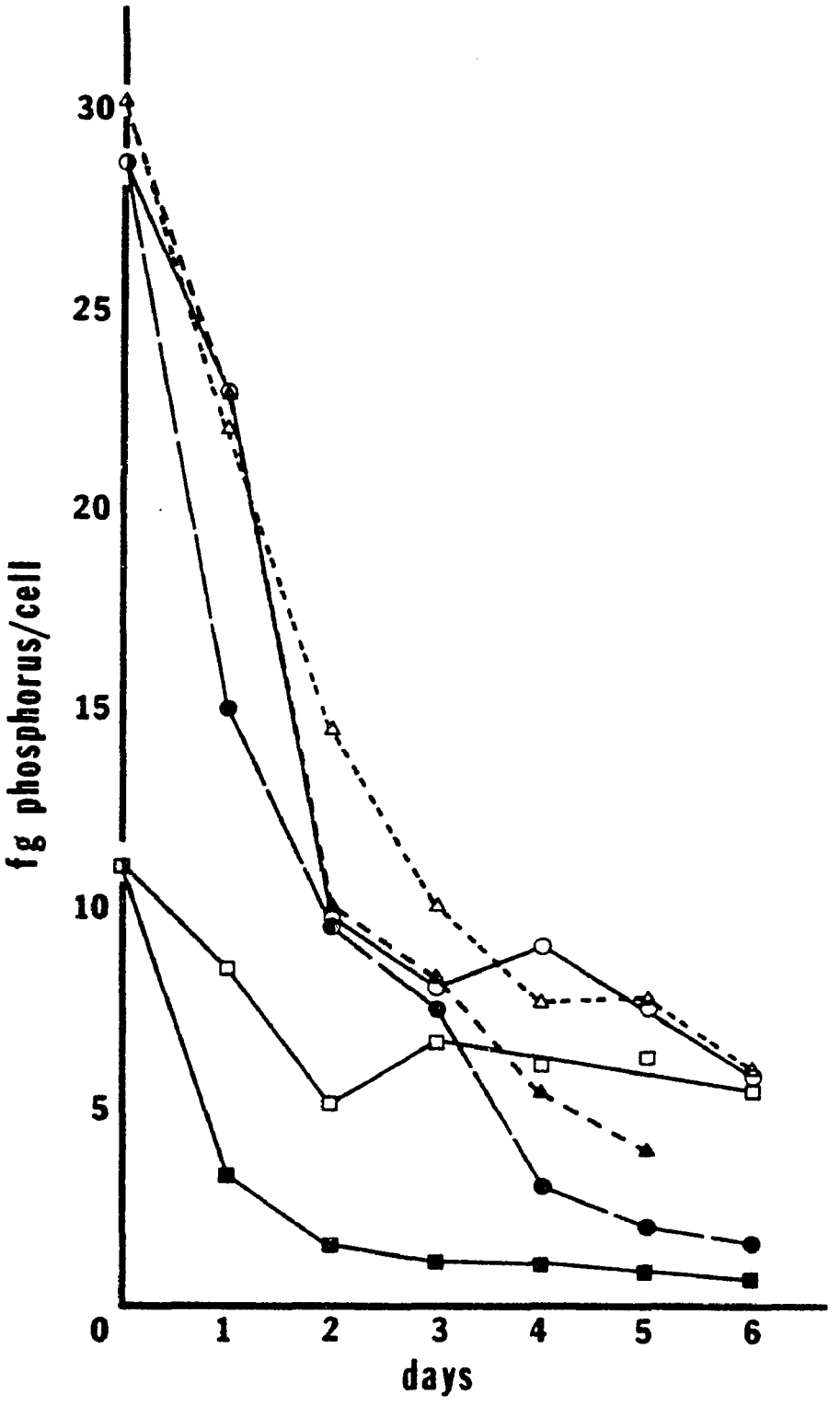
A daily accounting of exogenous and cellular phosphorus per ml (Table 3) shows increases in total amount with time (cf. Tables 1, 2). These discrepancies were probably the result of evaporation of water, and the shrinkage in volume of the solvent/suspending medium. In cases where exogenous phosphate was available, significant increases in cellular phosphorus per ml are evident: control cells almost 1100%, starved cells 365%, and CAP-pretreated starved cells 340%. Per ml phosphorus values of orthophosphate-deprived cells increased only 0-25% in the same time period.

Figures 40-43 are thin sections of sulfur-deprived cells fixed 46 hours after the restoration of exogenous sulfate. The number of peripheral thylakoids was still below normal and polyphosphate bodies were larger than normal and somewhat increased in number, but the cells were quite obviously recovering, irrespective of the presence or absence of exogenous phosphorus, or pretreatment with CAP. It can be seen that some of the polyphosphate bodies were porous.

Figure 44 shows thin sections of non-sulfur-starved cells deprived of exogenous phosphorus for 46 hours. These cells lacked both polyphosphate bodies and a significant degree of cytoplasmic integrity. Figure 45 is a thin section of a control cell grown in fresh complete medium for 46 hours.

Figure 46 displays thin sections of sulfur-depleted cells 98 hours after transfer to sulfur-sufficient, phosphorus-deficient medium. These

Figure 39. Decreases in total phosphorus by S-depleted cells transferred to medium containing 0.31 mM sulfate. S-starved cells were transferred either directly to medium containing 0.22 mM orthophosphate (○) and medium lacking it (●), or treated overnight with 10 µg/ml CAP, and transferred to medium containing 0.22 mM orthophosphate (△) and medium lacking it (▲). Cells grown originally in sulfur-sufficient medium were transferred to fresh medium containing 0.22 mM orthophosphate (□), and to medium lacking it (■).


30
25
20
15
10
5
0
1
2
3
4
5
6
fg phosphorus/cell
days

recovery medium		day zero	day one	day two	day three	day four	day five	day six
zero S + P	exogenous P/ml	7.14	7.14	7.39	7.48	7.21	6.58	6.36
	cellular P/ml	0.68	0.77	0.77	1.06	1.47	2.25	3.16
	total P/ml	7.82	7.91	8.16	8.54	8.68	8.83	9.52
zero S - P	exogenous P/ml	--	0.29	--	0.41	0.18	0.18	--
	cellular P/ml	0.57	0.50	0.54	0.65	0.62	0.64	0.71
	total P/ml	0.57	0.79	0.54	1.06	0.80	0.82	0.71
zero S + P (CAP)	exogenous P/ml	7.17	7.14	7.59	7.37	7.88	7.49	7.64
	cellular P/ml	0.68	0.64	0.58	0.73	1.10	1.75	2.99
	total P/ml	7.85	7.78	8.17	8.10	8.98	9.24	10.63
zero S - P (CAP)	exogenous P/ml	--	0.15	0.29	--	0.24	0.24	--
	cellular P/ml	0.57	0.44	0.35	0.46	0.58	0.67	--
	total P/ml	0.57	0.59	0.64	0.46	0.82	0.91	--
control + P	exogenous P/ml	6.96	6.73	6.33	6.12	5.49	4.67	4.15
	cellular P/ml	0.34	0.51	0.84	1.42	1.96	3.11	4.01
	total P/ml	7.30	7.24	7.17	7.54	7.45	7.78	8.16
control - P	exogenous P/ml	0.15	0.27	0.15	--	--	--	0.27
	cellular P/ml	0.33	0.25	0.27	0.28	0.31	0.33	0.33
	total P/ml	0.48	0.52	0.42	0.28	0.31	0.33	0.60

Table 3

An accounting of total phosphorus per ml culture when various recovery media were provided to sulfur-starved cells

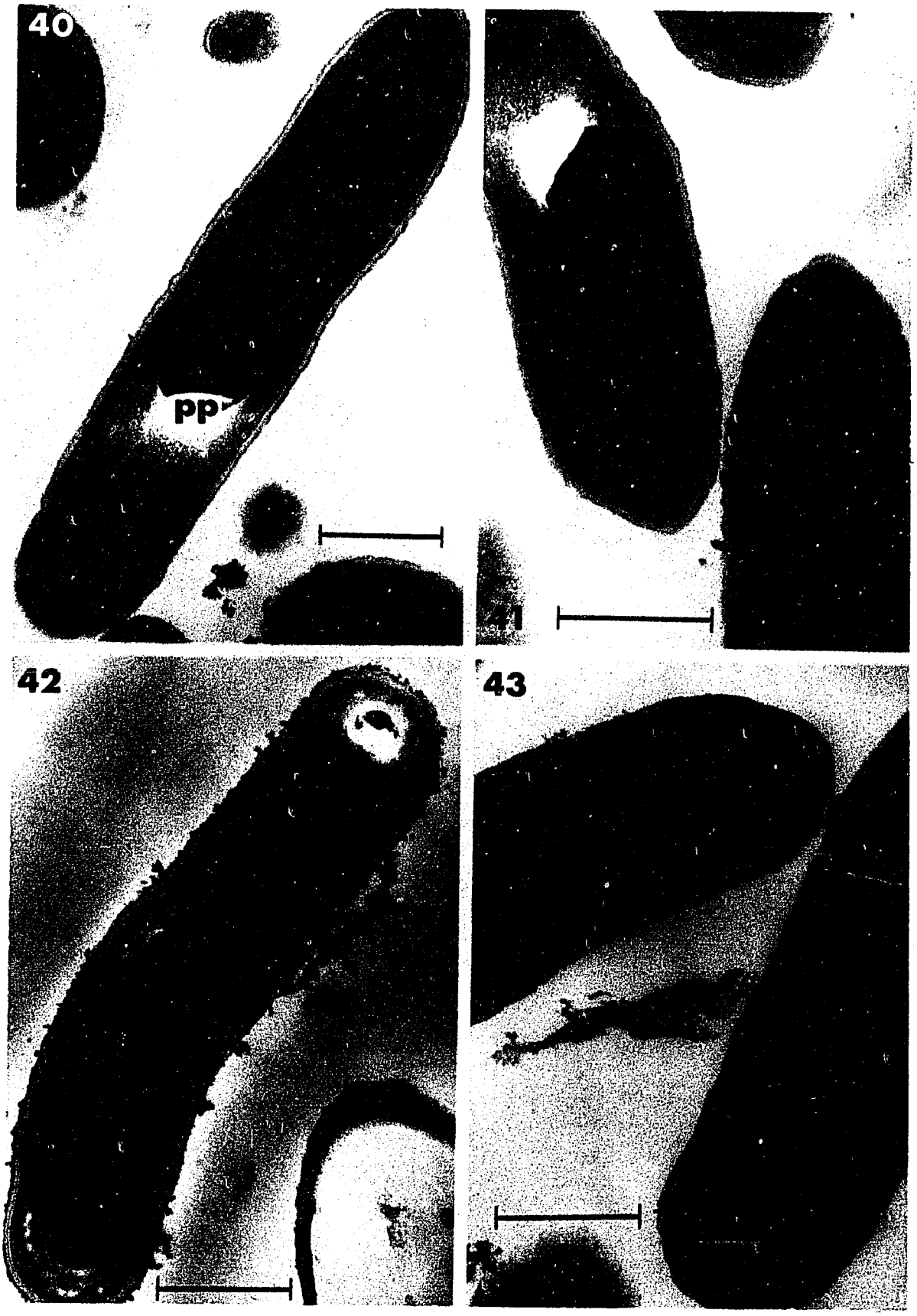
Figures 40-43. Thin sections of sulfur-deficient cells 46 hours after they were transferred to medium containing 0.31 mM sulfate. Polyphosphate bodies (pp) are evident; marker bars are equivalent to 500 nm.

Figure 40. Cells transferred to medium lacking phosphorus.

Figure 41. Cells transferred to medium containing 0.22 mM orthophosphate.

Figure 42. Cells pretreated with CAP and transferred to medium lacking phosphorus.

Figure 43. Cells pretreated with CAP and transferred to medium containing 0.22 mM orthophosphate.


40
PP
42
43

Figures 44, 45. Thin sections of sulfur-sufficient cells 46 hours after they were transferred to fresh medium containing 0.31 mM sulfate. Polyphosphate bodies are labelled pp; marker bars are equivalent to 500 nm.

Figure 44. Cells transferred to medium lacking phosphorus. The inclusions are polyhedral bodies.

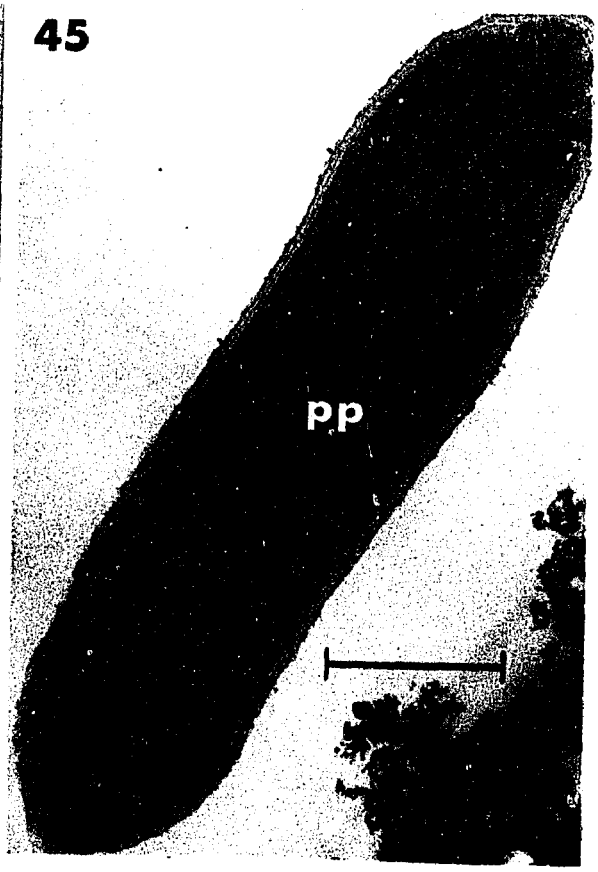
Figure 45. Cells transferred to medium containing 0.22 mM orthophosphate.

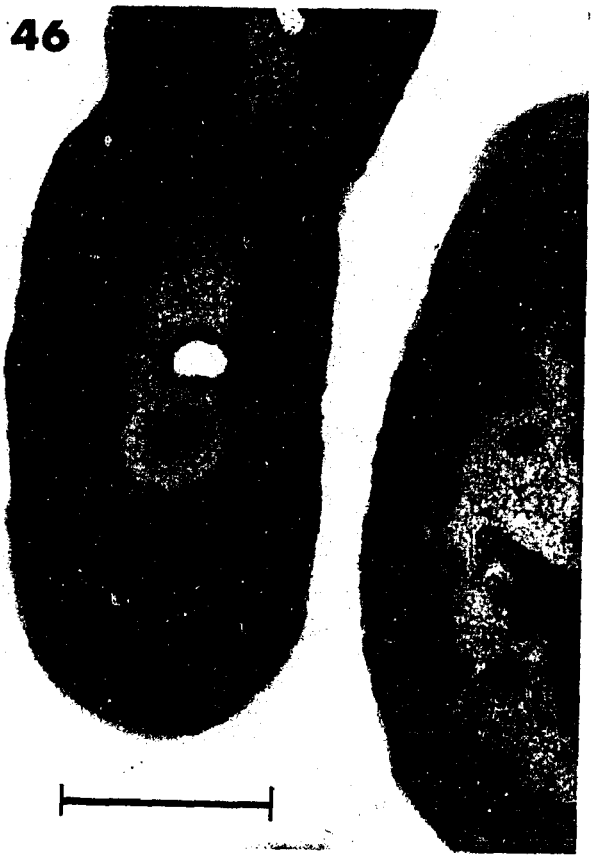
Figures 46, 47. Thin sections of sulfur-deficient cells 98 hours after they were transferred to medium containing 0.31 mM sulfate. The marker bars are equivalent to 500 nm.

Figure 46. Cells transferred to medium lacking phosphorus.

Figure 47. Cells transferred to medium containing 0.22 mM orthophosphate.


44


45
pp


46


47

cells were beginning to show the first effects of cellular phosphorus depletion (small polyphosphate bodies, cytoplasmic deterioration, etc.), but extent varied from cell to cell in the fields observed. Figure 47 is a thin section of a sulfur-depleted cell 98 hours after transfer to complete medium. The single polyphosphate body seen is extensively porous.

Figure 48 shows thin sections of sulfur-depleted, CAP-treated cells 98 hours after transfer to sulfur-sufficient, phosphorus-deficient medium. Polyphosphate bodies present had a porous appearance. Figure 49 shows thin sections of similar cells 98 hours after transfer to complete medium. Large membranous elaborations can be noted in both types of CAP-treated cells.

Figure 50 displays thin sections of non-sulfur-starved cells 98 hours after transfer to phosphorus-deficient medium. Polyphosphate bodies were not visible and cell walls were commonly not flush with the periphery of the cytoplasm. Figure 51 shows thin sections of control cells grown in fresh, complete medium for 98 hours.

Figure 52 includes thin sections of sulfur-starved cells 144 hours after transfer to sulfur-sufficient, phosphorus-deficient medium. Polyphosphate bodies were absent, thylakoids were reduced in number, and the cytoplasm was deteriorating. Figure 53 shows thin sections of sulfur-starved cells 144 hours after transfer to complete medium. These cells were by then quite normal in appearance ultrastructurally.

Figure 54 shows thin sections of sulfur-starved, CAP-treated cells 144 hours after transfer to complete medium. The cells were normal in appearance except for the continued presence of elaborate membranous inclusions. Figure 55 is a thin section of a control cell grown in fresh,

Figures 48, 49. Thin sections of sulfur-deficient cells 98 hours after they were pretreated with CAP and transferred to medium containing 0.31 mM sulfate. Polyphosphate bodies are designated pp; marker bars are equivalent to 500 nm.

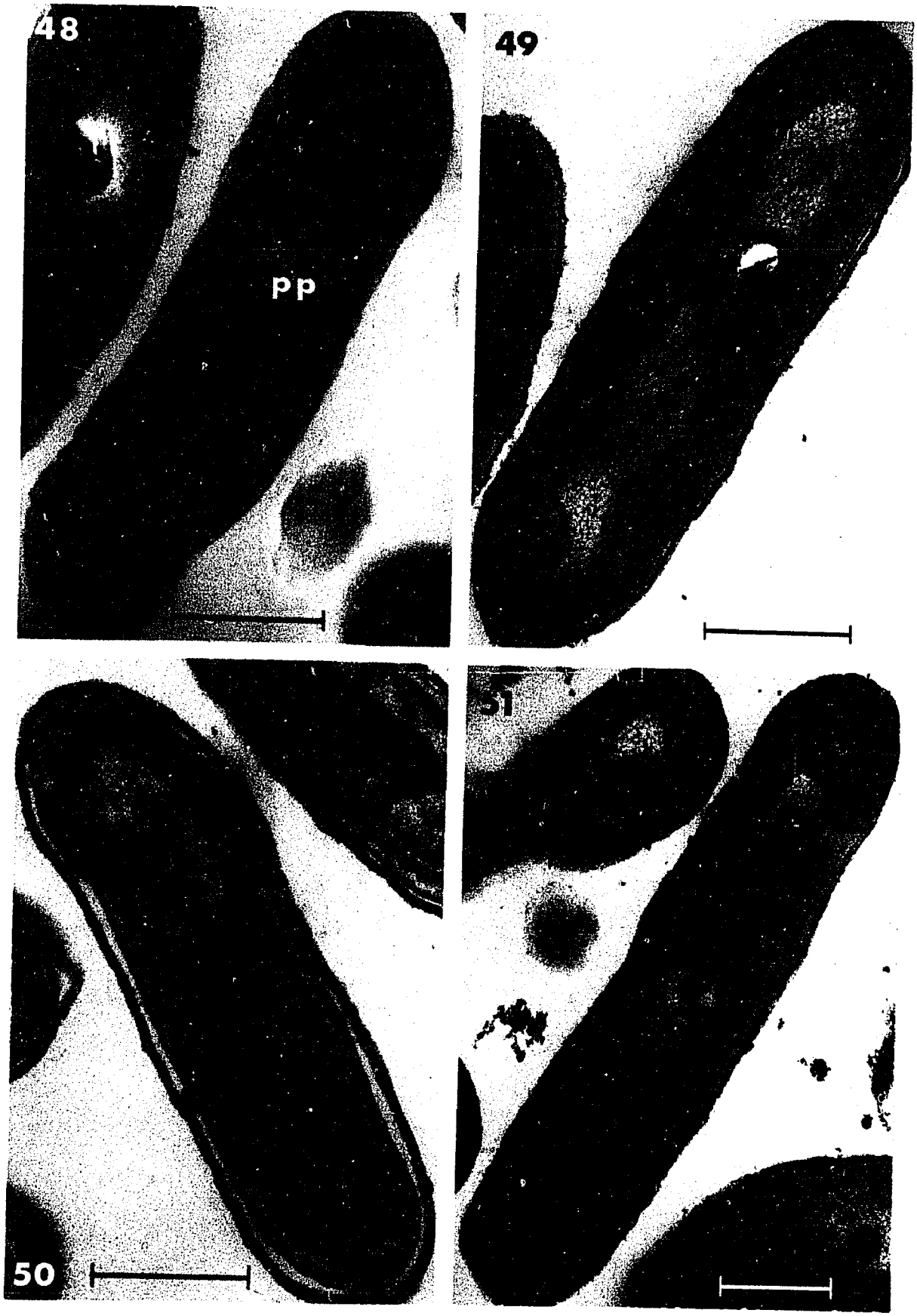
Figure 48. Cells transferred to medium lacking phosphorus.

Figure 49. Cells transferred to medium containing 0.22 mM orthophosphate.

Figures 50, 51. Thin sections of sulfur-sufficient cells 98 hours after they were transferred to fresh medium containing 0.31 mM sulfate. The marker bars are equivalent to 500 nm.

Figure 50. Cells transferred to medium lacking phosphorus. The inclusion is a polyhedral body.

Figure 51. Cells transferred to medium containing 0.22 mM orthophosphate.


48
PP
49
50
51

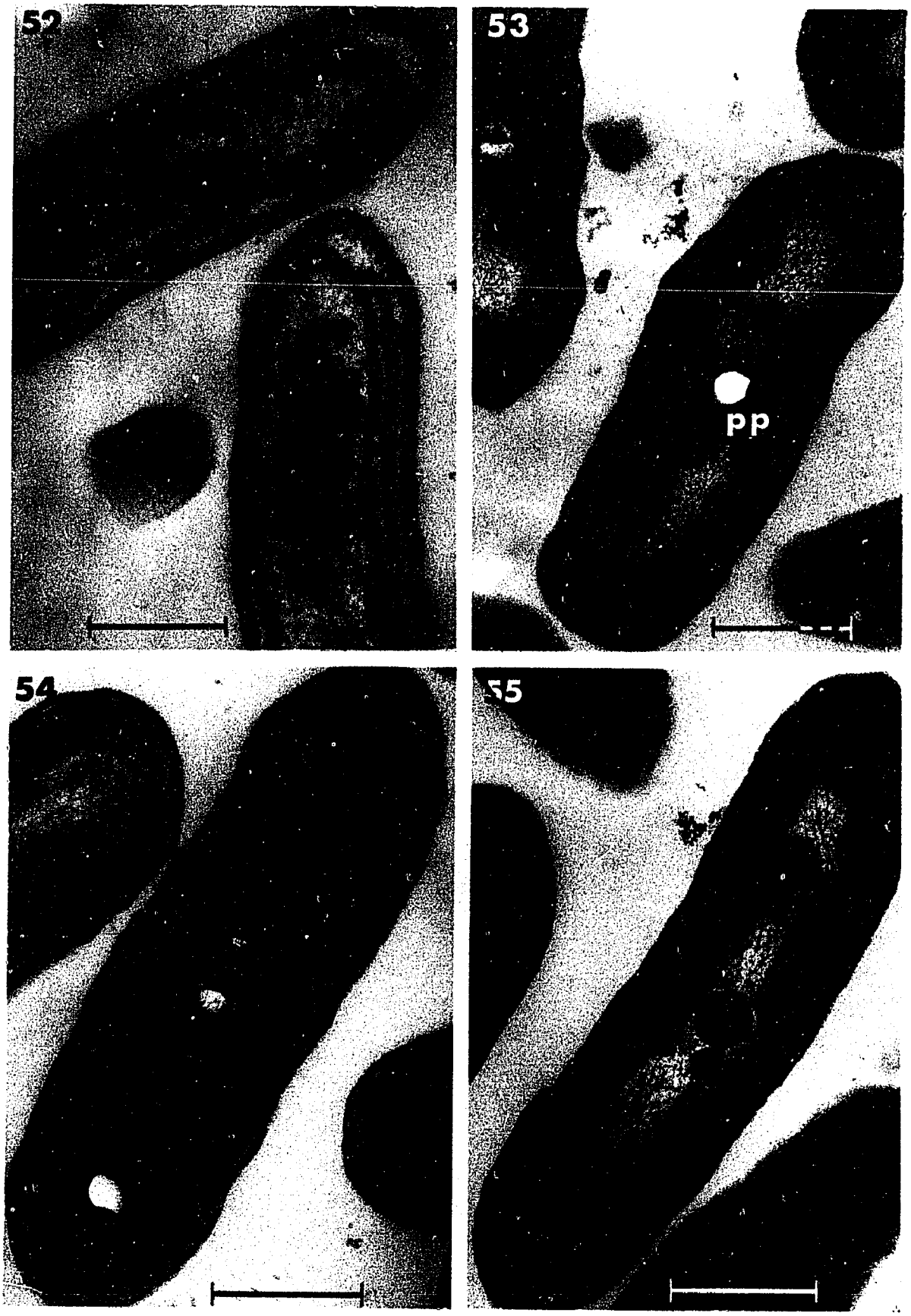
Figures 52-55. Thin sections of cells 144 hours after they were transferred from and to varying media. Polyphosphate bodies (pp) are evident; marker bars are equivalent to 500 nm.

Figure 52. Sulfur-deficient cells transferred to medium containing 0.31 mM sulfate but lacking phosphorus. The inclusions are polyhedral bodies.

Figure 53. Sulfur-deficient cells transferred to medium containing 0.31 mM sulfate and 0.22 mM orthophosphate.

Figure 54. Sulfur-deficient cells pretreated with CAP and transferred to medium containing 0.31 mM sulfate and 0.22 mM orthophosphate.

Figure 55. Sulfur-sufficient cells transferred to fresh medium containing 0.31 mM sulfate and 0.22 mM orthophosphate.


52
53
pp
54
55

complete medium for 144 hours.

D. Organic Sulfur Sources

Table 4 compares the R_f values of the oligopeptide and sulfur amino acids obtained during paper chromatography with those given by Smith (1960).

Figures 56 and 57 represent the growth responses of cells to a variety of sulfur amino acids and reduced glutathione. All compounds were tested in two successive six-day periods. During the initial period each potential sulfur source was present in the medium at an equivalent concentration of 0.31 mM sulfur. Each sulfur component was increased during the second period according to the performance of the culture during the first period.

The thioether DL-lanthionine (Figure 56), the disulfide L-cystine (Figure 57) and the tripeptide glutathione (Figure 57) allowed growth somewhat less or greater than of control in all phases. The thioacetal L-djenkolic acid (Figure 56) resulted in sulfur limitation and abrupt cessation of growth at a concentration of 0.31 mM sulfur, but allowed near-normal growth at 0.93 mM sulfur (n.b. discontinuities in growth data, Figure 57). The disulfide DL-homocystine (Figure 57) provided for intermediate growth at a concentration of 0.31 mM sulfur, but did not allow long-term growth, even when its concentration was tripled. The remainder of the sulfur amino acids, the thioether L-methionine (Figure 56) and the sulfonic acids, taurine and L-cysteic acid (Figure 57), supported only poor growth irrespective of concentration, in most cases little better than medium lacking sulfur entirely.

Figures 58 and 59 show the levels of cellular phosphorus during each six-day period. Cells growing with L-cystine or glutathione (Fig-

	R_f in solvent of 12:3:5 n-butanol : acetic acid : water		
	Smith 1960	Observed	
		1st spot	2nd spot
L-cystathionine	10	--	--
L-cysteic acid	7	9.4	13.5
L-cysteine	8	--	--
L-cystine	5	9	--
L-djenkolic acid	9	8	--
glutathione	--	11.5	33
L-homocysteine	18	--	--
L-homocystine	18	17	--
L-lanthionine	6	7	--
L-methionine	50	58	--
taurine	20	20	--

Table 4

A comparison of R_f values of sulfur amino acids obtained using a solvent of n-butanol : acetic acid : water (12:3:5 v/v/v), with those given by I. Smith (1960)

Figure 56. Cell numbers per ml of cultures with one of several sulfur amino acids present as the sole exogenous sulfur source, during the first six-day period at a concentration of 0.31 mM sulfur, and during the second six-day period at an elevated concentration.

The first (leftmost) period included cultures with zero sulfur (○), 0.31 mM L-methionine (△), 0.31 mM DL-lanthionine (▲), 0.155 mM L-djenkolic acid (■), and 0.31 mM sulfate (●).

The second (rightmost) period included cultures with zero sulfur (○), 0.93 mM L-methionine (△), 0.62 mM DL-lanthionine (▲), 0.465 mM L-djenkolic acid (■), and 0.31 mM sulfate (●).

cells x 10⁶
0
2
4
6
8
10
12
days
0
1
2
3
4
5
6%
1
2
3
4
5
6
7

Figure 57. Cell numbers per ml of cultures with one of several short-chain organosulfur compounds present as the sole exogenous sulfur source, during the first six-day period at a concentration of 0.31 mM sulfur, and during the second six-day period at an elevated concentration.

The first (leftmost) period included cultures with 0.31 mM taurine (○), 0.31 mM L-cysteic acid (□), 0.155 mM DL-homocystine (△), 0.31 mM reduced glutathione (▲), 0.155 mM L-cystine (■), and 0.31 mM sulfate (●).

The second (rightmost) period included cultures with 0.93 mM taurine (○), 0.93 mM L-cysteic acid (□), 0.465 mM DL-homocystine (△), 0.62 mM reduced glutathione (▲), 0.31 mM L-cystine (■), and 0.31 mM sulfate (●).

cells x 10⁸
0
2
4
6
8
10
days
0
1
2
3
4
5
6%
1
2
3
4
5
6
7

ure 59) at either concentration exhibited near-control cellular phosphorus levels. After the initial two days L-djenkolic-acid-grown cells, and after three days DL-lanthionine-grown cells (Figure 58), increased in phosphorus levels, but returned to near-control values when the amino acids were tripled and doubled, respectively. Cells grown on DL-homocystine (Figure 59) exhibited a significant increase in phosphorus level during the first period, although the culture did not become fully chlorotic. When the disulfide was tripled in concentration cellular phosphorus level decreased by 50% then began a slow increase.

Cells grown on L-methionine (Figure 58) experienced a 50% decrease in phosphorus level, but became chlorotic on day three, and within another 24 hours cellular phosphorus began to increase steadily and markedly until the end of the second period. Such accumulation was unaffected by the tripling of the thioether. In the same experiment (Figure 58) cells grown in sulfur-free medium increased in phosphorus level, and continued to do so steadily except when fresh, sulfur-free medium triggered a sharp but momentary decrease. Cells grown on L-cysteic acid and taurine (Figure 59) exhibited very large increases in cellular phosphorus in both growth periods.

Figures 60 and 61 show thin sections of cells grown in medium lacking sulfur. Such cells displayed large and manifold central polyphosphate bodies, as well as terminal caps and bands of polyphosphate within and adjacent to the plasma membrane. Figures 62 and 63 show thin sections of sulfate-grown control cells after 144 hours of growth in each period. Thin sections of cells variously provided with each of the two specified concentrations of DL-lanthionine, L-djenkolic acid, L-methionine, DL-homocystine, L-cysteic acid, taurine, L-cystine, and reduced

Figure 58. Changes in total phosphorus by cells provided with one of several sulfur amino acids present as the sole exogenous sulfur source, during the first six-day period at a concentration of 0.31 mM sulfur, and during the second six-day period at an elevated concentration. Phosphorus level of inoculum cells previously maintained on zero exogenous sulfur for 34 hours (◎); the arrows indicate the days when those cultures became fully chlorotic.

The first six-day period included cultures with zero sulfur (○), 0.31 mM L-methionine (△), 0.31 mM DL-lanthionine (■), 0.155 mM L-djenkolic acid (▲), and 0.31 mM sulfate (●).

The second six-day period included cultures with zero sulfur (○), 0.93 mM L-methionine (△), 0.62 mM DL-lanthionine (■), 0.465 mM L-djenkolic acid (▲), and 0.31 mM sulfate (●).

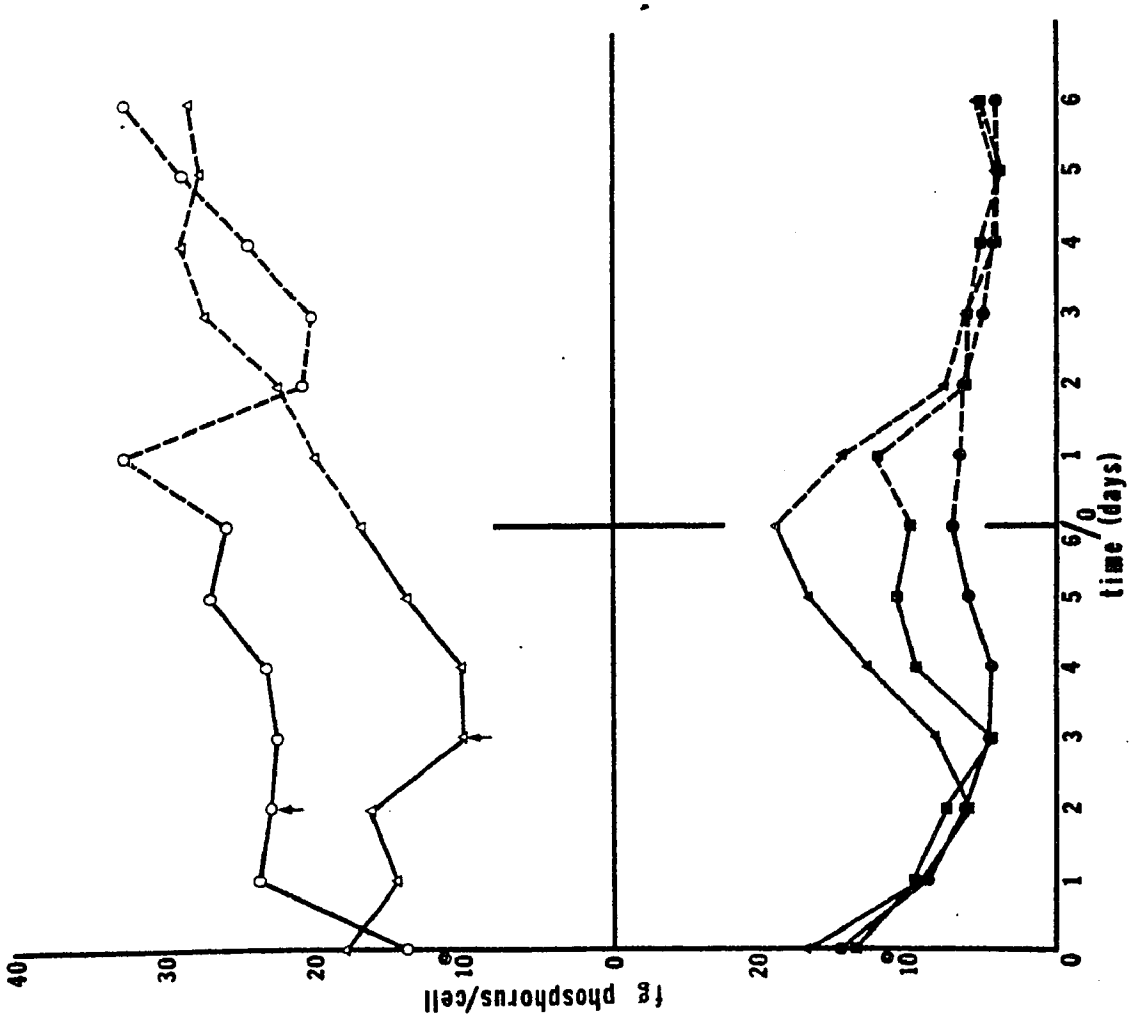
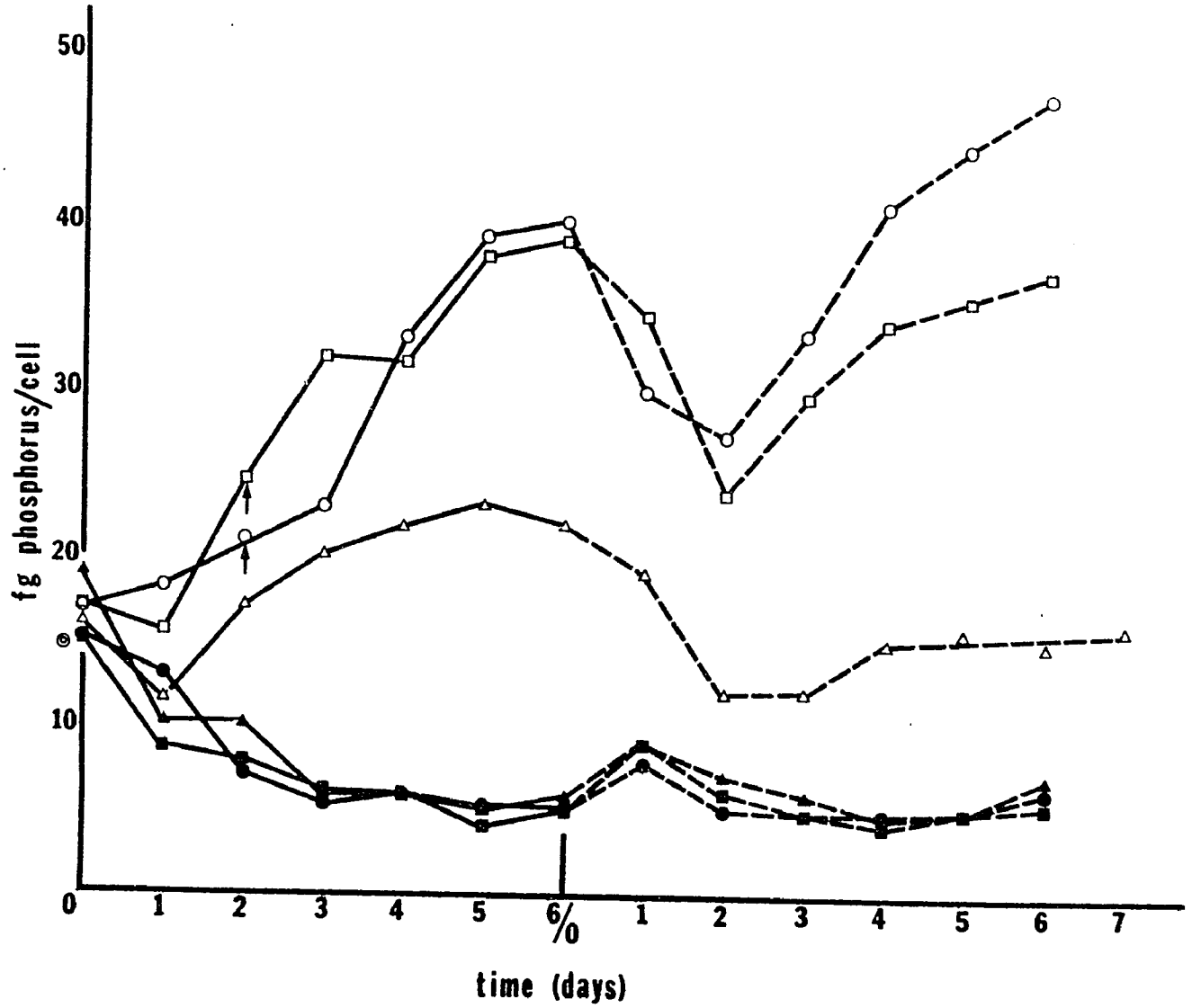

fg phosphorus/cell
40
30
20
10
0
20
10
0
0
1
2
3
4
5
6
time (days)

Figure 59. Changes in total phosphorus by cells provided with one of several short-chain organosulfur compounds present as the sole exogenous sulfur source, during the first six-day period at a concentration of 0.31 mM sulfur, and during the second six-day period at an elevated concentration. Phosphorus level of inoculum cells previously maintained on zero exogenous sulfur for 34 hours (◎); the arrows indicate the days when those cultures became fully chlorotic.

The first six-day period included cultures with 0.31 mM taurine (○), 0.31 mM L-cysteic acid (□), 0.155 mM DL-homocystine (△), 0.31 mM reduced glutathione (▲), 0.155 mM L-cystine (■), and 0.31 mM sulfate (●).

The second six-day period included cultures with 0.93 mM taurine (○), 0.93 mM L-cysteic acid (□), 0.465 mM DL-homocystine (△), 0.62 mM reduced glutathione (▲), 0.31 mM L-cystine (■), and 0.31 mM sulfate (●).


50
40
30
20
10
0
fg phosphorus/cell
0
1
2
3
4
5
6/0
1
2
3
4
5
6
7
time (days)

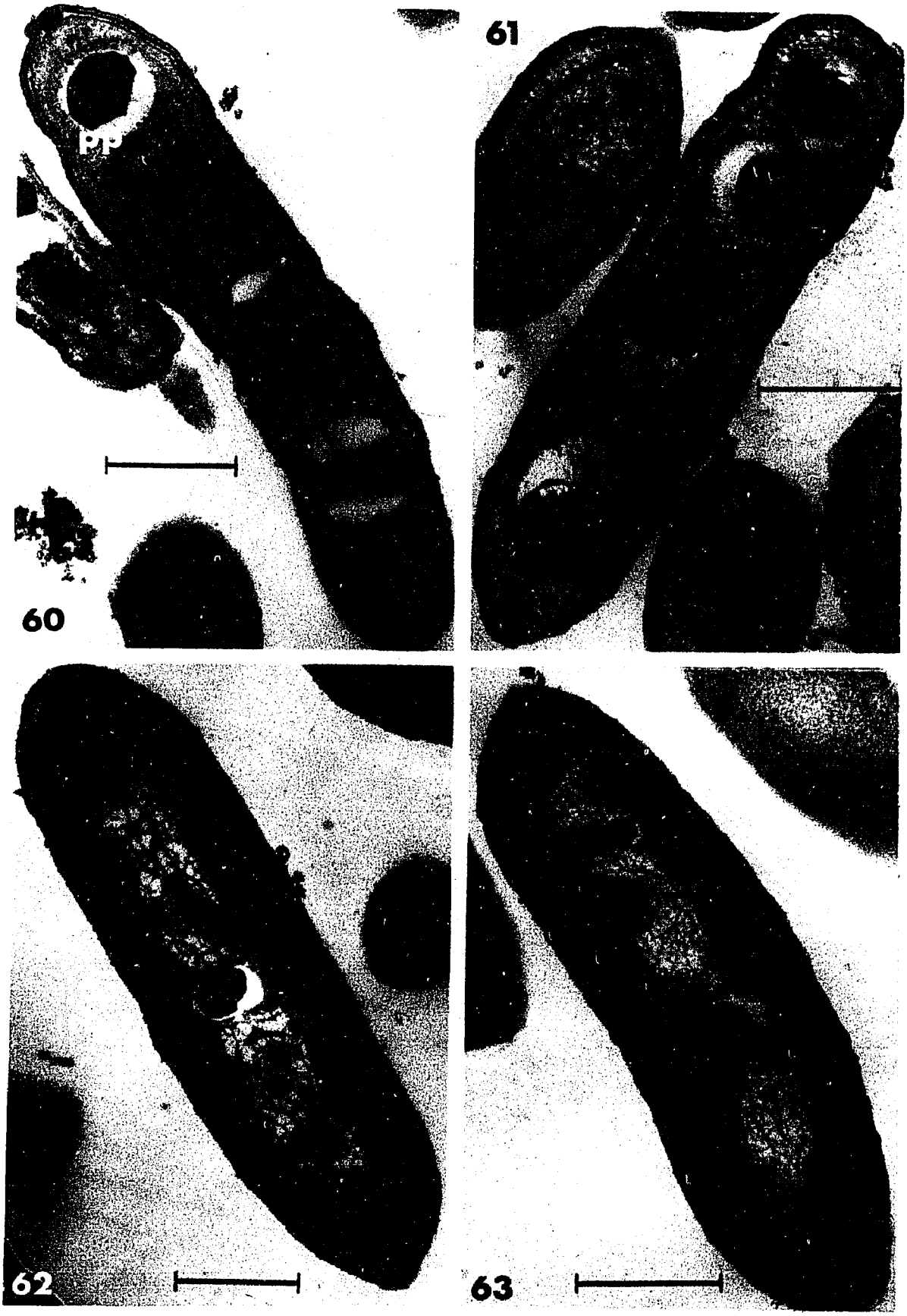
Figures 60-63. Thin sections of cells exhibiting the morphological extremes of sulfur nutrition 144 hours after the beginning of each six-day growth period. Polyphosphate bodies (pp) are evident; marker bars are equivalent to 500 nm.

Figure 60. Cells grown in medium lacking sulfur, first growth period.

Figure 61. Cells grown in medium lacking sulfur, second growth period.

Figure 62. Cells grown with 0.31 mM sulfate, first growth period.

Figure 63. Cells grown with 0.31 mM sulfate, second growth period.


PP
60
61
62
63

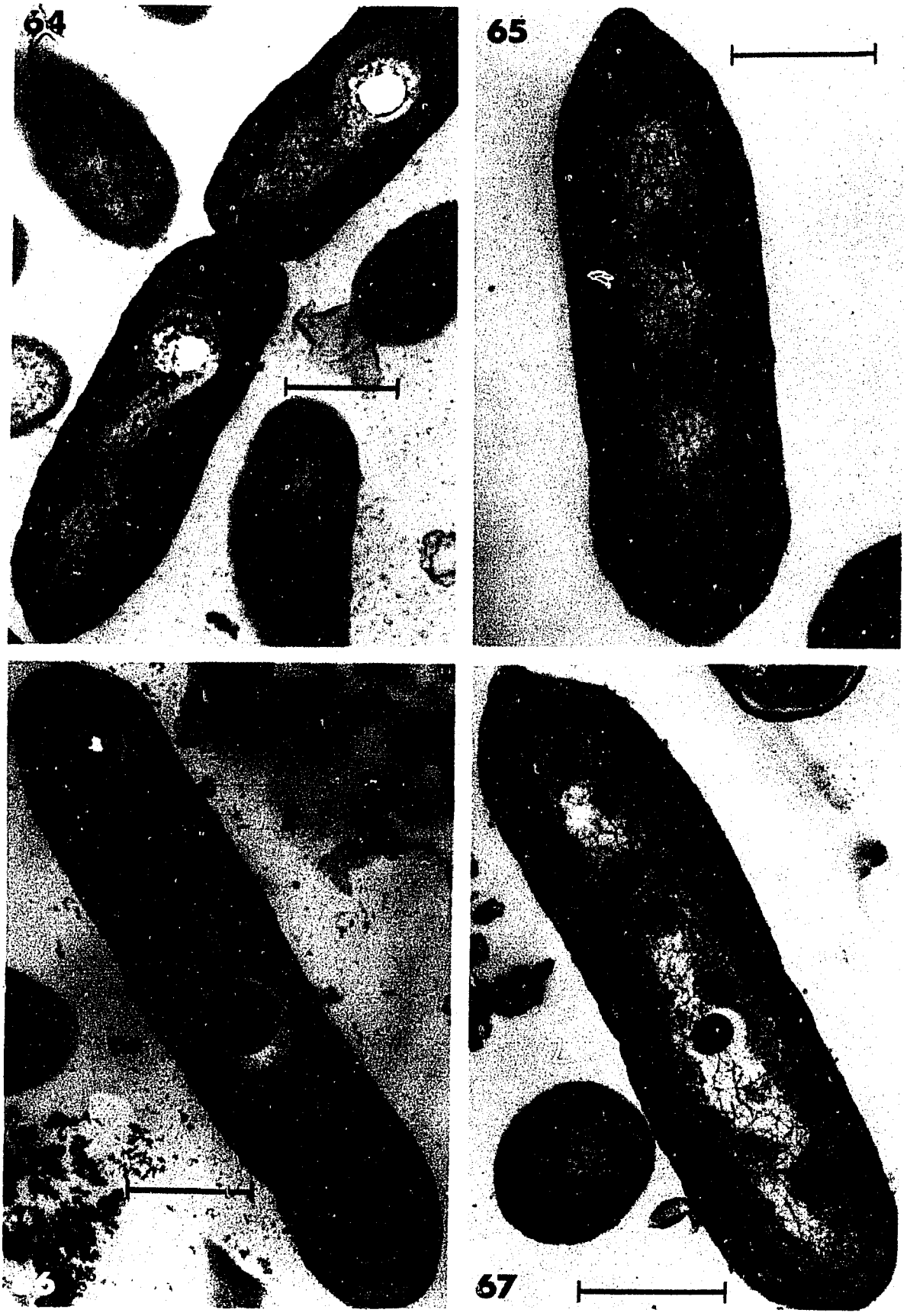
Figures 64-67. Thin sections of cells grown with a thioether or a thioacetal as the sole exogenous sulfur source 144 hours after the beginning of each six-day growth period. Polypohosphate bodies (pp) are evident; marker bars are equivalent to 500 nm.

Figure 64. Cells grown with 0.31 mM DL-lanthionine, first growth period.

Figure 65. Cells grown with 0.62 mM DL-lanthionine, second growth period.

Figure 66. Cells grown with 0.155 mM L-djenkolic acid, first growth period.

Figure 67. Cells grown with 0.465 mM L-djenkolic acid, second growth period.


64
65
67

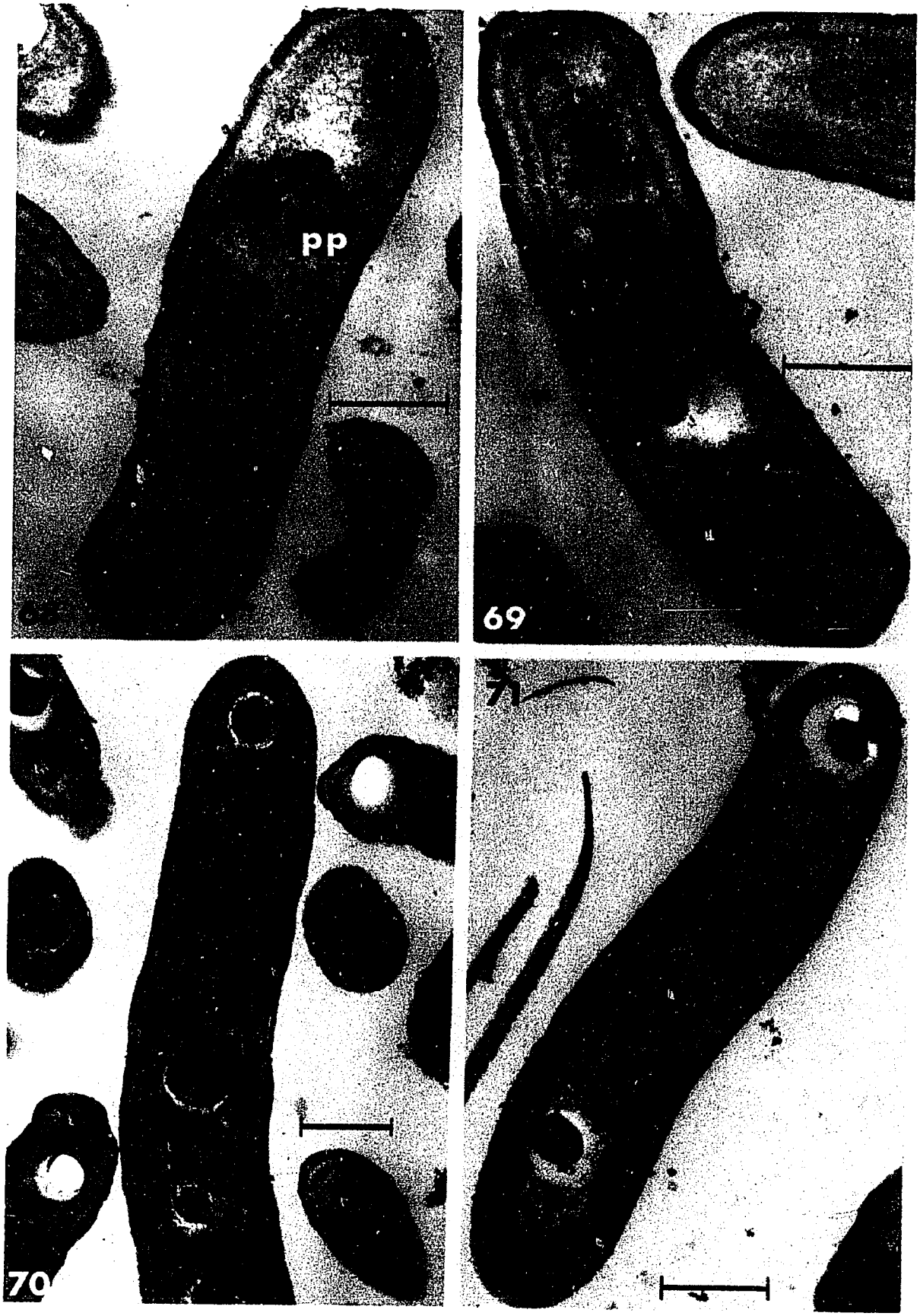
Figures 68-71. Thin sections of cells grown with a thioether or a disulfide as the sole exogenous sulfur source 144 hours after the beginning of each six-day growth period. Polyphosphate bodies are designated pp; marker bars are equivalent to 500 nm.

Figure 68. Cells grown with 0.31 mM L-methionine, first growth period.

Figure 69. Cells grown with 0.93 mM L-methionine, second growth period.

Figure 70. Cells grown with 0.155 mM DL-homocystine, first growth period.

Figure 71. Cells grown with 0.465 mM DL-homocystine, second growth period.


pp
69
70
71

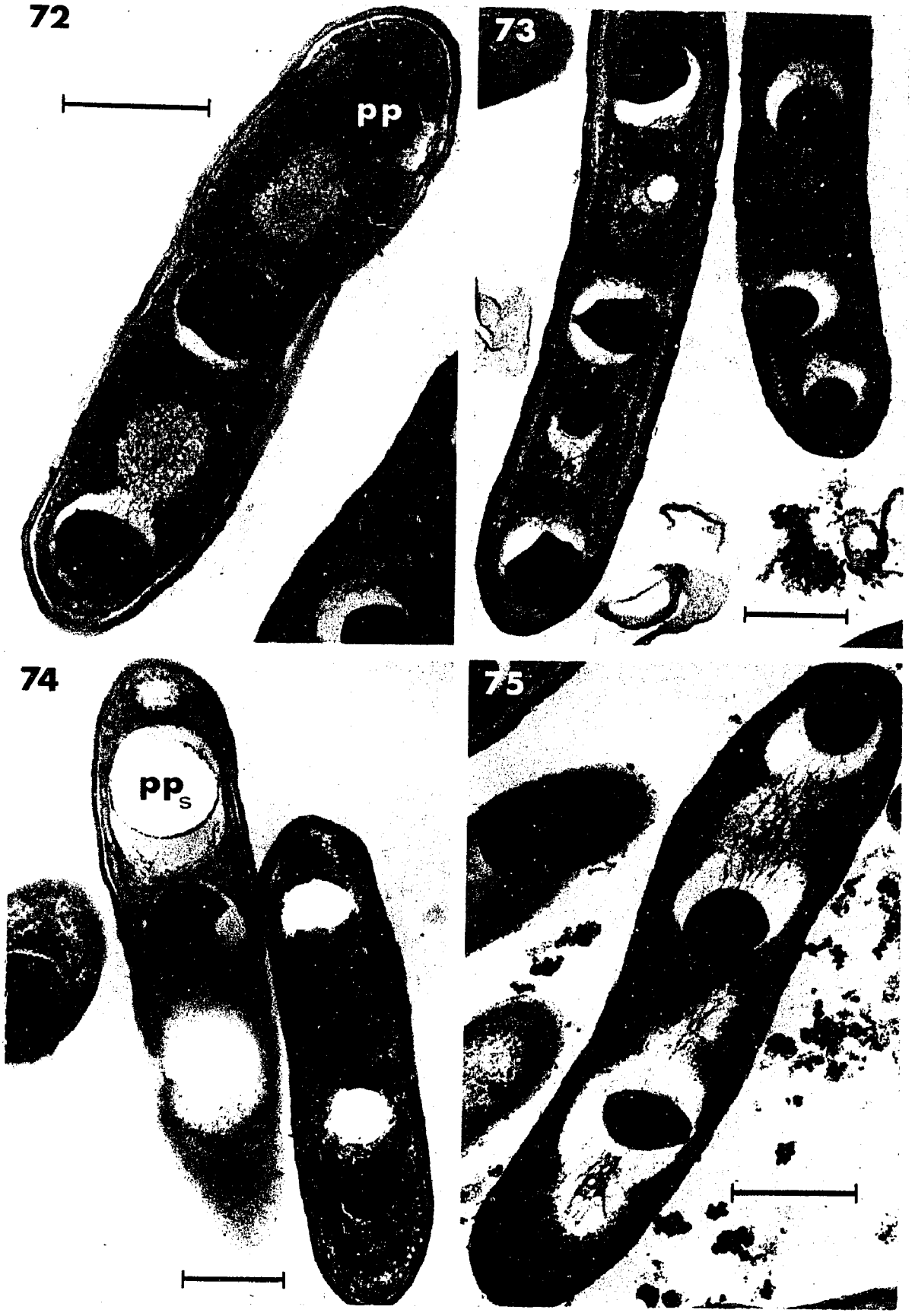
Figures 72-75. Thin sections of cells grown with a sulfonic acid as the sole exogenous sulfur source 144 hours after the beginning of each six-day growth period. Polyphosphate bodies are indicated as pp, and their remnant spaces as pp_s; marker bars are equivalent to 500 nm.

Figure 72. Cells grown with 0.31 mM taurine, first growth period.

Figure 73. Cells grown with 0.93 mM taurine, second growth period.

Figure 74. Cells grown with 0.31 mM L-cysteic acid, first growth period.

Figure 75. Cells grown with 0.93 mM L-cysteic acid, second growth period.


72
pp
73
74
pp$_s$
75

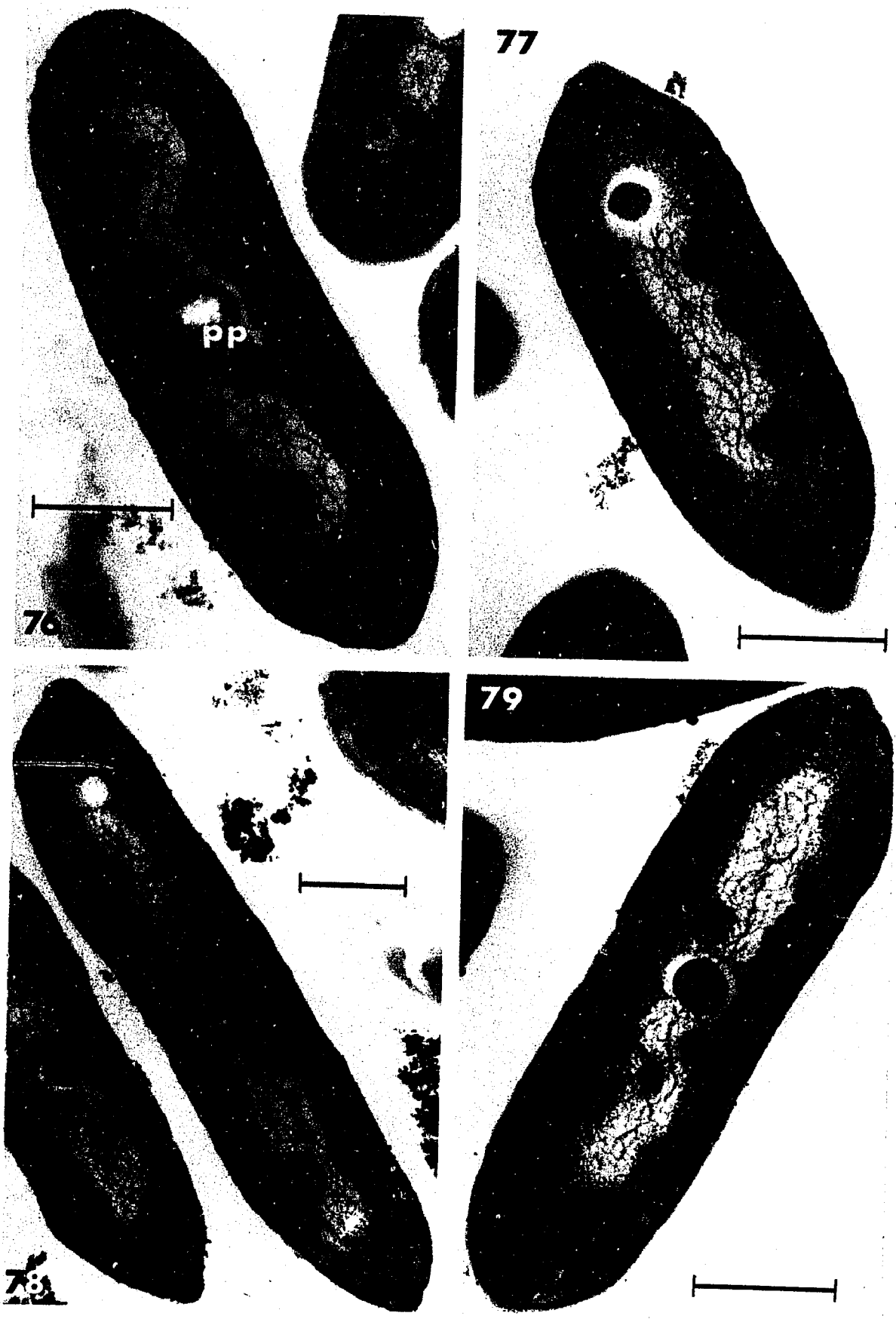
Figures 76-79. Thin sections of cells grown with a disulfide or an oligopeptide as the sole exogenous sulfur source 144 hours after the beginning of each six-day growth period. Polyphosphate bodies (pp) are evident; marker bars are equivalent to 500 nm.

Figure 76. Cells grown with 0.155 mM L-cystine, first growth period.

Figure 77. Cells grown with 0.31 mM L-cystine, second growth period.

Figure 78. Cells grown with 0.31 mM reduced glutathione, first growth period.

Figure 79. Cells grown with 0.62 mM reduced glutathione, second growth period.


pp
76
77
78
79

glutathione, and fixed after 144 hours of growth, are shown in Figures 64 through 79.

Preliminary investigation with a racemic mixture of cystathionine indicated both poor growth and marked polyphosphate accumulation when that thioether was used as the sole exogenous sulfur source. Therefore, L-cystathionine was provided exogenously at concentrations equivalent to 0.31, 1.0, and 2.31 mM sulfur. Figure 80 shows that growth with L-cystathionine, although better than cultures provided no exogenous sulfur, was considerably poorer than with sulfate as the sulfur source. Furthermore, growth did not increase with the increasing molarity of L-cystathionine in the different cultures. The pattern of cellular phosphorus accumulation is represented in Figure 81. Although such accumulation was sequential in direct order of cystathionine concentration in the medium, nonetheless final cellular phosphorus levels were virtually identical. Such levels were 400% greater than sulfate-grown cells, but 67% of that of sulfur-starved cells.

Figures 82-85 show thin sections of representative cells grown on L-cystathionine for 47 hours. The mean size of the polyphosphate granules was in inverse proportion to the exogenous L-cystathionine concentration. Figures 86-88 are thin sections of such cells grown for 144 hours. Deposition of polyphosphate in the cell displayed in Figure 86 resulted in the coalescence of two adjacent bodies. Figure 89 is a thin section of a cell maintained for 144 hours in sulfur-free medium.

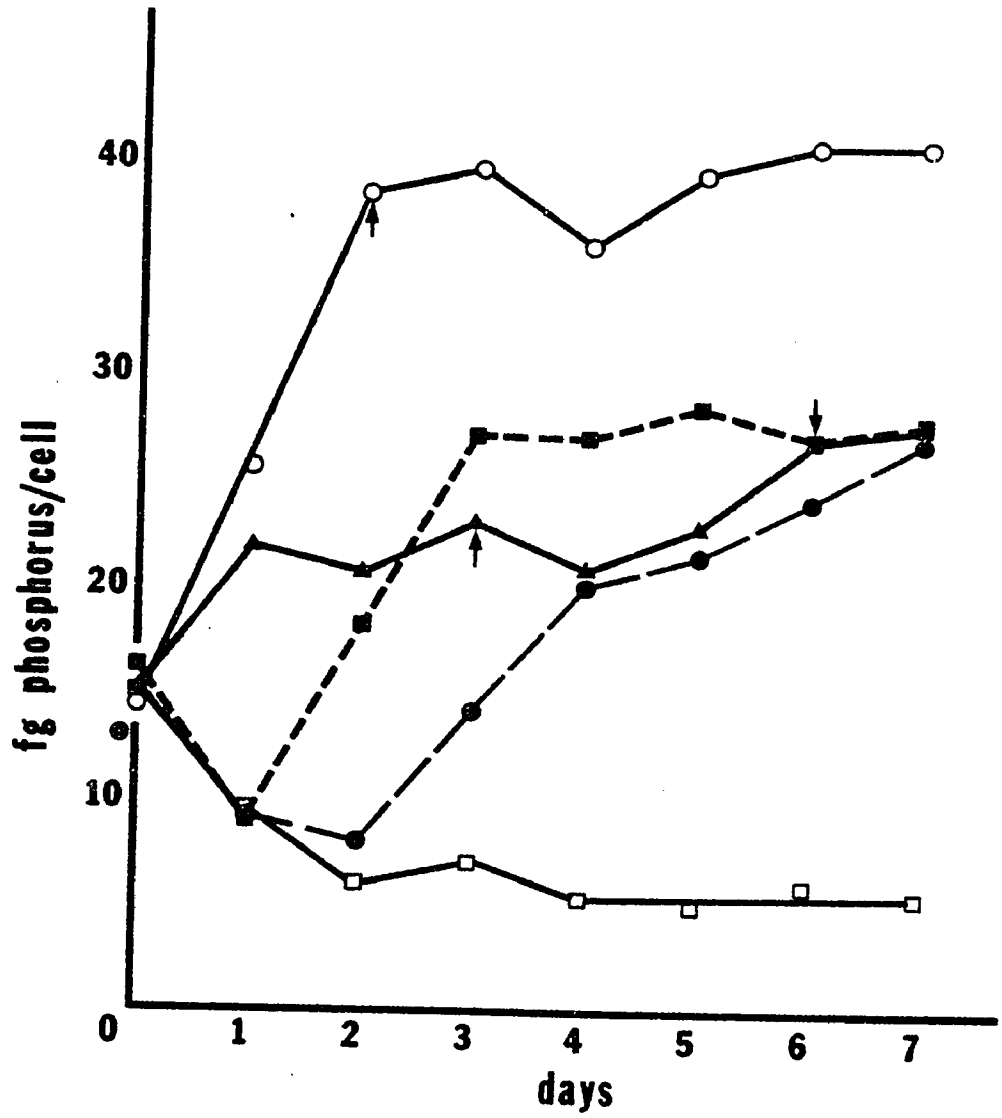
Energy Dispersive X-ray Microanalysis

Energy dispersive spectroscopy was done on many phosphate bodies of many cells in various nutritional states. Elemental contribution to the spectra obtained was remarkably similar. All spectra showed a very

Figure 80. Cell numbers per ml of cultures grown variously with: 0.31 mM L-cystathionine (▲); 1.0 mM L-cystathionine (■); 2.31 mM L-cystathionine (●); 0.31 mM sulfate (□); and zero sulfur (○).

cells x 10⁶
0
2
4
6
8
10
days
0
1
2
3
4
5
6
7

Figure 81. Changes in cellular phosphorus by cultures grown on an initial 0.32 mM exogenous orthophosphate. Phosphorus level of inoculum cells previously maintained on zero exogenous sulfur for 34 hours (◎); cells grown with 0.31 mM L-cystathionine (▲); cells grown with 1.0 mM L-cystathionine (■); cells grown with 2.31 mM L-cystathionine (●); cells grown with 0.31 mM sulfate (□); and cells grown with zero exogenous sulfur (○). The arrows indicate the days when those cultures became fully chlorotic.

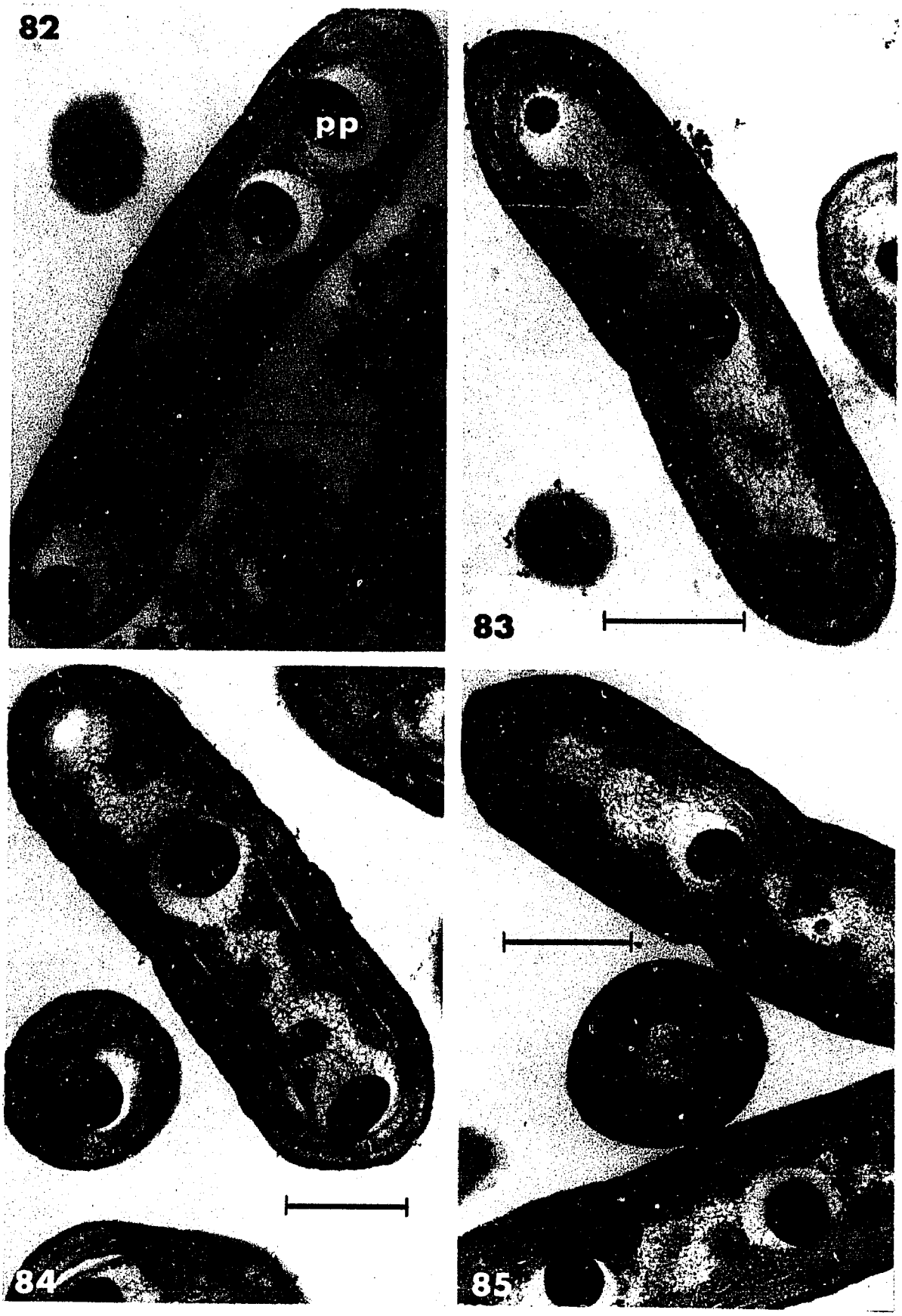

40
30
20
10
0
fg phosphorus/cell
1
2
3
4
5
6
7
days

Figures 82-85. Thin sections of cells grown for 47 hours with varying levels of the thioether L-cystathionine as the sole exogenous sulfur source. Polyphosphate bodies (pp) are evident; marker bars are equivalent to 500 nm.

Figure 82. Cells grown with 0.31 mM L-cystathionine.

Figures 83, 84. Cells grown with 1.0 mM L-cystathionine.

Figure 85. Cells grown with 2.31 mM L-cystathionine.


82
pp
83
84
85

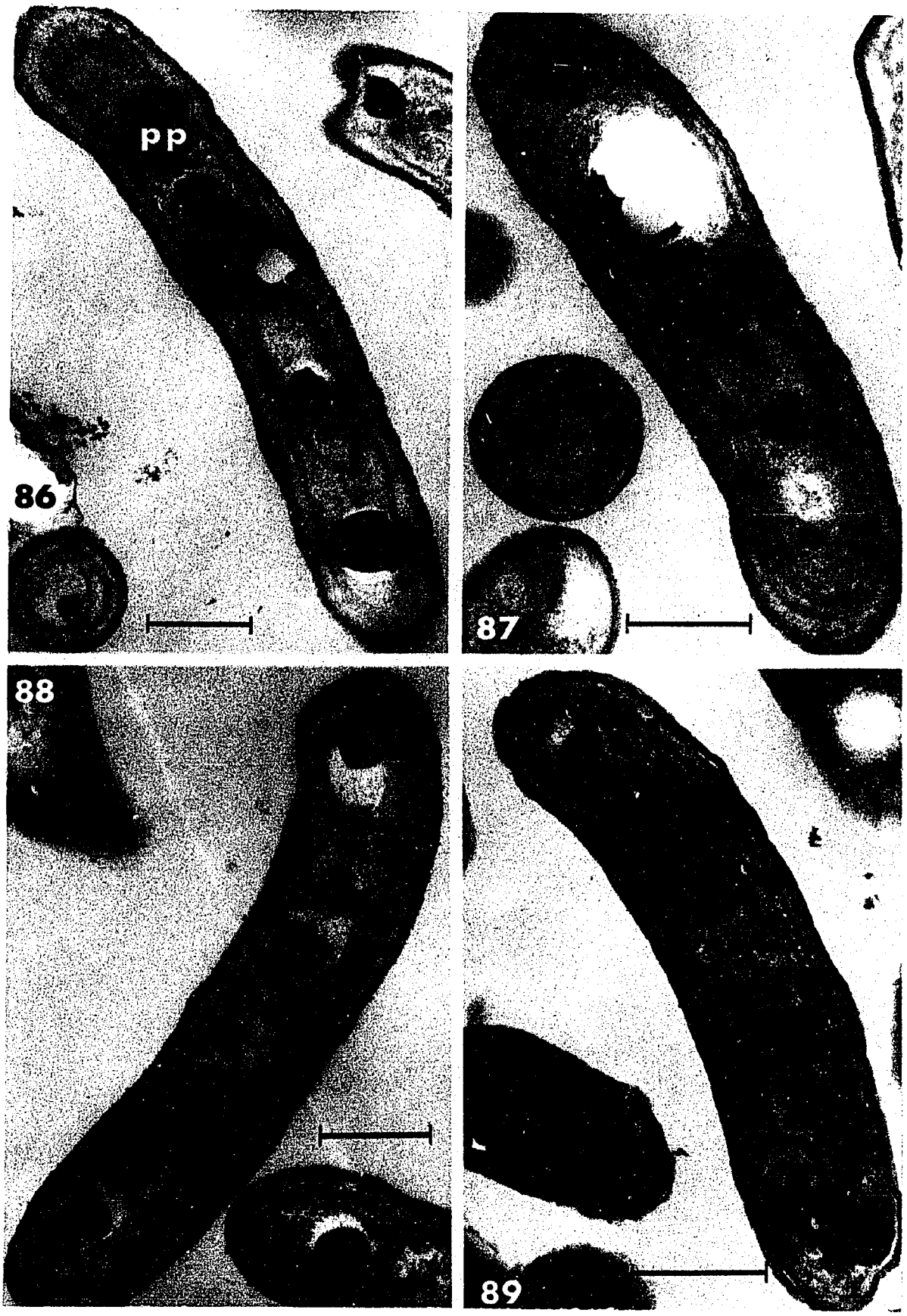
Figures 86-88. Thin sections of cells grown for 144 hours with varying levels of the thioether L-cystathionine as the sole exogenous sulfur source. Polyphosphate bodies (pp) are evident; marker bars are equivalent to 500 nm.

Figure 86. Cells grown with 0.31 mM L-cystathionine.

Figure 87. Cells grown with 1.0 mM L-cystathionine.

Figure 88. Cells grown with 2.31 mM L-cystathionine.

Figure 89. Thin sections of cells grown for 144 hours on medium lacking sulfur.


pp
86
87
88
89

strong phosphorus peak, as well as peaks of the major cations Mg^{2+}, Ca^{2+}, K^{+}, and frequently but not always Na^{+}. Very often evident was a small peak or shoulder for silicon. A sulfur peak was always present in polyphosphate bodies of control cells and those recovering from sulfur depletion, but never in those of sulfur-starved cells.

The most common variation was in the presence of calcium versus potassium. Almost invariably a strong peak for one cation was accompanied by a weak peak for the other. This reciprocity is shown in Figures 90 and 91. The polyphosphate bodies analyzed in these figures were both from sulfur-depleted cells 48 hours after transfer into sulfur-sufficient, phosphorus-deficient medium. The peak-to-background ratios for magnesium, phosphorus, potassium, and calcium are 4.8, 16.3, 4.7, and 2.0, in Figure 90, and 6.6, 27.0, 2.5, and 4.2 in Figure 91. Analysis of nearby areas of cytoplasm in the cells are shown in both figures.

For further comparison a polyphosphate body and cytoplasmic area were analyzed in a cell from a sulfur-depleted population 48 hours after transfer into sulfur-sufficient and phosphorus-sufficient medium (Figure 92). The peak-to-background ratios for magnesium, phosphorus, potassium, and calcium (of the granule) are 6.5, 24.0, 2.7, and 6.5.

Figure 90. The lowest energy portion of the x-ray spectrum from a polyphosphate body and the background cytoplasm of an air-dried cell of Synechococcus sp., 48 hours after transfer from sulfur-deficient, phosphorus-sufficient medium to sulfur-sufficient, phosphorus-deficient medium. The following elements and their K energy peaks are indicated thereon: sodium, 1.05-1.07 keV; magnesium, 1.26-1.28 keV; phosphorus, 2.02-2.14 keV; sulfur, 2.33-2.47 keV; potassium, 3.32-3.60 keV; and calcium, 3.70-4.02 keV. The ordinate displays the number of counts obtained in 100 seconds.

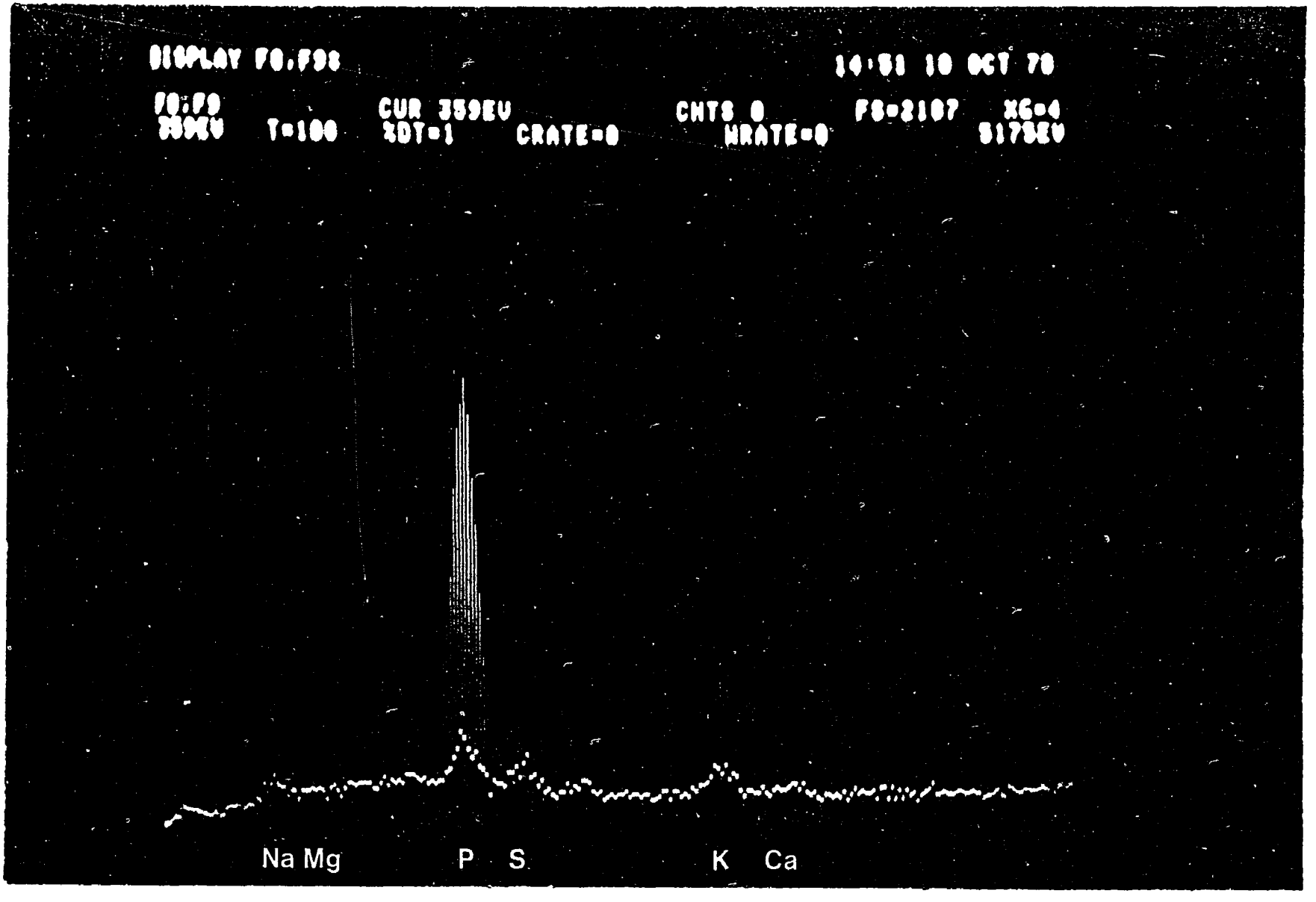

DISPLAY F8.F9S
14:51 10 OCT 78
F8:F9
359EV
T=100
CUR 359EV
ΔDT=1
CRATE=0
CNTS 0
NRATE=0
F8=2107
X6=4
517EV
Na Mg
P
S
K
Ca

Figure 91. The lowest energy portion of the x-ray spectrum from a polyphosphate body and the background cytoplasm of an air-dried cell of Synechococcus sp., 48 hours after transfer from sulfur-deficient, phosphorus-sufficient medium to sulfur-sufficient, phosphorus-deficient medium. The following elements and their K energy peaks are indicated thereon: sodium, 1.05-1.07 keV; magnesium, 1.26-1.28 keV; silicon, 1.72-1.82 keV; phosphorus, 2.02-2.14 keV; sulfur, 2.33-2.47 keV; potassium, 3.32-3.60 keV; and calcium, 3.70-4.02 keV. The ordinate displays the number of counts obtained in 100 seconds.

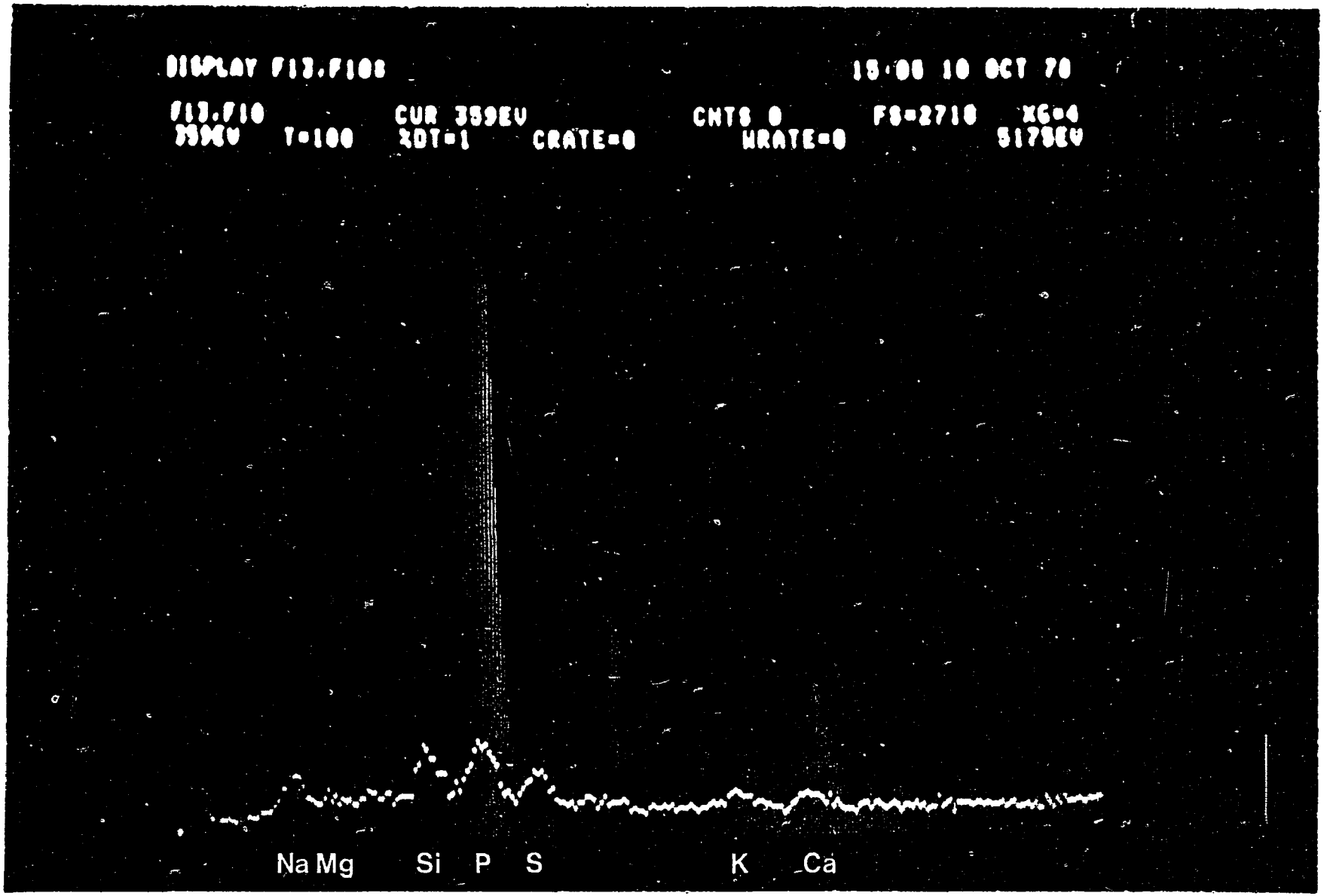
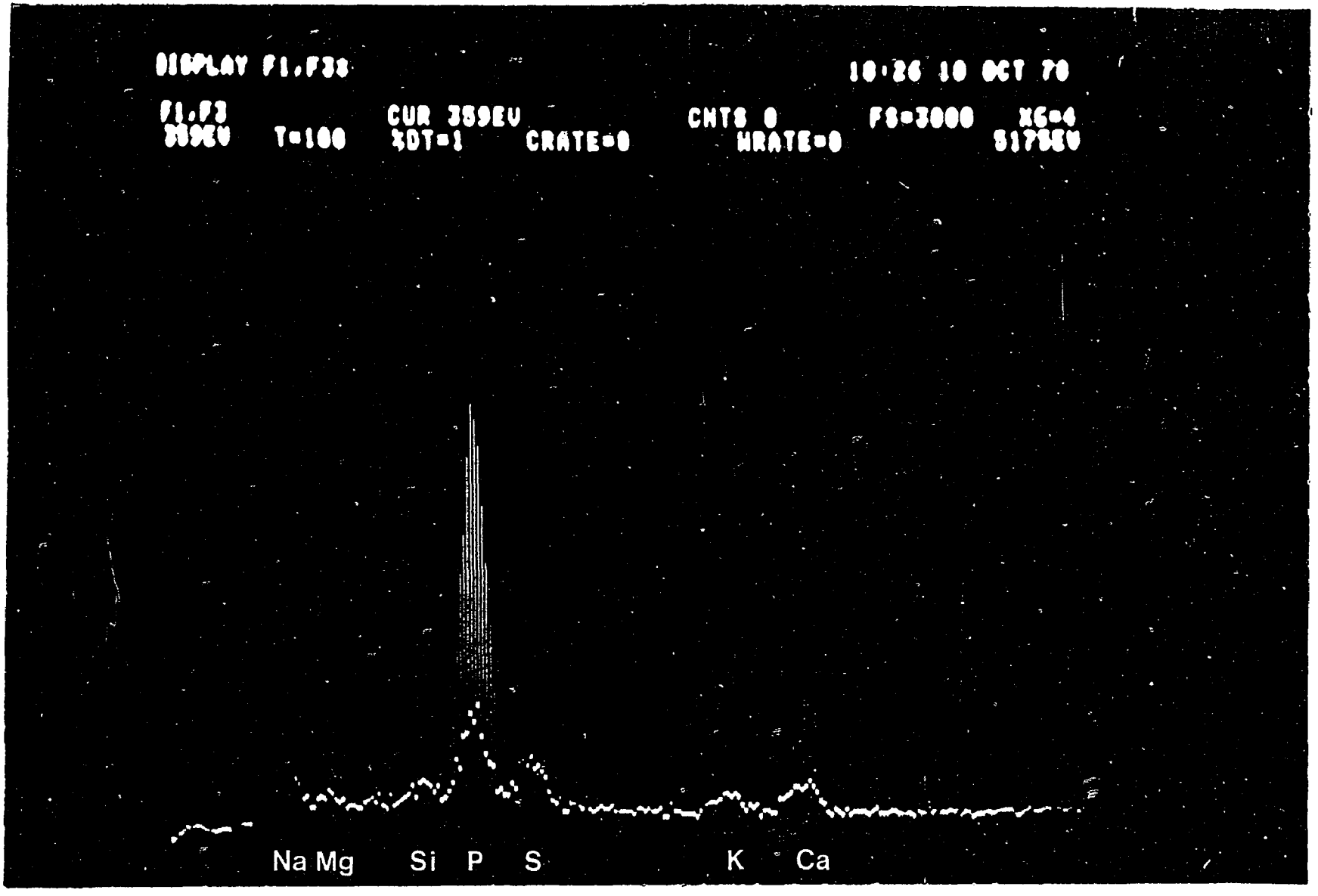

DISPLAY F13.F10B
15:08 10 OCT 78
F13.F10
359EV
Y=100
CUR 359EV
XDT=1
CRATE=0
CNTS 0
HRATE=0
FS=2710
XG=4
5179EV
Na Mg
Si
P
S
K
Ca

Figure 92. The lowest energy portion of the x-ray spectrum from a polyphosphate body and the background cytoplasm of an air-dried cell of Synechococcus sp., 48 hours after transfer from sulfur-deficient medium to complete medium (i.e. both phosphorus-sufficient). The following elements and their energy peaks are indicated thereon: sodium, 1.05-1.07 keV; magnesium, 1.26-1.28 keV; silicon, 1.72-1.82 keV; phosphorus, 2.02-2.14 keV; sulfur, 2.33-2.47 keV; potassium, 3.32-3.60 kev; and calcium, 3.70-4.02 keV. The ordinate displays the number of counts obtained in 100 seconds.


DISPLAY F1,F3X
18:26 10 OCT 76
F1,F3
309EV
Y=100
CUR 309EV
ΔDT=1
CRATE=0
CNTS 0
HRATE=0
FS=3000
XS=4
5175EV
Na Mg
Si
P
S
K
Ca

DISCUSSION

It is clear from the short-term experiments (Fig. 2,3) that phosphorus-depleted cells of Synechococcus sp. are capable of rapid uptake. Such uptake is largely complete after one hour, similar to findings obtained with cells of Plectonema boryanum (Jensen and Sicko, 1974; Sicko-Goad and Jensen, 1976). The amount of phosphorus taken up is at least in part a function of the concentration of exogenous inorganic phosphate, as is shown by the results after phosphorus-starved cells of Synechococcus were exposed to 0.16 mM and 0.06 mM orthophosphate. Cells suspended in medium containing the former concentration accumulated 40-50% more cellular phosphorus than in the latter during the first hour.

Normal phosphate uptake in cells of this species is energy-dependent and carrier-mediated, with an optimum likely in the pH 8.0-8.5 range (Falkner et al., 1974; Simonis et al., 1974; Ullrich-Eberius and Yingchol, 1974). Results of the present study indicate a similar pH range for optimal rapid phosphate uptake. As shown by the Henderson-Hasselbalch equation, $pH - pK_a = \log \frac{[salt]}{[acid]}$, the predominant anionic species of orthophosphate in this range is HPO_4^{2-}, and its concentration increases well into alkaline pH. If the increased uptake with pH were simply a function of greater availability of the dianion, the greatest uptake within the range tested should be shown at pH 9. Instead a substantial dropoff occurs at this pH, suggesting the involvement of a carrier molecule in rapid phosphate uptake, although not necessarily the same one(s) functioning in normal phosphate uptake.

In the short-term experiment during which exogenous sulfur concentration was varied (Fig. 3) the narrow range of phosphorus accumulation

measured and the similar size of polyphosphate granules observed preclude conclusive statements. However failure to note an immediate decline in phosphorus accumulation and polymerization in the cell population treated with 10 µg/ml chloramphenicol, suggests that proteins having a role in both orthophosphate transport and polymerization are relatively long-lived. Similar results were observed in cells of Enterobacter aerogenes by Harold (1963).

Harold (1963) predicted that polyphosphate is likely the 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, present findings and those of Niemeyer and Richter (1969) are to the contrary. Cells of the A. nidulans 625 strain of Synechococcus elaborate polyphosphate bodies whose mean size increases with both the duration of phosphorus starvation and the concentration of exogenous phosphate during replenishment.

The exposure of cells sulfur-starved for 24 hours to a reduced sulfur anion requires them to use that anion as is, if they are able to do so, and not after it has been oxidized to sulfate. Additionally the act of sulfur deprivation very likely either increases the amounts of proteins involved in the transport and assimilation of such anions, or triggers their activity, or both. No differences were observed in total phosphorus accumulation (Fig. 10) or in the size of polyphosphate inclusions (Fig. 11-14) when that sulfur anion was sulfate, thiosulfate,

or metabisulfite. That other sulfur anions can freely substitute for sulfate is hardly unexpected. Utkilen et al. (1976) have found evidence of a common carrier for sulfate, sulfite, and thiosulfate, and Utkilen (1976) has further determined that thiosulfate can serve as an electron donor for photosynthesis, in cells of Synechococcus sp. Such photoreduction by thiosulfate in this species is accompanied by the appearance of intracellular refractile granules, likely elemental sulfur (Peschek, 1978).

In the long-term starvation study the concurrent loss of orthophosphate from the growth medium (Fig. 16) and the decline in cellular phosphorus levels (Fig. 17) in the first eight hours 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 disappearance. 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 (Fig. 16). This concentration was not limiting until the fourth day, 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 nearly the

same (Fig. 15).

From the ultrastructural standpoint it is apparent that much of the newly obtained orthophosphate is polymerized into condensed phosphate. Comparing samples taken at 47 and 120 hours (Fig. 18-25), cells grown on sulfate concentrations differing successively by one order of magnitude began more to resemble cells with no exogenous sulfur and less so the control cells (initial concentration 0.31 mM sulfate), both in size of polyphosphate bodies and of cellular deterioration. In this particular early experiment most of the polyphosphate accumulation was accounted for by a substantial increase in size of extant bodies. Normal cells routinely exhibit two or three polyphosphate granules. If two, they are approximately one-third and two-thirds the distance along the cell's long axis; if three, two are in polar and one in central position. The normal size of such bodies is 200 nm D.

With time and experience it became possible to increase the severity of starvation effects while still retaining cell viability. Under such conditions additional polyphosphate was deposited, first as additional granules between those already present, then as bands and rings encircling the cell just within the plasma membrane (Fig. 86, 89).

The cell volume of Synechococcus sp. can be calculated roughly by considering the cell to be a cylinder capped by two hemispheres. The average dimensions of such cells are a diameter of 0.65 μm and a length of 2.5 μm. Each hemisphere then has a radius of 0.325 μm, and the cylinder remaining has a radius of 0.325 μm and a length of 1.85 μm. Total volume amounts to 0.758 μm^3, or 0.758 fl. If one assumes an approximate specific density of 1.05 pg/fl for the protoplasm encompassed, cell mass is very nearly 800 fg.

A total cellular phosphorus varying between 5 and 10 fg for normal cells would be equivalent to between 0.6 and 1.25% fresh weight, or between 1.56 and 3.12% fresh weight when expressed as phosphate. Sulfur-deficient cells which accumulated 40 fg total phosphorus contained 5% of their weight as phosphorus or 12.8% as phosphate. When compared to the large-sized granules seen ultrastructurally, the mass of phosphorus seems deceptively small. However Smith et al. (1954) obtained similar results with cells of Enterobacter (Aerobacter) aerogenes. Granules in sulfur-starved cells occupied up to 20% of cell volume, but the polyphosphate measured was less than 1% of the dry weight.

The intimate association of polyphosphate and fibrils of nucleic acid (Fig. 27) reported previously in other prokaryotic cells by Voelz et al. (1966) and Jensen and Sicko (1974), may have some bearing on the apparent ease by which phosphate is exchanged between nucleic acid and condensed phosphate within the cell (Correll and Tolbert, 1962; Harold, 1963; Harold and Sylvan, 1963).

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, 1964a,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 (Voladin, 1970). The discovery that sulfur deficiency in cells of Enterobacter aerogenes increased 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 photoautotrophic Synechococcus sp. respond to a deficiency of sulfur in a manner very similar to that of cells of the heterotrophic bacteria previously investigated.

The reciprocity of uptakes between orthophosphate and sulfate is likely due to a common transporter (or transporters) of these anions. Both phosphate and sulfate uptakes in cells of Synechococcus sp. are energy-dependent and carrier-mediated, with an optimum pH range between 7.5 and 8.5 (Falkner et al., 1974; Simonis et al., 1974; Ullrich-Eberius and Yingchol, 1974; Utkilen et al., 1976; Jeanjean and Broda, 1977). Utkilen et al. (1976) have found that sulfate uptake is competitively inhibited by sulfite and thiosulfate. Crompton et al. (1975) have demonstrated that the dicarboxylate carrier of the rat 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 con-

densed phosphate deposition. Not only is glutathione an important component in eukaryotic cells, but also it has been shown by Fahey et al. (1978) to constitute a substantial proportion of the soluble cellular thiol in most gram negative bacteria, including the cyanobacterium Nostoc muscorum.

The first suggestion that cells of Synechococcus sp. were less immediately susceptible to exogenous phosphorus depletion than to that of exogenous sulfur was the observation that phosphorus-deprived cells generally did not become as intensely or as quickly chlorotic as did sulfur-deprived ones. Two explanations were possible: (a) the cells were adversely affected physiologically by the loss of exogenous phosphorus, but did not suffer rapid chlorosis since unlike sulfur, phosphorus is not a constituent of the photosynthetic pigment proteins; (b) the cells were not immediately affected physiologically because an internal phosphorus reservoir exists to offset conditions of limiting phosphorus.

That such a phosphorus reservoir is polyphosphate was not unexpected. Phorphorus-deprived cells drawing upon this polymer would hardly be as metabolically impaired as sulfur-deprived cells which, using their phycobiliproteins as a sulfur source, would reduce the photoautotrophic capacity. The literature provides a few examples of the apparent utilization of polyphosphate as an internal phosphorus source. Baker and Schmidt (1964) demonstrated a decrease in polyphosphate over ten hours in synchronously grown cells of Chlorella abruptly deprived of exogenous phosphorus. The immediate decline in the cellular level of the polymer suggested use via constitutive phosphatases or reversed polyphosphate kinase. Stewart and Alexander (1971) showed that not only does detergent phosphorus rapidly increase the polyphosphate content of phosphorus-starved

cells of Anabaena flos-aquae, but also that such cells can assimilate enough detergent phosphorus in a day to permit a week's growth under phosphorus-limiting conditions.

The trivial observations mentioned above are borne out by Figures 36 and 37. Previously normal cells deprived of exogenous phosphorus for a period of six days contained a 25% greater level of chlorophyll a, but 100% greater level of c-phycocyanin, than cells deprived of exogenous sulfur for a like duration. It has been suggested that phycocyanin serves as a reserve for both sulfur and nitrogen (Wolk, 1973). Furthermore cells deprived of exogenous sulfur for six days, then restored to exogenous sulfur sufficiency but exogenous phosphorus deficiency, in three days accumulated levels of chlorophyll a and c-phycocyanin slightly higher than those of the control cells at the beginning of the restoration period. The subsequent pigment losses were about 33% of peak values, but levels were still considerably higher than those of the phosphorus-deficient control cells at the end of the six-day restoration period.

Taken together the pigment and cellular phosphorus levels form a meaningful pattern. Cells starved for sulfur for six days had cellular phosphorus levels three times that of non-starved control cells (Figure 39), as well as polyphosphate inclusion granules considerably increased in size (Figures 22, 25, 26). During recovery cellular phosphorus levels of populations previously starved of sulfur and that of control cells presently deprived of phosphorus fell rapidly. The porosity and decreasing size of the polyphosphate bodies in recovering cells strongly implies that the polymer was being hydrolyzed (Figures 40-55). Thus it can reasonably be concluded that the very large polyphosphate bodies induced by a period of sulfur starvation contribute to the maintenance and/or recovery of the cells whether exogenous phosphorus is then present or not.

The effect of chloramphenicol is not altogether clear. Certainly from the short-term studies it can be shown that the transporter involved in rapid phosphate uptake is long-lived. In the long-term studies recovering populations previously starved of sulfur and then treated for 12 hours with 10 µg/ml CAP did grow more slowly than their non-treated counterparts, but this effect was likely a general one by the inhibitor of prokaryotic cellular protein synthesis. Recovering cells treated with CAP appeared to take up no exogenous orthophosphate over the six-day period (Figure 38), whereas recovering, non-treated cells (whose population numbers were only somewhat greater) did show orthophosphate uptake after three days. Non-starved control cells transferred to fresh, complete medium took up an appreciable amount of orthophosphate over the entire six-day period.

However when one contrasts these results with Table 3, it becomes apparent that a discrepancy exists. All three populations provided with exogenous phosphate substantially increased in cellular phosphorus per ml suspension, despite decreases in cellular phosphorus per cell. Accompanying increases in cellular phosphorus per ml by populations deprived of exogenous phosphate were much less, and in one case exhibited no increase whatsoever. For this reason one can rule out some problem with obtaining a true estimation of cellular phosphorus by the method used, which depends upon an initial oxidation of the washed cells by $K_2S_2O_8$ under heat and pressure. Therefore the increases must have arisen from uptake of orthophosphate, despite the lack of a concomitant decrease in exogenous level in all cases.

In spite of the equivocal results in respect to the long-term effect of CAP on the phosphate transporter(s), population growth did occur, so

the cells had recovered in other ways. After a comparison of all the data at hand, it is concluded that recovering cells of Synechococcus sp. draw upon polyphosphate as their sole or primary phosphorus source for three or four days, then switch over to exogenous orthophosphate. The choice of phosphate source would of course depend on the energy investment required to procure the anion.

The large polyphosphate bodies resulting from a period of sulfur starvation represent both a convenient and a mandatory source of phosphate for cellular needs, because of the necessity of reducing the size of these voluminous inclusions to resume normal cell structure and function. The significantly smaller polyphosphate bodies of normal cells provide for a lesser period of grace in recovery, but nonetheless they represent a phosphorus reserve against limiting phosphate. Occasions of phosphate limitation in natural waters are far more frequent than those of, say, sulfate, against which little protection is afforded.

The obligatory photoautotrophy of small, unicellular, and likely primitive cyanobacteria of the "Anacystis nidulans" variety suggests that such organisms are most efficiently programmed to acquire and assimilate inorganic ions. Initial investigations on the biochemical basis of such obligate autotrophy were performed by Smith et al. (1967), and further studies have been done since that time.

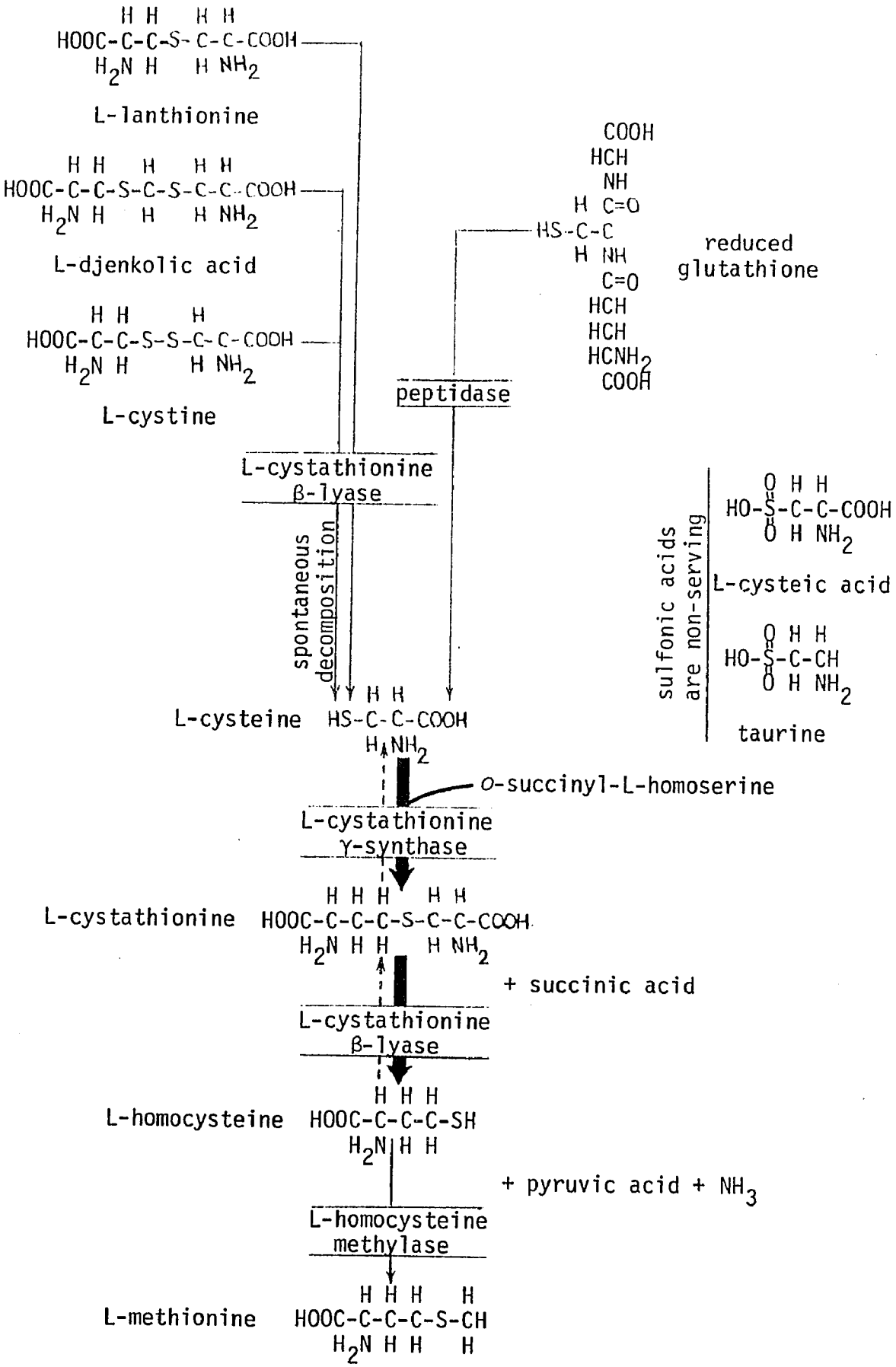
Several non-sulfur amino acids are taken up by species of the Synechococcus type (Maclean et al., 1965; Ingram et al., 1972; Singer and Doolittle, 1975; Kaney and Jhabvala, 1975), and a methionine-requiring mutant of A. nidulans has been isolated (Delaney et al., 1973), so it appears no barrier exists to amino acid transport into these cyanobacte-

rial cells. The pattern of sulfur amino acid utilization in the present study in no way contradicts this impression. For example, that an "exotic" amino acid such as L-djenkolate serves as a sole sulfur source, while the commonly occurring L-methionine cannot, makes a barrier explanation very unlikely.

The shortchain organosulfur compounds tested are shown in Figure 93. Those serving as sole sulfur sources -- L-cystine, DL-lanthionine, L-djenkolic acid, and glutathione (Figures 56-59, 65, 67, 76-79) -- have in common a potential L-cysteine moiety. Both of the known cystathionases, E.C. 4.4.1.1 and 4.4.1.8, probably are specific for cysteine or a close derivative as a point of attack (Brinkley, 1950; Cavallini et al., 1960; Flavin, 1962; Greenberg et al., 1964; Delavier-Klutchko and Flavin, 1965a; Giovanelli and Mudd, 1971). The speed and efficiency of enzymatic cleavage vary among organosulfur substrates and also among microbial species. Such variances most likely underlie the differences in the concentrations required to permit normal growth and morphology in cells of Synechococcus sp.

The enzyme present in heterotrophic prokaryotes such as Escherichia coli and Salmonella typhimurium is L-cystathionine-β-lyase, E.C. 4.4.1.8 (Wijesundera and Woods, 1962; Delavier-Klutchko and Flavin, 1965a,b). The presence of this enzyme in cells of Synechococcus sp. is inferred with confidence because: (a) neither L-methionine nor DL-homocystine was able to act as a sole sulfur source (Figures 56-59, 68-71), whereas L-cystine was able to do so (Figures 57, 59, 76, 77); (b) L-cystathionine, although promoting a greater time differential in the onset of increased phosphate accumulation as a function of exogenous level (Figure 81), clearly was unable to provide for general cellular sulfur requirements

Figure 93. The suggested scheme for a unidirectional transsulfuration in cells of Synechococcus sp., including the structures of all potential organic sulfur sources tested. Skeletons in red denote either L-cysteine, cysteinyl moieties, or somewhat larger residues hydrolyzed by cystathionase which decompose spontaneously to yield L-cysteine. Both L-cysteine and L-methionine are required in the synthesis of cellular peptides and proteins.


L-lanthionine
L-djenkolic acid
L-cystine
reduced glutathione
peptidase
L-cystathionine β-lyase
spontaneous decomposition
sulfonic acids are non-serving
L-cysteic acid
taurine
L-cysteine
O-succinyl-L-homoserine
L-cystathionine γ-synthase
L-cystathionine
+ succinic acid
L-cystathionine β-lyase
L-homocysteine
+ pyruvic acid + NH3
L-homocysteine methylase
L-methionine

at physiological concentrations (Figures 86-88).

Such a pattern strongly points toward a unidirectional transsulfuration, from L-cysteine to L-methionine, as occurs in heterotrophic prokaryotes (Datko et al., 1974b, 1977; Flavin, 1975) and the photoautotrophic eukaryote (green alga), Chlorella sorokiniana (Giovanelli et al., 1978). Contrasted to values obtained with cells grown with zero exogenous sulfur, L-cystathionine-grown cells exhibited twice the growth (Figure 80), accumulated about 65% the total phosphorus (Figure 81), and in the case of those grown with 2.31 mM L-cystathionine, still retained a rather normal morphology after 144 hours (Figures 88, 89). Therefore it is very likely that a small but constant amount of the pivotal compound, L-cystathionine, is transsulfurated to L-cysteine.

The reason for this paradox lies in the lack of absolute specificity of at least some cystathionine-metabolizing enzymes. Guggenheim and Flavin (1969) determined that cystathionine-γ-synthase (E.C. 4.2.99.9) from Salmonella cell extract catalyzes not only a γ-replacement by L-cysteine of the succinyl moiety of *o*-succinyl-L-homoserine to form L-cystathionine, but also slow γ- and β-eliminations on L-cystathionine to yield, respectively, L-cysteine and L-homocysteine. The enzyme thus seems efficient for carrying out a general base attack on an amino acid β-hydrogen.

Ultrastructurally, in addition to the obvious deposition of incremental condensed phosphate and general cytoplasmic degeneration, sulfur-depleted cells underwent a transient lengthening (two to three times that of normal cells) and narrowing. With time and particularly, fresh medium, even if sulfur-free, the cells returned to their former configuration. Consequently the mass/volume calculations made earlier are applicable when

comparing cells grown with 0.31 mM sulfate and zero sulfur, but only during the second six-day period, when the sulfur-deficient cells had reverted to control size.

It is concluded that the Anacystis nidulans strain of Synechococcus is capable of utilizing certain shortchain organosulfur molecules as a source for cellular sulfur needs, and thus does demonstrate a limited heterotrophic capacity. Sulfate contamination of the amino acids can be ruled out, as manufacturers' specifications indicated the highest contamination was 0.055%, occurring in non-serving L-methionine. Prolonged sulfur deficiency, whether operating via a decrease in glutathione or sulfated nucleoside phosphates or enzyme sulfhydryl groups or cysteine/cystine components in the general protein population, induces a marked increase in orthophosphate uptake, condensed phosphate deposition, and the size and number of polyphosphate inclusion bodies.

Despite initial evidence from glutaraldehyde-fixed cells that large polyphosphate bodies induced by sulfur starvation differed in their phosphorus : calcium ratio from smaller ones of normal cells, x-ray energy dispersive analysis of air-dried specimens indicated no fundamental differences exist between the polyphosphate bodies from these two sources. Both exhibited a strong phosphorus component, as well as either a moderately strong calcium or potassium peak (Figures 90-92). The dichotomy thus appears to be between the calcium versus potassium contribution in bodies from different individual cells, rather than between bodies from sulfur-deficient and sulfur-sufficient cells.

Rosenberg (1966) determined by chemical means that polyphosphate bodies isolated from cells of the hymenostome ciliate, Tetrahymena pyri-

formis, consisted of a calcium-magnesium pyrophosphate complex. Coleman et al. (1972) submitted polyphosphate bodies from this protozoon to electron probe analysis and found two distinct types of granules on the basis of calcium : phosphorus and magnesium : phosphorus ratios (potassium was the least variable constituent). As with the pattern reported above in Synechococcus sp., a single granule type occurred per individual cell. Nilsson and Coleman (1977) demonstrated that calcium-rich granules from Tetrahymena cells were membrane-bounded, and the cation was present in an apatite-like array.

Very similar combinations arise among photoautotrophic cells. Fuhs (1969), using interference microscopy, reported that polyphosphate bodies from Oscillatoria borneti and Anabaena cylindrica were identical to synthetic potassium polyphosphate. Peverly et al. (1978) determined that polyphosphate in Chlorella pyrenoidosa was most intimately associated with potassium, and little of the polymer formed when potassium was absent. Omission of calcium or magnesium did not bring about a like effect. On the other hand in analyzing polyphosphate bodies of glutaraldehyde-fixed and sectioned cells of Plectonema boryanum by SEM and x-ray energy analysis, Sicko-Goad et al. (1975) found a significant calcium peak in addition to that of phosphorus. Kessel (1977), using a TEM-based system, failed to show any cationic contribution whatsoever in bodies within similarly prepared cells of the very same organism.

Since polyphosphate is a polyanionic and strongly basophilic substance, an association with cationic species is to be expected. The reason for the differences in the prevalent cation present, potassium, calcium, or magnesium, is yet undetermined. Very likely it will in time be shown to be a variation on the same fundamental structure.

SUMMARY

Maximum phosphate accumulation during rapid uptake into phosphorus-depleted cells of the unicellular cyanobacterium, Synechococcus sp. (Anacystis nidulans), occurred in the pH 7.5-8.5 range. As shown both chemically and ultrastructurally, overnight exposure to 10 μg/ml chloramphenicol had no immediate effect on either uptake or deposition, suggesting long-lived protein molecules are involved in both processes.

A decrease in exogenous sulfate level increased orthophosphate uptake and condensed phosphate deposition, thus offering an effective means to identify those sulfur-containing substances which would satisfy all cellular sulfur requirements, while at the same time obviating the well known difficulties inherent in sulfur biochemistry. Sulfate (SO_4^{2-}), thiosulfate ($S_2O_3^{2-}$), and metabisulfite ($S_2O_5^{2-}$) were for all purposes of the same facility in serving as a sulfur source. Some sulfur amino acids and an oligopeptide also served, which will be reviewed shortly.

Cells subjected to phosphorus sufficiency/sulfur deficiency took up much orthophosphate and elaborated massive polyphosphate bodies (confirmed by electron microscopy), which then served as phosphate reservoirs when the cells were shifted to sulfur sufficiency/phosphorus deficiency. Control cells suffered a general pigment decline and cytoplasmic deterioration within 24 hours, but cells previously starved for sulfur recovered and were able to survive in the absence of exogenous phosphorus for three or four days. If exogenous phosphate was provided, such recovery was complete.

By the means described previously a limited ability for heterotrophy was revealed. Shortchain organosulfur compounds, L-cystine, L-djenkolic acid, DL-lanthionine, and glutathione, served as sole sulfur sources,

whereas L-cystathionine, DL-homocystine, L-methionine, L-cysteic acid, and taurine were not capable of doing so. Such a pattern is consistent with a unidirectional transsulfuration from L-cysteine to L-methionine, as has been demonstrated in heterotrophic bacteria and green plants, and it is inferred that the keystone enzymes are L-cystathionine-γ-synthase (E.C. 4.2.99.9) and L-cystathionine-β-lyase (E.C. 4.4.1.8).

It is further suggested that, in common with other prokaryote-like systems studied recently, the orthophosphate and sulfate anions are taken into the cyanobacterial cell by the same porter molecule(s). The absence of one anion would subject the system to input overload by the other. However such an overload would be relieved or precluded by an alternate (e.g. organic) source of the missing anion.

NOTE ADDED IN PROOF

Using extract from bean hypocotyls, DeSantis et al. (1976) determined that intramitochondrial sulfate transport in exchange with external sulfate, sulfite, phosphate, and dicarboxylates is catalyzed, as in the rat liver mitochondrion, by the dicarboxylate carrier. On the other hand Abou-Khalil and Hanson (1979) have reported that energy-linked sulfate in isolated corn mitochondria is via the phosphate transporter. In either event these studies provide further proof that the sulfate and phosphate anions are carried across membrane barriers by the very same porter molecules.

DeSantis, A., Arrigoni, O., and Palmieri, F. 1976. Carrier-mediated transport of metabolites in purified bean mitochondria. Plant Cell Physiol. 17: 1221-1233.

Abou-Khalil, S. and Hanson, J. B. 1979. Energy-linked sulfate uptake by corn mitochondria via the phosphate transporter. Plant Physiol. 63: 635-638.

APPENDICES

Appendix A

Composition of the culture medium designed by Hughes et al. (1958), modified by Allen (1968), and modified again by Lawry:

	g/liter
$NaNO_3$	1.50
K_2PO_4	0.039-0.056
$MgSO_4 \cdot 7H_2O$	0.077
Na_2CO_3	0.05
$CaCl_2$	0.027
$Na_2SiO_3 \cdot 9H_2O$	0.058
Na_2EDTA	0.001
Citric acid	0.006
Fe citrate	0.006
Microelements	1 ml/liter

Microelements (sulfur-free)

	g/liter
H_3BO_3	2.86
$MnCl_2 \cdot 4\ H_2O$	1.81
$ZnCl_2$	0.105
$Na_2MoO_4 \cdot 2\ H_2O$	0.391
$Cu(NO_3)_2 \cdot 3\ H_2O$	0.0765
$Co(NO_3)_2 \cdot 6\ H_2O$	0.0494

Add 1 g/liter glycylglycine and adjust to pH 8.3.

Appendix B

Reagents and preparation for the Murphy-Riley orthophosphate determination (Murphy and Riley, 1962):

(a) 5N sulfuric acid -- dilute 70 ml concentrated H_2SO_4 to 500 ml

(b) ammonium molybdate -- dissolve 20 g $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ and dilute to 500 ml

(c) potassium antimonyl tartrate -- dissolve 0.5486 g $KSbC_4H_4O_7 \cdot \frac{1}{2}\ H_2O$ and

200 ml (1 mg/ml Sb)

(d) 0.1 M ascorbic acid (freshly made on day of use) -- dissolve 1.32 g ascorbic acid and dilute to 75 ml

Mix thoroughly reagents a, b, c, d in the volumetric proportions 10:3:1:6. The working solution keeps for 24 hours. Whenever it is necessary to make up new sulfuric acid reagent, a standard curve must be run. This can be done conveniently by dissolving 0.1757 g KH_2PO_4 and diluting to one liter (= 40 µg/ml phosphorus as orthophosphate). A reliable standard curve is generated in the 0.05-0.8 ml range using this stock solution.

Appendix C

Reagents and preparation for the Michaelis-buffered fixative:

(a) stock Michaelis buffer -- dissolve 1.94 g sodium acetate, 2.94 g sodium barbital, and 3.4 g NaCl and dilute to 100 ml

(b) 1% Bactotryptone solution -- dissolve 1 g Bactotryptone and 0.5 g NaCl and dilute to 100 ml

(c) Michaelis working buffer -- mix thoroughly 5 ml stock Michaelis buffer, 2 ml 0.1 N HCl, 18 ml dH_2O, and 0.25 ml 1 M $CaCl_2$; adjust to pH 8.0

Dilute the final working buffer 1:1 with 2% OsO_4, and add 0.1 ml Bactotryptone solution per ml fixative.

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