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ASPECTS OF THE ALKALINE PHOSPHATASE OF *PLECTONEMA BORYANUM* AND OTHER SELECTED CYANOBACTERIA

by

Barbara Blaho Doonan

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

1978

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ABSTRACT

ASPECTS OF THE ALKALINE PHOSPHATASE OF PLECTONEMA BORYANUM AND OTHER SELECTED CYANOBACTERIA

by

Barbara Blaho Doonan

Advisor: Professor Thomas E. Jensen

The alkaline phosphatase(s) of *Plectonema boryanum* show a considerable increase in activity following placement of the cyanobacterial cells in a phosphate free medium. Five days of phosphate starvation resulted in a fourteen fold increase of alkaline phosphatase activity. Growth in the presence of inhibitors of transcription and translation plus the immediate inhibition of enzyme activity through addition of orthophosphate indicate that the synthesis of the enzyme is "*de novo*".

Enzyme was extracted from *P. boryanum* with lysozyme or polymyxin B treatment in order to make comparative studies of cell bound and cell free enzyme. The effects of various ions on enzyme activity were tested and calcium was found to enhance activity to the greatest degree. The optimum pH, temperature stability, substrate specificity and Michaelis constant were determined.

Histochemical techniques applied at the ultrastructural

level have clearly established the periplasmic space as the site of enzyme activity in _P_. _boryanum_, _Anabaena_ _cylindrica_ and _Coccochloris_ _peniocytis_. Localization of activity was investigated by a modification of the method of Costerton (1973). Unfixed cells were reacted with calcium nitrate, which acted as the initial capture reagent. After this deposition, the cells were suspended in 2% lead nitrate which reacted with newly produced phosphate to form an electron dense precipitate of lead phosphate. The majority of activity appeared to be associated with layer 3 of the cell wall. In _A_. _cylindrica_ a secondary site of activity has been demonstrated in the sheath.

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TABLE OF CONTENTS

		Page
1.	List of tables and figures	1
2.	Introduction	8
3.	Literature Review	10
	A. Phosphatases	10
	B. Occurrence of alkaline phosphatase	11
	C. Characteristics of the enzyme	13
	D. Proposed assembly of the *E. coli* enzyme. . .	14
	E. Algal surplus phosphates and alkaline phosphatase	15
	F. Alkaline phosphatase - inhibition.	16
	G. Secretion of alkaline phosphatase.	17
	H. Cell wall structure	18
	I. Location of activity	23
	J. Cytochemistry	24
	K. Ecological aspects.	25
4.	Materials and Methods	27
	A. Growth conditions	27
	B. Dry weight determinations	28
	C. Alkaline phosphatase assay and calibration. .	29
	D. Starvation conditions and testing for induction of alkaline phosphatase	33
	E. Other isolates of blue-green bacteria - alkaline phosphatase	34
	F. Enzyme specific inhibitors	34
	G. Transcription - translation inhibitor studies.	35

Page

H. Temperature studies 37

I. Extraction of enzyme 37

J. pH effects, ion effects and substrate specificity 38

K. Kinetics of alkaline phosphatase 39

L. Cytochemistry 40

5. Results and Observations 42

A. Determination of maximum alkaline phosphatase activity in *P. boryanum* 42

B. Presence of alkaline phosphatase in seventeen blue-green isolates 45

C. Extraction of enzyme from *P. boryanum* . . . 48

D. Inhibitor studies 56

E. Glutaraldehyde as enzyme inhibitor 60

F. Temperature effects 63

G. pH optimum and ion effects 63

H. Substrates hydrolyzed by *P. boryanum* alkaline phosphatase 83

I. Kinetics of alkaline phosphatase 85

J. Cytochemistry 85

6. Discussion. 115

A. Determination of maximum alkaline phosphatase activity in *Plectonema boryanum* 115

B. Alkaline phosphatase levels in seventeen isolates of blue-green bacteria 117

C. Extraction of enzyme from *Plectonema boryanum*. 118

D. Inhibitor studies 122

Page

E. Temperature effects, pH optimum, substrates hydrolyzed. 125

F. Kinetics 132

G. Cytochemistry of alkaline phosphatase in cyanobacteria 132

7. Summary 140

8. Appendices 142

9. Literature cited 145

LIST OF TABLES

Table | Page
1. Alkaline phosphatase activity of various cyanobacteria 46
2. Effect of inhibitors of transcription and translation on activity of alkaline phosphatase in *P. boryanum* 57
3. Effect of various enzyme inhibitors on alkaline phosphatase activity 59
4. Relative hydrolysis of substrates by cell-free enzyme. 84

LIST OF FIGURES

Figure		Page
1.	Hydrolysis of a monophosphoric ester	10
2.	Induction of alkaline phosphatase	44
3.	_P_. _boryanum_ cell with all four layers of the wall missing following polymyxin treatment . .	50
4.	_P_. _boryanum_ cell treated with polymyxin shows alteration of the cell wall	50
5.	Irregular wavelike appearance of outer layers of the wall and ballooning of the layers into small blebs following polymyxin treatment	52
6.	_P_. _boryanum_ cell. Same description as Figure 5	52
7.	_P_. _boryanum_ cell. Enlarged view of wall showing entire wall peeled back from the cell. . . .	52
8.	_P_. _boryanum_ cell. Same description as Figure 7	52
9.	_P_. _boryanum_ cell. Remnants of cells following lysozyme treatment	55
10.	_P_. _boryanum_ cell. Following lysozyme treatment a mass resembling a cell remains	55
11.	The effect of orthophosphate on cell bound and cell free activity	62
12.	Inhibition of _P_. _boryanum_ alkaline phosphatase activity by 2.5% glutaraldehyde.	62
13.	Temperature stability of cell bound _P_. _boryanum_ alkaline phosphatase	65

Figure		Page

14. Temperature stability of cell free P. boryanum alkaline phosphatase activity 67

15. pH optimum of cell free P. boryanum alkaline phosphatase activity 69

16. pH optimum of cell bound P. boryanum alkaline phosphatase activity 71

17. The effect of Ca^{++}, Mg^{++} and Zn^{++} on cell bound alkaline phosphatase activity in P. boryanum 73

18. The effect of Co^{++}, Fe^{++} and Mn^{++} on P. boryanum cell bound alkaline phosphatase activity 75

19. The effect of K^{+} and Na^{+} on P. boryanum cell bound alkaline phosphatase activity 75

20. The effect of Ca^{++}, Mg^{++} and K^{+} on P. boryanum cell free alkaline phosphatase activity. . . 78

21. The effect of Fe^{++} and Zn^{++} on P. boryanum cell free alkaline phosphatase activity 80

22. The effect of Na^{+}, Co^{++} and Mn^{++} on P. boryanum cell free alkaline phosphatase activity. . . 80

23. The effect of molybdenum on cell bound and cell free P. boryanum alkaline phosphatase activity 82

24. Lineweaver-Burk plot of P. boryanum cell free alkaline phosphatase activity in the presence of varying concentrations of p-nitrophenol

Figure | Page

phosphate with and without added ortho-phosphate. 87

25. Lineweaver-Burk plot of *P. boryanum* cell bound alkaline phosphatase activity in the presence of varying concentrations of p-nitrophenol phosphate. 89

26. *P. boryanum* typical control cell treated for alkaline phosphatase localization. 91

27. *P. boryanum* control cell showing thylakoidal precipitate 91

28. *P. boryanum* control cell showing clumps of precipitate probably due to nonspecific lead salt deposition. 91

29. *P. boryanum* control cell treated with substrate and no lead. 93

30. *P. boryanum* cell treated with complete reaction mixture. Tangential section showing cell covered with precipitate 93

31. *P. boryanum* control cell prefixed in paraformaldehyde 96

32. *P. boryanum* cell treated with complete reaction mixture following prefixation in paraformaldehyde. 96

33. *P. boryanum* control cell prefixed in glutaraldehyde 98

34. *P. boryanum* cell treated with complete

Figure		Page

reaction mixture following prefixation in glutaraldehyde. 98

35. P. boryanum control cell prefixed in glutaraldehyde 101

36. P. boryanum cell treated with complete reaction mixture following prefixation in glutaraldehyde in the presence of substrate 101

37. P. boryanum cell. Same description as Figure 36

38. P. boryanum cell derepressed and unfixed, treated with complete reaction mixture. Upper cell wall shows activity in the wall; lower cell apparently not derepressed 103

39. P. boryanum section of a portion of a filament showing activity in the outer walls. Same treatment as Figure 38 103

40. P. boryanum cells treated as described in Figure 38. Cell shows light granular precipitate in cell wall 105

41. Enlarged view of wall clearly showing precipitate in periplasmic space (layer 3). The plasma membrane and the 4 layers of the wall are shown. 105

42. Enlarged view showing aggregates of precipitate in the wall. 105

43. Enlarged view of wall showing dense deposit of precipitate. 105

44. Enlarged view of wall showing more grainlike

Figure | Page

appearance of precipitate in periplasmic space and apparent small amount of precipitate on the outer surface of layer 4. . . . 105

45. *Anabaena cylindrica* cell derepressed and unfixed treated with complete reaction mixture. Cell shows activity in the wall and in the sheath. 108

46. *A. cylindrica* enlarged view of the wall shows precipitate in the wall and in the sheath. Cell treated as in Figure 45 108

47. *A. cylindrica* enlarged view of the wall shows precipitate clearly in periplasmic space. Cell treated as in Figure 45 108

48. *A. cylindrica* cells showing activity in end walls, sheath and some activity in cross walls. Cells treated as in Figure 45 110

49. *A. cylindrica* control cell with no precipitate and normal inclusions. 110

50. *Coccochloris peniocytis* log phase cells Control cell showing no reaction product . . 112

51. *C. peniocytis* log phase cell unfixed and treated with complete reaction mixture showing activity in the periplasmic space. 112

52. *C. peniocytis* cell showing encrustation of reaction product on cell surface. Cell treated as in Figure 51. 114

Figure Page

53. *C. peniocytis* cell with reaction product in wall and reaction product on cell debris probably due to free enzyme. Cell treated as in Figure 51. 114

INTRODUCTION

When microorganisms such as the cyanobacteria are placed in an environment where orthophosphate (the preferred form of phosphorus) is not available or is present in low concentration, the cells begin to produce an alkaline phosphatase. This enables them to obtain orthophosphate from the hydrolysis of organic phosphates. In this way the phosphorus requirements created by phosphate starvation or phosphate limitation can be fulfilled. The mechanism, as elucidated from *Escherichia coli*, seems to entail derepression (Echols *et al*., 1961; Willsky *et al*., 1973) of a specific portion of the genome. This is followed by transcription and translation of the pertinent region resulting in an increase of alkaline phosphatase.

The alkaline phosphatases of certain bacteria, *e.g.* *Escherichia coli* have been extensively studied (Torriani, 1960; Schlesinger *et al*., 1968). Although some work has been carried out using blue-greens, no study has dealt with more than a few aspects of the enzyme (Healey, 1973; Ihlenfeld and Gibson, 1975). It is for these reasons that studies were initiated to closely examine the alkaline phosphatase of *Plectonema boryanum Gomont*. The experiments were designed to elucidate the pattern of induction, ultrastructural localization of activity in the cell, and the effects of various environmental parameter changes on the enzyme both in cell bound form and in a cell free form. A

survey of seventeen isolates was also undertaken in order to determine the extent and causative conditions of this enzyme in a variety of cyanobacteria.

LITERATURE REVIEW

Phosphatases

Phosphatases are a large and complex group of enzymes functioning over a wide pH range (pH 4-11). One of these is alkaline phosphatase, an orthophosphoric monoester phosphohydrolase. As shown in Fig 1, a general phosphatase reaction is the hydrolysis of monoesters of phosphoric acid to release an alcohol and inorganic orthophosphate (P_i). The symbol P_i implies a mixture of $H_2PO_4^-$ and $HPO_4^=$. The ratio of $H_2PO_4^-$ to $HPO_4^=$ is dependent on the pH of the specific reaction medium. Phosphatases have also been known to act as transferases by catalyzing the transfer of phosphate groups from one substance to another (Stadtman, 1961.)

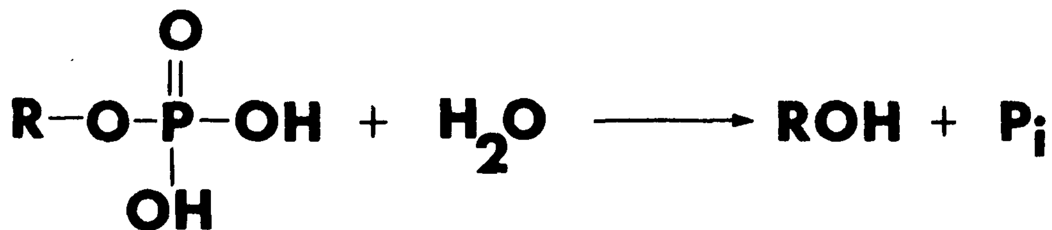


Figure 1 - Hydrolysis of a monophosphoric ester.

Occurrence of Alkaline Phosphatase

The enzyme alkaline phosphatase has been found to occur at all phylogenetic levels (Torriani, 1960; Bone, 1971; Bowers and Korn, 1973; Day and Ingram, 1973; Nose *et al*., 1973). Early reports on the occurrence of alkaline phosphatase (APase) in procaryotic organisms were made in studies by Pett and Wynne, (1933;1938) on the alkaline phosphatases of *Clostridium acetobutylicum Weizmann*, *Propionibacterium Jensenii van Neil*, *Aerobactor aerogenes*, *Acaligenes faecalis* and *Bacillus subtilis*. Further detailed information on APase in procaryotes did not appear in the literature until the late 1950's (Horiuchi *et al*.,1959). One reason for the apparent paucity of information may be due to the fact that a significant amount of the enzyme is not formed in many organisms unless a condition of limiting inorganic phosphate exists (Horiuchi *et al*.,1959; Torriani, 1960; Cashel and Freese, 1964; Fitzgerald and Nelson, 1966; Cheng; Ingram and Costerton, 1970; Okabayashi *et al*., 1974).

In some procaryotes, specific loci of the genome may be derepressed when the supply of inorganic phosphate becomes depleted (Echols *et al*., 1961; Garen and Echols, 1962), and the organism produces APase which makes it possible to use organic phosphates to satisfy its phosphorus needs. A system of this type has been found in the bacterium *Escherichia coli* (Echols et al., 1961; Garen and Echols, 1962).

Fitzgerald and Nelson (1966) reported that when

Microcystis aeroginosa (Wis. 1036), *Aphanizomenon flos-aquae* (Wis. 1062), *Gleotrichia echinulata* (Wis. 1052) and *Nostoc muscorum* (Wis. 1013) were grown in a medium containing a normal amount of phosphorus (7.2 mg/l), APase activity was very low. However, when the algae were grown in a medium with reduced phosphorus (0.4 mg/l) the enzyme activity was increased up to 25 times. They further reported that mixed cultures (collected from a Wisconsin lake and thus not pure) of *Microcystis* alone or with *Lyngbya birgei* had low alkaline phosphatase activities. When the cultures were placed in a medium containing no phosphorus, activity was greatly increased. In later investigations by Bone (1971), Healey (1973) and Ihlenfeld and Gibson (1975), it was confirmed that blue-green bacterial alkaline phosphatase activity was low in a culture medium with normal phosphorus content and that activity greatly increased when phosphorus was low. Bone (1971) also reported that when 15 m*M* KNO_3 was present, even higher levels of alkaline phosphatase were found. The pattern of appearance of alkaline phosphatase observed by these authors seems to indicate that an induction system similar to that found in *E. coli* (Garen and Echols, 1962) is probably functioning here as well, but as yet such a system has not been demonstrated. It is of interest to note that Kuenzler and Perras (1965) list a marine *Synechococcus* as alkaline phosphatase negative.

Certain bacteria have been found which seem to produce

the enzyme constitutively. This appears to be the case in the marine pseudomonad B-16 studied by Costerton (1973) and in a species of anaerobic rumen bacteria (Cheng and Costerton, 1973). No blue-greens have been reported to produce significant amounts of the enzyme constitutively.

Characteristics of the enzyme

The alkaline phosphatase of *E. coli* has been studied most intensively. The pH optimum found for the alkaline phosphatase of most organisms studied is within the range pH 8 to pH 11. *E. coli* alkaline phosphatase has a pH optimum between pH 8.5 and 9.5. When cells were heated at 100° C for 5 minutes only 50% of the total alkaline phosphatase activity was lost, indicating that the enzyme is fairly stable (Torriani, 1960). The enzyme is able to hydrolyze monohexose phosphates, mononucleotides and synthetic monoesters. Maximum activity is found using p-nitrophenol phosphate as substrate (Torriani, 1960). In the cyanobacteria Healey (1973) reported that the enzyme from *Anabaena variabilis* showed maximum activity at pH 8.3, while Ihlenfeld and Gibson (1975) reported that the alkaline phosphatase from *Anacystis nidulans* has a broad pH optimum of 8 to 9.5.

The purified *E. coli* enzyme has been shown to be a stable, globular zinc metalloprotein consisting of two identical subunits having a total molecular weight of 86,000 (Schlesinger *et al.*, 1969). It has also been suggested that the enzyme may act as a phosphotransferase,

since a phosphorylated enzyme has been isolated from E. coli (Anderson and Nordlie, 1967). In the enzyme from E. coli there are four zinc ions per dimer, one pair of these ions is essential to catalytic activity and the other pair plays a structural role (Anderson and Vallee, (1975). These authors have substituted other ions in place of zinc but obtain a catalytically active enzyme only with molybdenum. Chappelet-Tordo et al. (1975) have shown that only one site of the active dimer functions at any given time demonstrating "absolute negative cooperativity".

Since a need for zinc had been shown for the E. coli enzyme, it was somewhat unexpected to find that the cellular enzyme in Anabaena required calcium. Healey (1973) found enzyme activity to depend not only on the concentration of substrate but also on the concentration of calcium. The Staphylococcus aureus enzyme (Davies and James, 1974) was found to be stimulated by magnesium and Vibrio parahaemolyticus was also stimulated 2.5 fold using 10^{-3} M magnesium or zinc. The Anabaena requirement for calcium could not be replaced by magnesium. Ihlenfeld and Gibson (1975) observed stimulation of the enzyme in Anacystis nidulans by molybdenum and manganese and an unexpected inhibition of the enzyme with zinc. Therefore, it appears that the ion requirements for enzyme functioning are quite variable.

Proposed Assembly of E. coli enzyme

Schlesinger et al. (1969) have postulated the assembly

of alkaline phosphatase in the bacterial cell as follows: subunits in the folded state are transported from polyribosomes through the cell membrane into the periplasmic space of the cell wall where dimerization and activation with zinc occurs. Using an analogue of the amino acid histidine which caused the formation of inactive alkaline phosphatase, it was suggested that histidine may play a role in the catalytic site (Schlesinger *et al.*, 1969). This aspect of alkaline phosphatase has not been dealt with in blue-green bacteria.

Algal surplus phosphates and alkaline phosphatase

In a study of limiting and surplus phosphate in various algae, Fitzgerald and Nelson (1966) observed alkaline phosphatase activity in four species of blue-green algae. An inverse relationship was found between alkaline phosphatase activity and the amount of phosphate in the medium. As an attempt at further correlation of the data, these authors used a 60 min boiling water bath extraction procedure in order to measure the orthophosphate of the algal cells cultured under conditions of surplus or limited phosphorus. The procedure is thought to separate essential phosphate compounds from surplus stored compounds. Surplus phosphorus is taken into cells and stored in an osmotically inert condensed form referred to as "polyphosphate bodies" (Harold, 1966; Jensen and Sicko-Goad, 1976). Using boiling water extraction of surplus phosphate it was found that the average extractable orthophosphate decreased from a value of

0.43 to 0.038 mg P/100 mg algae, and alkaline phosphatase activity increased from 500 units/mg algae to 2,800 units/mg algae as the algae were placed under conditions of limiting phosphorus. Thus, a relationship does seem to exist between extractable phosphate and alkaline phosphatase activity.

Alkaline Phosphatase - Inhibition

Various inhibitors of enzyme activity have been used both in purified preparations and in living cultures. Although the enzyme has been shown to be unusually heat stable, chelating agents, 6 *M* guanidine HCl, and low pH will reversibly inactivate the enzyme. Inactivation is due to removal of essential metals or by dissociation of subunits (Schlesinger, *et al*., 1969). Torriani (1960) found the cellular *E. coli* enzyme to be totally uneffected by treatment with sodium fluoride. However, addition of orthophosphate to a culture of *E. coli* showing high alkaline phosphatase activity causes an immediate strong inhibition of the enzyme. This inhibition is nearly complete when the phosphate concentration reaches 100 m*M*. In *Anabaena variabilis* the enzyme showed 50% inhibition with inorganic phosphate (0.25 m*M*) and was also inhibited to the same extent with molybdenum (Healey, 1973). Both inhibitors were found to be of a competitive nature. Fitzgerald and Nelson (1966) working with *Microcystis aeruginosa* (Wis. 1036), *Aphanizomenon flos-aquae* (Wis. 1062) *Gleotrichia echinulata* (Wis. 1052) and *Nostoc muscorum* (Wis. 1015) observed an inhibition of enzyme activity

following addition of increasing amounts of orthophosphate to the medium. However a later report (Fitzgerald, 1969) showed an increase in extractable (surplus) phosphate two hours after addition of inorganic phosphate to a phosphorus-limited culture with no decrease in alkaline phosphatase activity. This led to the conclusion that decreases in activity as a result of added inorganic phosphate were probably a result of dilution of existing activity due to growth rather than a total inactivation of existing enzyme. This would seem to indicate that inhibition is at the transcriptional or translational level preventing further synthesis of enzyme.

Secretion of Alkaline Phosphatase

Another interesting aspect of alkaline phosphatase production by procaryotic organisms is the tendency for certain of these organisms to secrete the enzyme into the surrounding medium. In *E. coli* (Torriani, 1960) only 10 - 15% of the activity seems to be released from the cells and *S. marcesens* releases no enzyme at all (Bhatti, 1974). Cashel and Freese (1964) working with *B. subtilis* found excretion of the enzyme into the surrounding medium to be a normal occurrence. Secretion of the enzyme seems also to be the case in *S. aureus* (Okabayashi *et al.*, 1974), *V. parahaemolyticus* (Sakaguchi et al., 1972), *P. aeruginosa* (Costerton, 1970) and marine Pseudomonad B-16 (Costerton, 1973). Of the blue-green bacteria studied a small amount of enzyme release was observed by Healey (1973) in *A. variabilis* and a very small amount of alkaline phosphatase

was observed in the growth medium of *Oscillatoria woronichinii* (Kuenzler and Pellas, 1965). These observations of blue-green activity were made under phosphate limited conditions.

Cell Wall Structure

As most of the procaryotic alkaline phosphatase activity studied seems to be found in an extracellular location, a thorough understanding of basic procaryotic cell wall makeup is essential. In gram-negative bacteria and blue-green bacteria the cell walls have been found to be similar four layered structures (Murray *et al*., 1965; Jost, 1965; Jensen and Sicko, 1971; Bayer, 1974). This description refers to the longitudinal walls of filamentous blue-green bacteria and the entire wall of single cells. Cross walls of filamentous blue-green bacteria differ as to the arrangement of layers as will be explained later.

Beginning at the plasma membrane and moving outward, layer one (L1) is an electron transparent layer immediately adjacent to the plasma membrane. Occasionally this layer, which ranges in thickness from 3 - 10 nm (Jost, 1965; Jensen and Sicko, 1971) has been difficult to separate from the plasma membrane. Jost (1965), using freeze-etch replicas, clearly demonstrated its presence in *Oscillatoria rubescens*. A specific function for L1 has not been determined. De Petris (1967) suggested a placement of cell wall building enzymes in loose association with the plasma membrane within L1. Giesbrecht *et al*., (1977) have demonstrated the presence of

an interlayer between the cytoplasmic membrane and the cell wall proper of *Staphylococcus aureus*. These authors suggest the importance of such an interlayer for the neoformation of bacterial cell walls: *e.g.* as a needed site for attachment of long murein chains and to catalyze cross linking of new murein. Studies of wall formation in *Micrococcus luteus* (Weston *et al.*, 1977) lend some support to such a suggestion.

The second layer (L2) is an electron dense layer which measures approximately 10 nm. in thickness as observed in cross sections of *Oscillatoria rubescens* (Jost, 1965) and *Cylindrospermum sp.* (Jensen and Sicko, 1971) and 7 nm. in sections of *Symploca muscorum* (Pankratz and Bowen, 1963). L2 of blue-green bacteria is generally thicker than that of gram negative bacteria. The L2 of *Oscillatoria princeps* measures from 120 to 250 nm. in thickness (Jensen, 1965). When the layers beyond L2 are removed from *Tolypothrix tenuis* (Hocht *et al.*, 1965), *Anacystis nidulans* and *Gloeocapsa alpicola* (Allen, 1968) or pseudomanad B-16 (Costerton, 1973) it is found that this layer (L2) is responsible for preserving the shape of the cell. This mucopolymer or peptidoglycan layer is made up of a repeating sequence of N-acetylmuramic acid and N-acetylglucosamine forming the backbone which is linked together by peptide chains of 3-4 amino acids. In *A. nidulans* these have been found to be primarily glutamic acid, diaminopimelic acid and L and D forms of alanine (Golecki, 1977). L2 has been found to be the portion of the blue-green bacterial cell wall susceptible

to lysozyme treatment as shown by Jensen and Sicko (1971) in Cylindrospermum sp. and by Lindsey et al., (1971) in A. nidulans. This enzyme splits the 1, 4 glycoside linkage between N-acetylmuramic acid and N-acetylglucosamine (Fuhs, 1958; Crespi et al., 1962). It is also this layer which renders blue-green bacteria susceptible to penicillin treatment through inhibition of the peptide cross linkages (Krauss, 1962; Kumar, 1964; Vance, 1966; Srivistava, 1969).

The third layer of the wall (L3) which varies in thickness from 3 and 10 nm (Pankratz and Bowen, 1963; Jensen and Sicko, 1971), appears electron transparent in thin section. In a model of the cell wall of E. coli proposed by Schnaitman (1971) trypsin sensitive proteins project from the mucopolymer layer (L2) through layer 3 to penetrate the outer double track layer (L4).

L4, made up of protein and phospholipid is described as a membrane-like, hexagonally close-packed bilayer with lipopolysaccharide as an integral part of the layer (Korn, 1969). In addition, other carbohydrates, amino acids and fatty acids have been found (Frank et al., 1962; Drewes and Gollwitze 1965; Golecki, 1977). Among the substances identified from extracts of A. nidulans are glucosamine, 2-amino-2-deoxyheptose, 2-keto-3-deoxyoctonate, mannose, glucose, galactose, rhamnose, leucine, and lysine (Weise et al., 1970). The lipopolysaccharide components of the cell wall seem to form a closely associated network through a degree of ionic cross linkages between them.

This was indicated by the successful disruption and release of lipopolysaccharide components when P. aeruginosa cells were treated with 0.2 M $MgCl_2$ (Ingram et al., 1973). The excess of Mg^{++} or other cations could function by increasing the number of repulsive positive charges and dissociating the lipopolysaccharide cross linkages. The finding that the barrier function of the gram-negative envelope is disrupted by treatment of cells with EDTA emphasizes the role of cations in maintaining the integrity of the cell wall (Leive, 1974).

Due to the work of Schnaitman, Osborne and others (Korn, 1969 review) on various gram-negative bacteria, additional components of L4 have been identified as 3-4 polypeptides, phosphatidyl ethanolamine, phosphatidyl glycerol and cardiolipin. A further means of maintaining the formal structure of the lipopolysaccharide seems to be via pyrophosphate bridges. According to the model proposed by Schnaitman (1971) lipopolysaccharide also extends beyond the outer double track. This is the portion of the cell wall responsible for antigenic specificity.

A few studies of gram-negative bacteria have shown either additional cell wall layers or have described unusual arrangements of layers. As mentioned earlier Giesbrecht et al., (1977) demonstrated an interlayer between the plasma membrane and the cell wall proper in Staphylococcus aureus consisting of clusters of embedded ring-like disks. Fischman and Weinbaum (1966) found a hexagonal pattern of

subunits on the mucopolymer layer of E. coli B made up of protein and/or lipoprotein. In Ectothiorhodospira mobilis Pelsh, Remsen et al. (1968) observed a scalloped appearance of repeating 50 A subunits on the surface of the outer double track. These may be similar to the peg-like subunits on the outer layer described by Thornley and Glauert (1968) in Acinetobacter and by Murray (1963) in Spirillum serpens. In a study of the blue-green bacterium Gloeocapsa alpicola four additional layers beyond L1 through L4 are described (Jensen and Sicko, 1972). This is the only blue-green in which such an observation has been made.

The cross walls differ from end walls in the number and arrangement of wall layers. A study of peptidoglycan metabolism by Sturgeon et al. (1975), using ethanol to stimulate and D-cycloserine to inhibit, localized regions of synthesis and/or degradation in the blue-green Agmenellum quadriplicatum with spacing similar to that expected for cross wall formation. In thin sections layers L1 and L2 together with the cytoplasmic membrane are repeated between adjoining cells (Frank et al., 1962; Pankratz and Bowen, 1963; Lamont, 1969 a,b). Either L3 and L4 are never a part of the cross wall as in the Oscillatoriaceae (Halfen and Castenholz, 1971) or they appear just prior to separation of adjoining cells in other filamentous blue-greens (Ris and Singh, 1961; Ingram and Thurston, 1970).

External to the cell wall a layer of mucilage or sheath material surrounds the cells or filaments of many blue-green

bacteria (Wolk, 1973). Some gram-negative bacteria such as the genus Sphaerotilus, a filamentous bacterium are similarly found to be enclosed within a sheath or surrounded by a capsule or slime layer which often exceeds the diameter of the bacterium itself. Among the polysaccharides present in the blue-green bacterial sheath are: glucose, galactose, xylose, uronic acids and glucuronic acids. The sheath in thin section has a fibrillar appearance (Leak, 1967; Lamont, 1969; Tuffery, 1969). It has been suggested (Lange, 1976) that a voluminous sheath may provide a micro-environment containing a concentrated supply of readily available essential nutrients.

Location of Activity

Active alkaline phosphatase has been determined for several gram-negative bacteria to be located in some part of the periplasmic space between the plasma membrane and the outer double track layer, or it is found to be firmly attached to the cell wall as in E. coli (Wetzel et al., 1970), pseudomonad B-16 (Costerton, 1973) and P. aeruginosa (Cheng and Costerton, 1973). The peripheral location of the enzyme in E. coli (Neu and Heppel, 1965; Nossal and Heppel, 1966) and P. aeruginosa (Cheng et al., 1970) was determined by selective release upon spheroplast production using lysozyme and EDTA, or using osmotic shock. Cheng et al., (1971) working with P. aeruginosa suggested an association of enzymes such as alkaline phosphatase with lipopolysaccharide on both the inner and outer aspects of the outer double track layer of the gram-negative cell wall. This association

seems to protect the enzyme from low pH denaturation (MacAlister _et al_., 1977). Removal of the outermost layers, L3 and L4, of pseudomonad B-16 (Costerton, 1973) and _P. aeruginosa_ (Ingram _et al_., 1973) resulted in the release of all of the alkaline phosphatase activity and a proportional amount of lipopolysaccharide. _In vitro_ studies using a lipopolysaccharide cell wall complex seem to show an association of this type to be very likely _in vivo_. (Day and Ingram, 1974).

Cytochemistry

Studies using both light microscopy (Wetzel _et al_., 1970) and electron microscopy (Done _et al_., 1965; Costerton, 1973; Cheng _et al_., 1976) strengthen the biochemical evidence indicating localization of alkaline phosphatase within the periplasm of _E. coli_ (Done _et al_., 1965; Wetzel _et al_., 1970), pseudomonad B-16 (Costerton, 1973) and _Pseudomonas aeruginosa_ (Cheng _et al_., 1970) with cytochemical evidence. For ultrastructural localization of activity, various modifications of the Gomori method (Gomori, 1939, 1952; Takamatsu, 1939; Wetzel et al., 1967) were employed in which calcium is used as the capture reagent for the orthophosphate released through enzyme activity. Further treatment with lead nitrate produces an extremely electron dense precipitate of lead phosphate at the site of activity. Costerton (1973) working with marine pseudomonad B-16 has found that incubation of test cells in substrate medium prior to fixation with glutaraldehyde and then followed by post fixation in

osmium tetroxide gives good reproducible results. Other workers (Done et al., 1965; Wetzel et al., 1970) have found prefixation with a mixture of pure glutaraldehyde, or a mixture of pure glutaraldehyde and paraformaldehyde before treatment with a substrate containing medium, a preferable method. Prefixation causes cross linkage of proteins in such a way as to possibly lessen their diffusion from the active site (Barrow and Holt, 1971). However, glutaraldehyde is known to inhibit enzyme activity (Widnell, 1972). McNicholas and Hulett, (1977) found 3% glutaraldehyde to have caused a loss of 68% of the alkaline phosphatase activity of *B. licheniformis* within 5 minutes. Nevertheless, glutaraldehyde remains a fixative of choice primarily due to its fine preservation of ultrastructure. Cytochemical procedures have not previously been applied to blue-green alkaline phosphatase(s).

Ecological Aspects

Of major concern today is the problem of eutrophication of natural waters. It is of importance to further clarify the relationship between available nutrients and the growth of organisms which normally inhabit these waters. In their natural environment cyanobacteria may be subjected to conditions of surplus nutrients such as phosphate not only in the form of orthophosphate which they take in preferentially but also very likely in an organic form. Organic phosphates may come from a number of sources, one of which is secretion by algae into their environment as reported by Kuenzler (1970).

As many studies have indicated, induction of alkaline phosphatase in procaryotes takes place when the preferred orthophosphate is not available (Torriani, 1960; Fitzgerald and Nelson, 1966). In this way the organisms can make use of organic substrates to yield needed orthophosphate. The kinds of phosphorus containing compounds which can be utilized, the conditions under which the enzyme is induced, and the factors controlling its functioning may help our understanding of the nutritional responses of these cells. In this way it may be more possible to control the growth of blue-green bacteria. Thus, better measures to deal with "blooms" of these bacteria could be developed. It may also be possible to stimulate the growth of blue-green bacteria in environments such as rice paddies where the nitrogen fixing ability of many species is directly related to crop production. Recently it has been reported that when the aquatic water fern *Azolla* containing the blue-green bacterium *Anabaena* is placed in a rice paddy, yields are increased from 50 to 100% (Galston, 1975; Ashton and Walmsley, 1976). Thus, this study which is designed to elucidate some aspects of the physiological response of blue-green bacteria to changing environmental conditions, is also a means to increase our understanding of these organisms and their possibilities for further future economic uses.

MATERIALS AND METHODS

Growth Conditions

The primary test organism used in this study was *Plectonema boryanum Gomont* (UTEX 581). All cultures were obtained from the Starr Culture Collection (Starr, 1964; 1971). In addition to *P. boryanum*, the following blue-green bacteria were tested for the presence and/or the inducibility of alkaline phosphatase:

1. *Oscillatoria sp.* (UTEX 1543)

*2. *Oscillatoria prolifera* (UTEX 1270)

3. *Plectonema calothricoides* (UTEX 598)
4. *Nostoc commune* (UTEX 584)
5. *Nostoc muscorum* (UTEX 486)
6. *Nostoc foliaceum* (UTEX 1624)
7. *Gleocapsa sp.* (UTEX 1938)
8. *Lyngbya sp.* (UTEX 487)
9. *Lyngbya sp.* (UTEX 621)
10. *Anabaena spiroides* (UTEX 1552)
11. *Anabaena cylindrica* (UTEX 629)
12. *Microcystis aeruginosa* (UTEX 1939)
13. *Coccochloris peniocytis* (UTEX 1548)
14. *Phormidium luridum var. olivace* (UTEX 426)
15. *Scytonema hofmanni* (UTEX 1581)
16. *Fremyella diplosiphon* (UTEX 481)

17. *Chloroglea fritschii mitra* (Cambridge University culture collection No. LB 1411/1).

All cultures were grown in a defined medium, modified Fitzgerald (Fitzgerald *et al.*, 1952; Zehnder and Gorham, 1960) or *Bold mineral medium (Deason and Bold, 1960). (See appendices A and B for the composition of media.) A sample of each blue-green bacterium (from agar slants or liquid media) was transferred into culture flasks under sterile conditions. These flasks were then placed in a Sherer-Gillette growth chamber adjusted to: 1) 200 ft-candles of illumination (2152 lux) with a combination of incandescent and fluorescent sources, 2) 25°C and, 3) an alternating 12 h day/night cycle. Further transfers were made at weekly intervals until the cells were well acclimated to the growth conditions. Growth of experimental cultures was allowed to continue until the cells reached logarithmic phase. This was determined as a function of increase of dry weight per unit volume. Stock cultures were also maintained under the above described conditions. To maintain the cultures, transfers to fresh media were made regularly from cultures which were between two and four months old.

Dry Weight Determinations

Dry weight of the samples was found to be not only the most consistent and easiest way to determine growth of the blue-green bacteria, but also as a means to express specific activity of the cell bound enzyme. Many of the blue-greens tested for alkaline phosphatase activity are filamentous and

have sheaths which make their dispersal and rupture into individual cells difficult for counting or for spectrophotometric analyses. Prewashed Millipore filters, type HA with a pore diameter of 0.45 um were predried to constancy by heating to 100°C for 24 h and weighed after cooling in order to determine the dry weight. Volumes of blue-green bacterial suspensions equivalent to the volumes assayed for enzyme activity were syringed through 22 gauge needles and passed through the predried Millipore filters. After drying the filters under an incandescent lamp, the filters were then further dried under the previously described conditions, and weighed when cool. The difference in the weight of the filters was taken to be the dry weight of the cells. Normally an aliquot containing between 200 and 700 mg of blue-green bacteria per liter were assayed.

Alkaline Phosphatase Assay and Calibration

Alkaline phosphatase activity was monitored by two spectrophotometric tests. Appropriate dilutions were made of samples tested so that spectrophotometric absorbance values fell within the range 0.07 to 0.7 whenever possible. The two methods used to assay for phosphatase activity were the Murphy and Riley (1962) test for orthophosphate and a modification of the Bessey *et al*. (1946) test for free p-nitrophenol. See Appendix C for composition of the solutions.

Both methods are colorimetric tests; the Murphy and

Riley method (1962) depends upon the absorbance of a phosphomolybdate complex formed as a function of the orthophosphate concentration in solution. The colored complex formed during the Murphy-Riley determination is stable for a period of 24 h. One to four ml of the solution to be tested was mixed with glass distilled water and placed in a 50 ml graduated cylinder to a volume of 40 ml. Eight ml of the mixed reagent and two ml of glass distilled water were added so that the final volume of the solution was 50 ml. The solution was then mixed thoroughly and color development was allowed to proceed for 10 min to 1 h. Percent transmittance was read directly and converted to an absorbance value. All readings were taken at 880 nm on a B & L Spectronic 20 spectrophotometer equipped with infrared sensitive phototube and filter. Calibration curves were used to determine the sensitivity of the test. The absorbance value divided by the slope of the calibration curve gave a direct reading of phosphorus concentration as phosphate. All phosphate determinations were carried out in glassware which was acid washed and used only for these tests.

Calibration curves were determined in the following manner: A standard phosphate solution containing 0.1757 of potassium dihydrogen phosphate per liter H_2O was prepared. This solution then contained 40 mg phosphate (P) per liter. Dilutions of the stock solution were made to the following concentrations:

80ug P/l (3.2ug P/40 ml)
160ug P/l (6.4ug P/40 ml)
320ug P/l (12.8ug P/40 ml)
400ug P/l (16.0ug P/40 ml)
640ug P/l (25.6ug P/40 ml)

The percent transmittance values were determined, and converted to absorbance. The values were measured at 880 nm in 2.56 cm cuvettes. Beer's law was obeyed in this concentration range, and the method yielded an excellent reproducibility.

The second method of Bessey *et al.*, (1946) depends upon the fact that p-nitrophenol is yellow in color above a pH of 7.5. Therefore, usage of p-nitrophenol phosphate (pNPP) as substrate in the phosphatase assay results in the release of free p-nitrophenol (pNP). Calibration curves were used to determine the sensitivity of the test. The absorbance value divided by the slope of the calibration curve gave a direct reading of pNP in m*M*/l.

Calibration curves were determined in the following manner: A standard pNP solution containing 0.8346 g pNP was prepared in 0.001 N HCl. This solution contains 6 m*M* pNP per liter. Dilutions of the stock solution were made to the following concentrations:

6.0mM pNP/l
4.0mM pNP/l
2.0mM pNP/l
1.0mM pNP/l

Following the above dilutions 0.1 ml of each was then added to 10 ml 0.02 N NaOH, or to 10 ml 0.05 *M* Tris buffer (pH 8.5), and read at 420 nm. The percent transmittance values were determined and converted to absorbance, or

absorbance was read directly. The values were measured at 420 nm in 2.56 cm cuvettes (Spectronic 20) or in 1 cm cuvettes (Beckman 25). Beer's law was obeyed in this concentration range and the method yielded excellent reproducibility.

The basic assay for alkaline phosphatase was as follows: An aliquot of cells was removed from the culture flask placed in a centrifuge tube and spun at 2300 xg for 10 min. The supernatant was retained in addition to the pellet in order to test for enzyme activity in the medium. The cells were then washed two times in glass distilled water. A 1 ml sample of cells or medium supernatant was then added to a test tube containing 2 ml 0.05 M Tris buffer pH 8.5 and 1 ml 0.01 M $CaCl_2$. Then 1 ml of 0.04 M pNPP was added, and the entire contents were thoroughly mixed. The reaction was allowed to procede at 25°C for 15 min to test for cell-bound activity, and for 1 h to determine if there was any activity associated with the medium (cellular secretion).

2 ml 0.05 M Tris buffer pH 8.5

1 ml 0.01 M $CaCl_2$

1 ml algae resuspended in distilled water
or
1 ml medium

1 ml substrate (para nitrophenol phosphate)

---

5 ml total volume

At the end of the reaction time, the tubes containing cells were centrifuged at 2300 xg for 10 min and the supernatant was read directly at 420 nm if the Bessey *et al.* (1946)

method was used, or an aliquot of the supernatant was combined with reagent in the Murphy-Riley (1962) test and read at 880 nm. Those tubes containing samples of medium were either read immediately at 420 nm (Bessey _et_ _al_, 1946) or the reaction was stopped with 1 ml 8% trichloroacetic acid and an aliquot was combined with reagent in the Murphy-Riley test (1962) and read at 880 nm. Assay of cell free activity extracted from the cells was carried out at 39°C for one h and then treated in the above described manner.

Specific activity of cell-bound alkaline phosphatase is reported as µmoles pNP/ml x mg $cells^{-1}$ x min^{-1}, or, as ug PO_4 x mg $cells^{-1}$ x min^{-1} . Cell free enzyme activity is reported as nmoles pNP x ug $protein^{-1}$ x min^{-1}, or as umoles pNP x ml $medium^{-1}$ x min^{-1}.

Starvation Conditions and Induction of Alkaline Phosphatase

Cells of _Plectonema_ _boryanum_ were grown in modified Fitzgerald's medium containing 8-10 mg PO_4 per liter for 14 d at 25°C, 200 ft-Cd of illumination, and an alternating 12 h day/night cycle. At this time the culture is nearing the completion of its maximum growth, (Jensen and Sicko-Goad, 1976). After this growth period the cells were removed aseptically from the culture medium by centrifugation in a Sorvall refrigerated centrifuge (Model RC-2B) at 4°C and 12,100 xg for 10 min. The medium containing phosphate was decanted, and the cells were washed 3 times in sterile medium free of phosphate. After the final centrifugation, the cells were resuspended in phosphate free medium and then

placed under the original environmental conditions. Induction of alkaline phosphatase was assayed by testing *P. boryanum* cells over the period of 0 to 240 d of phosphate starvation. This was done in order to determine the appropriate times to test for alkaline phosphatase.

Other Isolates of Blue-green Bacteria - Alkaline Phosphatase Activity

The sixteen axenic cultures of blue-greens listed under growth conditions, obtained from the Starr culture collection (Starr, 1964, 1971;) plus *Chloroglea fritschii* (Cambridge Culture Collection, LB 1411/1), were treated in a manner similar to *P. boryanum*. These were grown in modified Fitzgerald's medium or in Bold's mineral medium at 25°C under 200 ft-cd in an alternating 12 h day/night cycle to the logarithmic growth phase. An aliquot of these cells and an aliquot of the growth medium was assayed for alkaline phosphatase activity at this time. The remainder of the cultures were harvested aseptically by centrifugation in a Sorvall refrigerated centrifuge at 4°C and 12,100 xg for 10 min, washed 3 times in sterile phosphate-free medium, resuspended in sterile phosphate-free medium and placed under the original environmental conditions. After five d of phosphate starvation an aliquot of these cells and an aliquot of the growth medium were tested for alkaline phosphatase activity.

Enzyme Specific Inhibitors

For studies involving the effect of various inhibitors

on cell bound enzyme activity, the cells were treated in the following manner. At the end of the starvation period, the cells were pelleted by centrifugation and resuspended in 0.05 M Tris buffer (pH 8.5) containing the appropriate concentration of inhibitor. The cells were then incubated in the presence of the inhibitor at 25°C for periods ranging from 0 min to 2 h prior to assay for alkaline phosphatase activity. The inhibitors tested were orthophosphate (0.1 - 100 mM), potassium cyanide (20 mM), mercuric chloride (10 mM) and sodium fluoride (10 mM).

The effect of inhibitors on cell free enzyme was studied in the following manner. The appropriate concentration of inhibitor was added to 0.05 M Tris buffer pH 8.5 containing 1 ml cell free extract for 0 - 10 min prior to assay for alkaline phosphatase activity. The inhibitors tested were orthophosphate (0.1 - 100 mM) and sodium fluoride (10 mM).

Transcription-Translation Inhibitor Studies

Inhibitors of nucleic acid and protein systhesis were tested in an attempt to determine the "*de novo*" nature of the enzyme. For these studies the blue-green bacteria were treated in the following way. After 14 d of normal growth the cells were harvested as previously described and prepared for phosphate starvation. The phosphate free washed cells were resuspended in phosphate-free medium containing an appropriate concentration of inhibitor. In order to distribute the cells as uniformly as possible, they

were syringed ten times through a #22 gauge needle and equal aliquots were then withdrawn. For each inhibitor tested an equal aliquot of cells was pretreated with 1 m*M* EDTA and shaken on a rotary shaker at 25°C one h before being resuspended in phosphate free medium with inhibitor. A duplicate was resuspended directly into phosphate-free medium containing inhibitor with no pretreatment. The cultures were then placed under the original environmental conditions for five d and then assayed for alkaline phosphatase activity. The inhibitors tested were actinomycin D (100 ug/ml), puromycin (200 ug/ml), chloramphenicol (200 ug/ml) and 6-methyl purine (200 ug/ml), (Wolfe and Hahn, 1965; Cundliffe and McQuillen, 1967; Doolittle, 1972).

Glutaraldehyde is a common fixative for electron microscopy as it gives excellent preservation of ultrastructure. However, its use could have resulted in an inhibitory action critical to these cytochemical studies (Widnell, 1972). Therefore, glutaraldehyde was tested for its effect on alkaline phosphatase activity. The procedure was as follows: Cells were removed from a five d starved culture, centrifuged, washed and resuspended in 2.5% pure glutaraldehyde made up in either 0.05 *M* Tris buffer (pH 8.5) or in 0.05 *M* barbital buffer (pH 8.5). At intervals ranging from 0 - 90 min , an aliquot of cells was removed and combined with 1 ml 0.01 *M* $CaCl_2$, 1 ml 0.04 *M* pNPP and 2 ml 0.05 *M* Tris buffer (pH 8.5), and assayed for APase activity.

Temperature studies

In order to test for the effect of temperature on alkaline phosphatase activity, starved cells or an aliquot of cell free extract were acclimated to the various temperatures for 10 min prior to assay. The assay was then carried out at the particular temperature. The temperature range included 4°, 22°, 30°, 40°, 50°, 70°, and $80^{\circ}C$. Cells and cell free extracts were also heated at $100^{\circ}C$ for 10 min, removed from the boiling water bath, and assayed at $25^{\circ}C$.

Extraction of Enzyme

Disruption of the cell wall was attempted by two methods. Method one was a modification of the lysozyme treatment of Murray *et al*. (1965) as used by Jensen and Sicko (1968). The phosphate starved cells were suspended in 0.3 *M* Tris buffer pH 8.5 containing 134 ug EDTA/ml, 68 ug lysozyme/ml and 10% w/v sucrose in Erlenmyer flasks and then placed on a rotary shaker at $25^{\circ}C$ overnight. After 18 h, the contents of the flasks were transferred to centrifuge tubes and centrifuged for 1 h at 37,000 xg and a temperature of $4^{\circ}C$. The supernatant was retained as the crude cell free enzyme extract and assayed for alkaline phosphatase activity and protein by the Lowry *et al*. method (1951). Method two made use of the antibiotic polymyxin B sulfate according to the procedure of Cerny and Teuber (1971). An aliquot of phosphate starved cells was suspended in 0.14 *M* NaCl containing 200 ug/ml polymyxin B sulfate in Erlenmyer flasks. These were placed on a rotary shaker overnight at

$25^{o}C$. In the morning the contents of the flasks were centrifuged at 37,000 xg for one h and the supernatant retained as the crude cell free enzyme extract. This extract was assayed for alkaline phosphatase activity and for protein (Lowry et al., 1951). Both extracts were either used immediately or stored frozen at $10^{o}C$ for future use.

pH Effects, Ion Effects and Substrate Specificity

The effects of various ions on alkaline phosphatase activity were determined by adding 1 ml of 0.1 mM, 10.0 mM, 10 mM or 100 mM ion to the assay mixture. The ions tested were Ca^{++}, Mg^{++}, Mn^{++}, Co^{++}, Mo^{++}, Fe^{++}, Zn^{++}, Na^{+}, and K^{+}. Tests were carried out on both cell bound and cell free enzyme. For studies to elucidate the optimal pH for enzyme activity in cell free and cell bound form, equal aliquots of cells or enzyme extract were assayed at a pH range of 7.2 to 10. Tris buffer (0.05 M) was used for the pH range from 7.2 to 9, and 0.05 M carbonate-bicarbonate buffer for the range from 9 to 10. Cell free extract or 1 ml of cells was suspended in 3 ml buffer and 1 ml 0.04 M substrate. Fifteen substrates in addition to pNPP were tested to determine which were most readily hydrolyzed. A 1 ml aliquot of each substrate was added to a mixture of 1 ml cell free extract and 3 ml 0.05 Tris buffer pH 8.5. The reaction was allowed to proceed for 1 h at $39^{o}C$. The substrates included:

1. p-nitrophenol phosphate
2. D-fructose-6-phosphate
3. β-glycerophosphate

4. D-glucose-6-phosphate
5. D-fructose 1,6 di-phosphate
6. D-galactose-6-phosphate
7. glucosamine-6-phosphate
8. cyclic AMP
9. D, Lα-glycerophosphate
10. 2, 3 - diphosphoglyceric acid
11. D(-)3-phosphoglyceric acid
12. DNA - calf thymus - type I
13. RNA - yeast - type X1
14. bis-nitrophenol phosphate
15. cyclic UMP

The concentration of all substrates was 0.04 M with the exception of DNA (50 ug/assay) and RNA (200 ug/assay) (Selitrennikoff and Sonneborn, 1977).

Kinetics of alkaline phosphatase

The dependence of alkaline phosphatase activity on the concentration of pNPP can be described on the basis of Michaelis-Menten kinetics. The concentration of substrate added to the assay mixture was varied over the range 0.1 mM to 100 mM pNPP. The velocity of the reaction expressed as uM pNP released x mg cells^{-1} x min^{-1} for cell bound enzyme, and nmoles pNP released x ug protein^{-1} x min^{-1} for cell free enzyme was plotted against substrate concentration. In order to determine the Km and Vmax of the reaction, a Lineweaver-Burk plot was made of the data from both the cell bound and cell free enzyme. Upon plotting $^{1}/_{v}$

versus $1/_s$, a straight line was obtained. Once the slope and the y-intercept were determined Km and Vmax could be calculated as the slope is mathematically equal to Km divided by Vmax.

These studies using the cell free enzyme extract were repeated in the presence of 10 m$\underline{M}$ PO_4 as K_2HPO_4 and the data were plotted as $1/_v$ vs $1/_s$. The slope and y-intercept were determined thus permitting the calculation of K_I and Vmax for inhibited enzyme.

Cytochemistry

Cytochemical localization was investigated using a modification of the method of Costerton (1973). The basic incubation solution contained the following: sodium β glycerophosphate 0.5% (0.23 $\underline{M}$); pNPP 0.001% ($8X10^{-3}\underline{M}$); sodium barbital 0.005 $\underline{M}$ or 0.024 $\underline{M}$; Ca $(NO_3)_2 \cdot 4H_2O$ 0.02 M; $CaCl_2$ 0.01 $\underline{M}$ and tris (hydroxymethyl) aminomethane 0.05 $\underline{M}$ at pH 8.5. The solutions were made up in distilled water. Controls in which only substrate had been omitted from the incubation solution were included for each preparation.

Unfixed cells were placed in the basic incubation solutions for 20 min and then removed by centrifugation at 2300 xg. The cells were then resuspended in an aqueous solution of 2% lead nitrate for 10 min in order to convert calcium phosphate to lead phosphate. A sample of the cells from each incubation solution was resuspended in barbital buffer (0.05 $\underline{M}$, pH 8.5) rather than lead nitrate as an additional control. Some cells were fixed in 2.5%

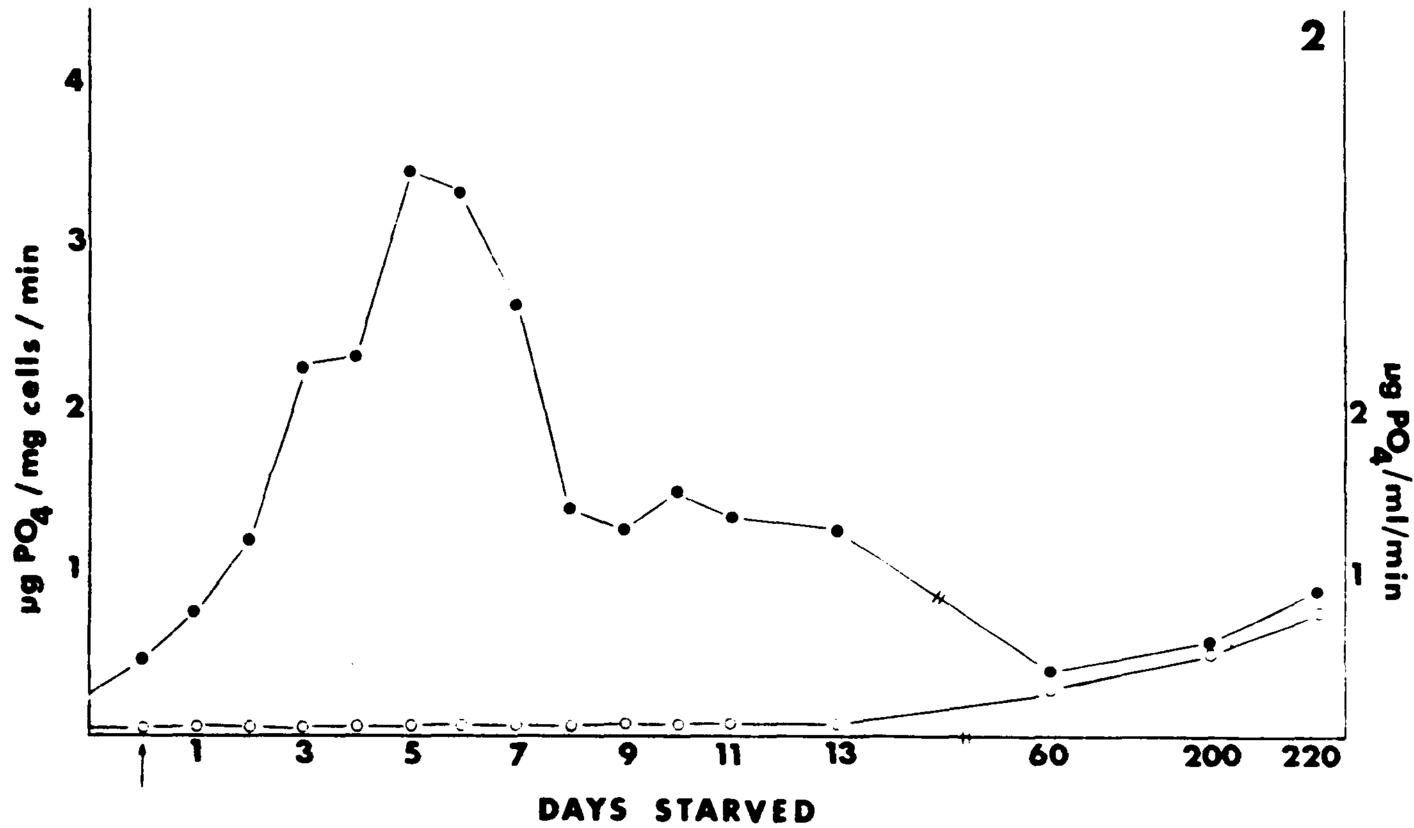
glutaraldehyde, or 1.5% glutaraldehyde in the presence of pNPP, or 4% paraformaldehyde in barbital buffer for 1 1/2 h and then treated in the above described manner. Following two washes in barbital buffer, fixation for electron microscopy was carried out in three ways: 1) osmium tetroxide by the method of Pankratz and Bowen (1963), or 2) 2.5% barbital-buffered glutaraldehyde for 1 1/2 h followed by osmium tetroxide for 1 1/2 h, or 3) 2.5% barbital-buffered glutaraldehyde for 1 1/2 h followed by osium tetroxide for 3 h. The cells were then dehydrated in a graded ethanol-propylene oxide series and embedded in Epon 812 according to the method of Luft (1961). Sections were cut on a Dupont diamond knife, collected on 300-mesh unsupported copper grids and stained with methanolic uranyl acetate, (Stempak and Ward, 1964) uranyl acetate plus lead citrate (Reynolds, 1963), or observed unstained. Micrographs were taken with a Hitachi HU-11E or a Hitachi HS-9 microscope, each operating at 75kV.

RESULTS AND OBSERVATIONS

Determination of Maximum Alkaline Phosphatase Activity in *P. boryanum*

Plectonema boryanum was cultured in modified Fitzgerald's medium containing 8-10 mg phosphate per liter to peak of logarithmic phase which occurs between days 14 and 17. On day 14 the cells were transferred to phosphate-free medium and then assayed on a daily basis in order to determine the amount of alkaline phosphatase activity present. Figure 2 shows an increase of alkaline phosphatase activity to its highest level after five d of phosphate starvation as determined by assay of specific activity. The activity remained at a fairly constant level beyond eight days of phosphate starvation reaching its lowest point as the culture reached two months of age. The level of enzyme activity begins to rise again in cultures over seven months old. Throughout the entire starvation period virtually no activity could be detected in the growth medium, (Fig 2). It was not until two mo of age that activity could be detected in the medium. The level of activity in the growth medium, taken from a seven mo culture, showed an increase paralleling the increase of activity found in the cells from the same culture.

Figure 2. Levels of alkaline phosphatase activity in *P. boryanum* following cell growth in P-free media. Closed circles (●——●) indicate cell bound activity; open circles (o——o) indicate cell free activity.


2
4
3
2
1
µg PO4 / mg cells / min
µg PO4/ml/min
2
1
1
3
5
7
9
11
13
60
200
220
DAYS STARVED

Presence of Alkaline Phosphatase in Seventeen Blue-green Isolates

In addition to *P. boryanum* seventeen other blue-green isolates were tested for the presence of alkaline phosphatase. In Table 1, it can be seen that all blue-greens tested appear to be alkaline phosphatase positive. All of the cultures were assayed both during the logarithmic phase of growth and also following the standard five d starvation procedure. Comparing the activity of the log phase cells with that of phosphate-starved cells eleven of the isolates may be considered to be inducible as there is a substantial increase in activity following their placement in phosphate-free medium. Ten of the isolates show some enzyme activity in the culture medium. *Nostoc muscorum*, *Anabaena spiroides* and *Coccochloris peniocytis* demonstrated the greatest amounts of cell-free activity. Variable amounts of cell free activity were detected in seven other blue-greens, *Oscillatoria sp*, *Nostoc foliaceum*, *Lyngbya sp*. (UTEX 487) and (UTEX 621), *Phormidium luridum var*. *olivace*, *Fremyella diplosiphon*, and *Chloroglea fritschii*. It is of interest to note that in comparing log phase cell-free activity with phosphate starved cell-free activity, many variations were found. In *Oscillatoria prolifera* (UTEX 1270) activity increases with starvation, *Anabaena spiroides* (UTEX 1552) shows a decrease under the same conditions and *Nostoc foliaceum* (UTEX 1624) shows equal activity under both conditions. *Oscillatoria prolifera*

TABLE 1

Alkaline Phosphatase Activity of Various Cyanobacteria

ORGANISM	LOG		PHOSPHATE STARVED	
	CELLS[a]	MEDIUM[b]	CELLS	MEDIUM
Oscillatoria sp (UTEX 1543)	2.05	0.14	3.0	0.0
Oscillatoria prolifera (UTEX 1270)	6.87	0.0	7.3	0.46
Plectonema calicothricoides (UTEX 598)	5.93	0.0	6.7	0.15
Nostoc commune (UTEX 584)	0.75	0.0	1.76	0.00
Nostoc muscorum (UTEX 486)	22.0	13.0	43.0	1.9
Nostoc foliaceum (UTEX 1624)	6.0	0.6	10.45	0.65
Gleocapsa sp (UTEX 1938)	0.46	0.0	1.8	0.0
Lyngbya sp (UTEX 487)	3.2	0.2	6.1	0.43
Lyngbya sp (UTEX 621)	2.9	0.25	29.6	0.1
Anabaena spiroides (UTEX 1552)	4.5	4.95	10.85	0.75

ORGANISM	LOG		PHOSPHATE STARVED	
	CELLS[a]	MEDIUM[b]	CELLS	MEDIUM
Anabaena cylindrica				
(UTEX 629)	1.7	0.0	7.4	0.13
Microcystis aeruginosa				
(UTEX 1939)	0.58	0.0	4.65	0.15
Coccochloris peniocytis				
(UTEX 1548)	2.55	3.93	2.43	1.77
Phormidium luridum var.olivace				
(UTEX 426)	2.7	0.12	3.0	0.16
Scytonema hofmanni				
(UTEX 1581)	1.8	0.0	17.8	0.2
Fremyella diplosiphon				
(UTEX 481)	3.53	0.83	12.55	0.83
Chloroglea fritschii mitra				
(LB 1411/1)	11.7	0.15	16.35	0.165

a. specific activity: umoles pNP released x mg cells^{-1} x min^{-1} at 25°C.

b. specific activity: umoles pNP released x ml medium^{-1} x min^{-1} at 25°C.

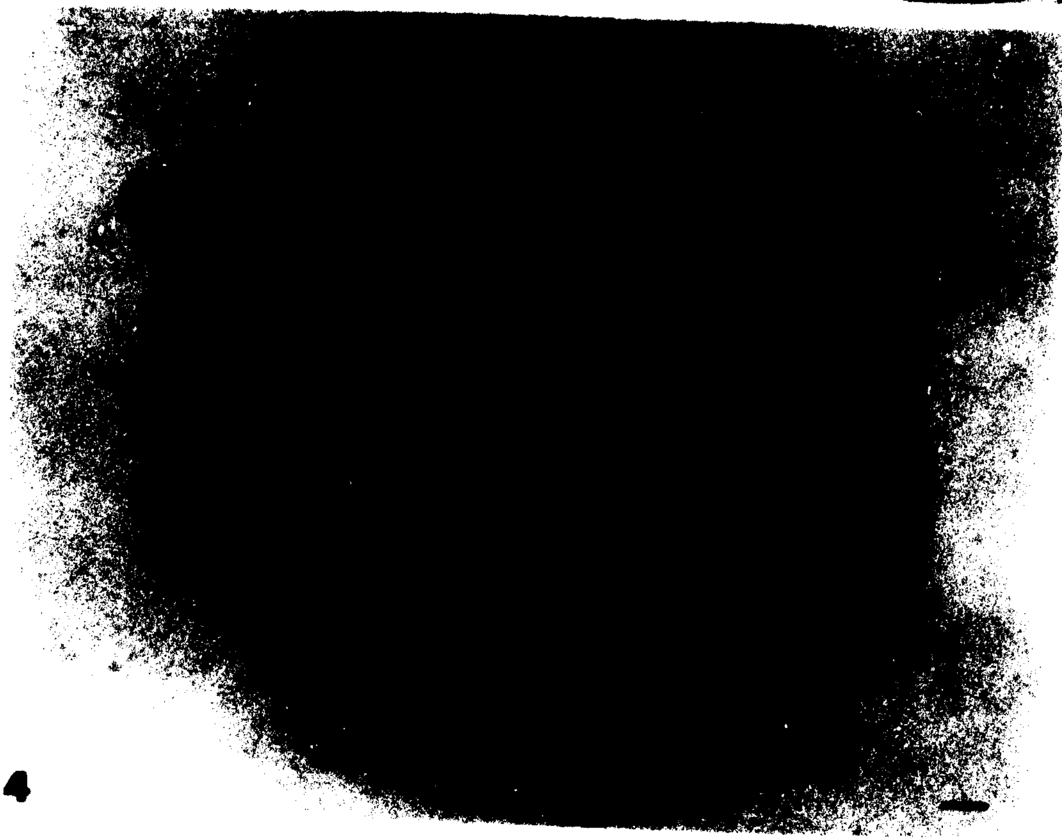
(UTEX 1270) and Scytonema hofmanni (UTEX 1581) which show no detectable cell free activity in log phase do have a small amount of cell free activity after starvation. Gloeocapsa sp. (UTEX 1938) and Nostoc commune (UTEX 584) do not seem to secrete any enzyme into the surrounding medium under either normal or starved conditions. Several of the isolates, Oscillatoria sp. (UTEX 1543), Plectonema calicothricoides (UTEX 598) and Coccochloris peniocytis (UTEX 1548), seem to be constitutive for the enzyme alkaline phosphatase, as there is no increase over previous activity brought about through phosphate starvation.

Extraction of Enzyme from P. boryanum

As Plectonema boryanum does not release alkaline phosphatase into the surrounding medium it was necessary to disrupt the cells in order to obtain a cell free extract. Of the two methods chosen to accomplish this task, polymyxin B sulfate was found to be less damaging to the cells (Fig 3-8). This particular antibiotic seemed to interact with gram-negative bacteria at the outer double track layer (L4) of the wall in such a way that breaks occurred and its normal positioning was interrupted. Cells of P. boryanum treated for 18 h with polymyxin B sulfate showed few ultrastructural changes. There was relatively little cell lysis possibly due to the high salt content of the polymyxin B sulfate mixture. Fig 3 shows a polymyxin treated cell missing its entire wall. In Fig 4 the arrow indicates an area where the outer double track has been peeled back, thus exposing layer

Figure 3. *P. boryanum* cell with all four layers of the wall missing following polymyxin treatment. Fixed in osmium Bar represents 0.1 um.

Figure 4. *P. boryanum* cell treated with polymyxin shows alteration of the cell wall; a section of the wall peeled back and a large bleb of outer wall material (arrows). Osmium fixation. Bar represents 0.1 um.



Figures 5-8. *P. boryanum* cells.

Figures 5-6. Irregular wavelike appearance of outer layers of the wall and ballooning of layers (arrows) into small blebs following polymyxin treatment. Osmium fixation. Bars represent 0.1 um.

Figures 7-8. Enlarged view of wall showing entire wall peeled back from cell (arrows). Large arrow indicates layer 4 (outer double track). Osmium fixation. Bars represent 0.1 um.

three. Once the firm interactions which hold the wall and its enclosed periplasmic enzymes together are disturbed, release of a portion of the alkaline phosphatase is obtained. Fig 5-6 show whole cells which appear normal except for a vesiculation of parts of the outer wall layers. The arrows in Fig 3-7 and 8 indicate further areas of disruption of the outer wall where entire portions have been pulled away from the cell.

Incubation of phosphate-starved cells with the lysozyme mixture described previously for 16-18 h also successfully released alkaline phosphatase. However, control of this method was difficult and generally treatment sufficient to release adequate alkaline phosphatase also resulted in the lysis of a majority of the cells. The crude enzyme extracts were various shades of blue as a result of pigment release indicative of cell lysis. Fig 9 and 10 show cells following 18 h lysozyme treatment. Only portions of cells were generally recognizable. In Fig 9 membranes and a polyhedral body which has apparently lost its center are visible (arrows). Breakdown of the mucopolymer layer (L2) which is the target of lysozyme yields osmotically fragile cells. In spite of suspension in varying sucrose concentrations, few spheroplasts were detected. Fig 10 shows what appears to be a cell but no normally positioned outer envelope is discernible.

Following either treatment the entire sample was subjected to high speed centrifugation at 37,000 xg for one h. The polymyxin B sulfate enzyme extracts were clear

Fig 9-10. *P. boryanum* cells.

Fig 9. Remnants of cells following lysozyme treatment. Membranes and a polyhedral body (Pb) which has lost its center are seen. Bar represents 0.1 um.

Fig. 10. Following lysozyme treatment a mass resembling a cell remains. Arrow indicates possible wall remnant. No normally positioned outer envelope is discernible. Osmium fixation. Bar represents 0.1 um.

and colorless in appearance and had specific activities ranging from 0.1-20 nmoles pNP released x ug protein^{-1} x min^{-1}. The specific activities for the lysozyme extracts were in the same range as the polymyxin extracts (0.1-20). Both extracts were used in studies of physiological aspects of *P. boryanum* cell free alkaline phosphatase. Thus, it was possible to compare free enzyme with *in situ* enzyme in the living cell.

Inhibitor Studies

Cells of *P. boryanum* were phosphate starved for five days in the presence of various inhibitors of transcription and translation as described previously. Table 2 lists the effect of these inhibitors on alkaline phosphatase activity. It was not unexpected to find an effect on cell growth due to the specific action of the inhibitors. Certain of the antibiotics were added to the prepared cultures following treatment with 10^{-3}*M* EDTA in order to facilitate penetration of the cell envelope. Actinomycin D, well known as an inhibitor of transcription due to its interference with DNA-dependent RNA polymerase, reduced cell growth to 60% of control level. Of the total cell sample assayed after five d incubation with this inhibitor, specific activity of alkaline phosphatase was reduced by 40% when compared with an equal size sample of control cells. Puromycin, which resembles an amino acyl transfer RNA, caused release of incomplete peptides from the ribosome thus interfering with protein synthesis. Without EDTA

TABLE 2

Effect of Inhibitors of Transcription and Translation on Activity of Alkaline Phosphatase in *P. boryanum*.

EFFECT ON CELL GROWTH

INHIBITOR	% OF CONTROL GROWTH	% APase ACTIVITY[a]
None	100	100
Actinomycin D (10^{-3} *M*) with EDTA pretreatment	60	60
Puromycin	100	70
Puromycin with (10^{-3} *M*) EDTA pretreatment	60	8
Chloramphenicol	68	14
6-methyl purine	60	42
6-methyl purine with (10^{-3} *M*) EDTA pretreatment	63	33

a - As related to control activity expected with respect to sample size at the end of five d in phosphate-free medium plus inhibitor.

pretreatment no effect on cell growth was observed, although there was a 30% reduction of alkaline phosphatase activity. However, when puromycin was added to an EDTA pretreated culture, growth was found to be 60% of control growth and there was a 92% reduction of enzyme activity when a comparison was made with enzyme activity of an equal sample size of control cells. It appears that pre-treatment with 10^{-3} M EDTA greatly enhanced the entrance of puromycin into the cells. All inhibitors of nucleic acid synthesis and protein synthesis tested caused a reduction in alkaline phosphatase activity.

Table 3 shows the effect of a number of specific enzyme inhibitors tested on both cell bound and cell free samples of alkaline phosphatase. Of the three inhibitors used on the cell bound enzyme, only mercuric chloride caused a significant reduction of activity from 1.8 to 0.03. Sodium fluoride had no effect on cell bound activity at all and potassium cyanide enhanced activity slightly. The cell free enzyme was inhibited only slightly in the presence of sodium fluoride when assayed as described. The mode of action of the inhibitors chosen is a reaction between inhibitor and the various ions which are essential to enzyme activity. Heavy metals such as mercury can also cause non-specific protein precipitation.

Another type of inhibitor, whose effect on the alkaline phosphatase of P. boryanum appears to be of key importance to the level of enzyme activity is orthophosphate

TABLE 3

Effect of Various Enzyme Inhibitors on Alkaline Phosphatase Activity

INHIBITOR PRESENT	CELL BOUND ACTIVITY[a]
None	1.8
10^{-2} M potassium cyanide	2.2
10^{-2} M sodium fluoride	1.8
10^{-2} M mercuric chloride	0.03

	CELL FREE ACTIVITY[b]
None	20.7
10^{-2} M sodium fluoride	18.6

a.-Specific activity: umoles pNP released x mg cells $^{-1}$ x min^{-1}

b.-Specific activity: nmoles pNP released x ug protein^{-1} x min^{-1}

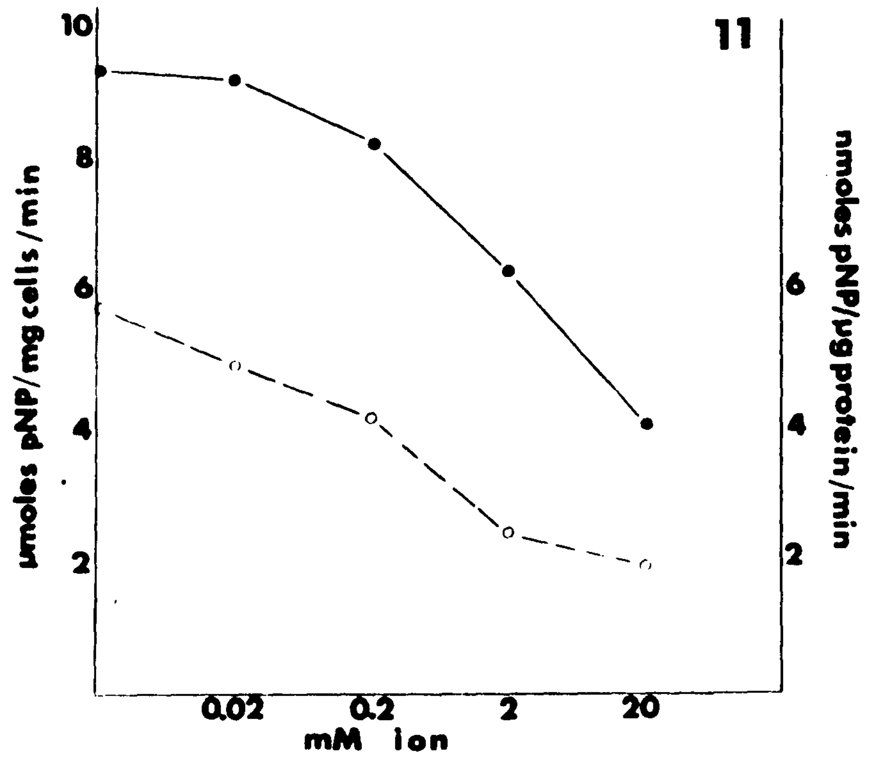
(P_i), the end product of the phosphatase reaction. Therefore, assays were conducted in the presence of increasing amounts of orthophosphate. As the concentration of P_i in mixtures containing either starved cells or a sample of cell free alkaline phosphatase was increased, inhibition of activity became more pronounced. Fig 11 shows that at a concentration of 20 m$\underline{M}$ P_i, cell bound activity, measured immediately after addition of phosphate, was decreased to 43% of the control value. Similarly, cell free activity, when measured after one h incubation was reduced to 32% of the control value at the same concentration of P_i.

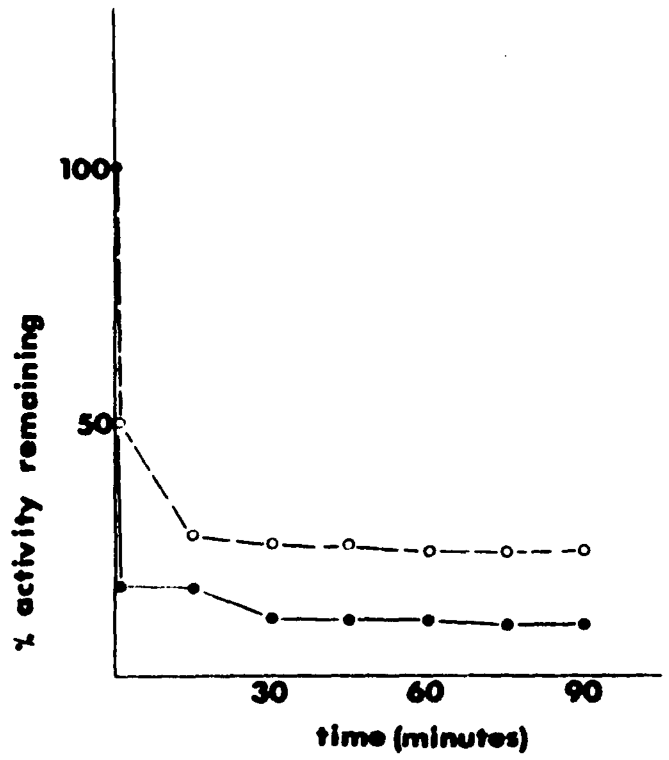
Glutaraldehyde as Enzyme Inhibitor

In attempting to localize alkaline phosphatase activity in several blue-greens at the ultrastructural level, one of the methods as described previously involves fixation of the cells with glutaraldehyde prior to application of histochemical techniques. Therefore, it was desirable to test for the effect of this fixative on alkaline phosphatase activity. Fig 12 shows that prefixation of *P. boryanum* cells in 2.5% glutaraldehyde in 0.05 $\underline{M}$ barbital buffer (pH 8.5) immediately reduces enzyme activity by one-half and in 30 min only 25% of control activity remains. When a similar test was run using 0.05 $\underline{M}$ Tris buffer (pH 8.5) activity was immediately reduced to 18% of control and in 30 min this was further decreased to a level of 10% of the control value where it remained for the duration of the 90 min prefixation period.

Figure 11. The effect of orthophosphate on cell bound alkaline phosphatase activity (closed circles ●——●) and on cell free activity (open circles o——o). P_i concentrations are plotted on a logarithmic scale.

Figure 12. Inhibition of *P. boryanum* alkaline phosphatase activity by 2.5% glutaraldehyde in 0.05 *M* Tris buffer (closed circles ●——●) and in 0.05 *M* Barbital buffer (open circles o——o).


11
µmoles pNP/mg cells/min
10
8
6
4
2
nmoles pNP/µg protein/min
6
4
2
0.02
0.2
2
20
mM ion


12
% activity remaining
100
50
30
60
90
time (minutes)

Temperature Effects

The temperature sensitivity of cell bound alkaline phosphatase and the cell free enzyme are quite different. As shown in Fig 13 there is an increase in cell bound activity from a low of 3 when assayed as previously described at $4^{o}C$ to a high of 22 at $40^{o}C$. Any further increase in temperature resulted in decreasing activity until no measurable amount of activity remains after heating the cells at $100^{o}C$ for 10 min. The maximum activity of the cell free enzyme was shifted upward (Fig 14). The enzyme appeared to be stable as it continued showing an increase in activity up to $70^{o}C$ under the described conditions. At $80^{o}C$ activity was at slightly above normal $39^{o}C$ assay levels. Heating the enzyme extract at $100^{o}C$ for 10 min probably denatured the protein and virtually all activity was lost.

pH Optimum and Ion Effects

The pH optima of both cell bound and cell free alkaline phosphatase were found to lie within the range pH 8.2-9.2 (Figs 15-16). As many metals have been found to play important roles in enzyme activity (see Literature Review), a number of ions thought to be of significance to *P. boryanum* alkaline phosphatase activity were tested. The response of cell bound activity to these ions is depicted in Figs 17, 18, 19, and 23. Of all the ions tested, calcium appeared to enhance activity to the greatest degree. The addition of 20 m*M* calcium stimulated enzyme

Figure 13. Temperature stability of cell bound *P. boryanum* alkaline phosphatase activity.

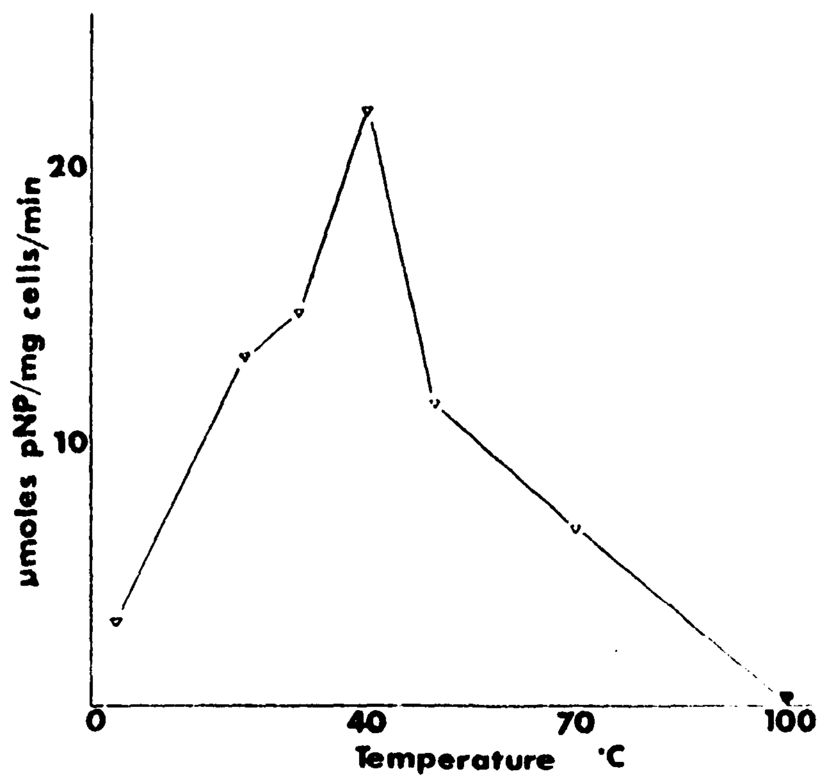

13
µmoles pNP/mg cells/min
20
10
0
40
70
100
Temperature °C

Figure 14. Temperature stability of cell free *P. boryanum* alkaline phosphatase activity.

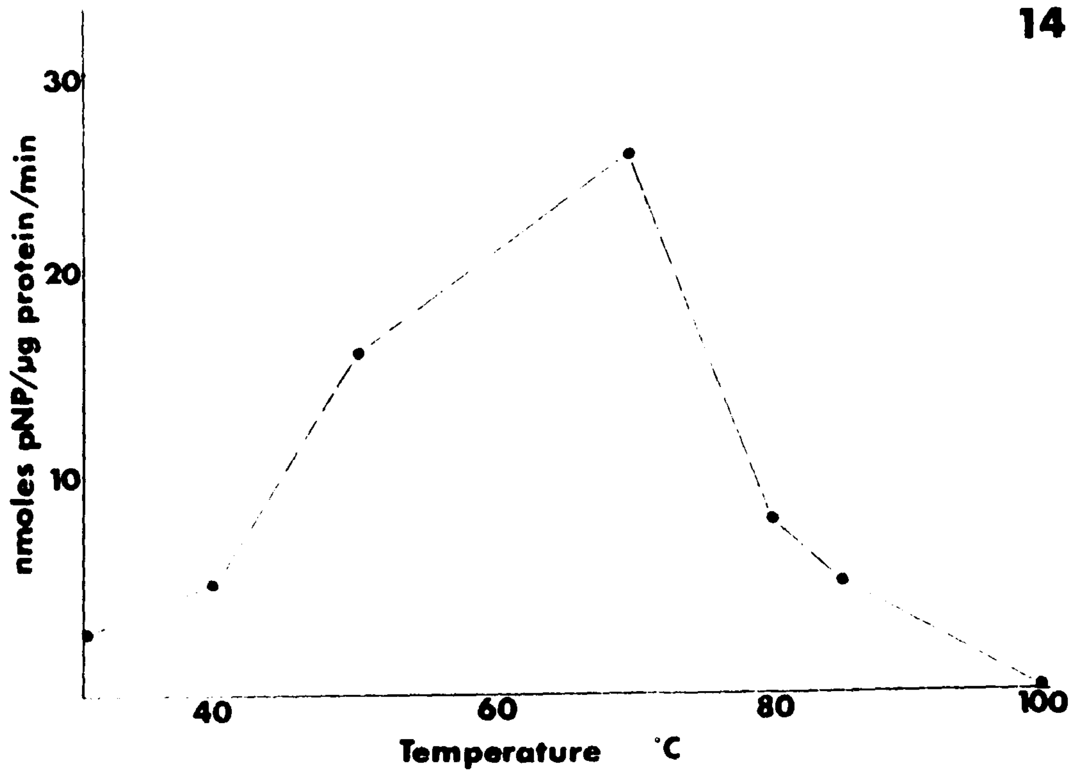

14
nmoles pNP/µg protein/min
30
20
10
40
60
80
100
Temperature °C

Figure 15. pH optimum of cell free *P. boryanum* alkaline phosphatase activity.

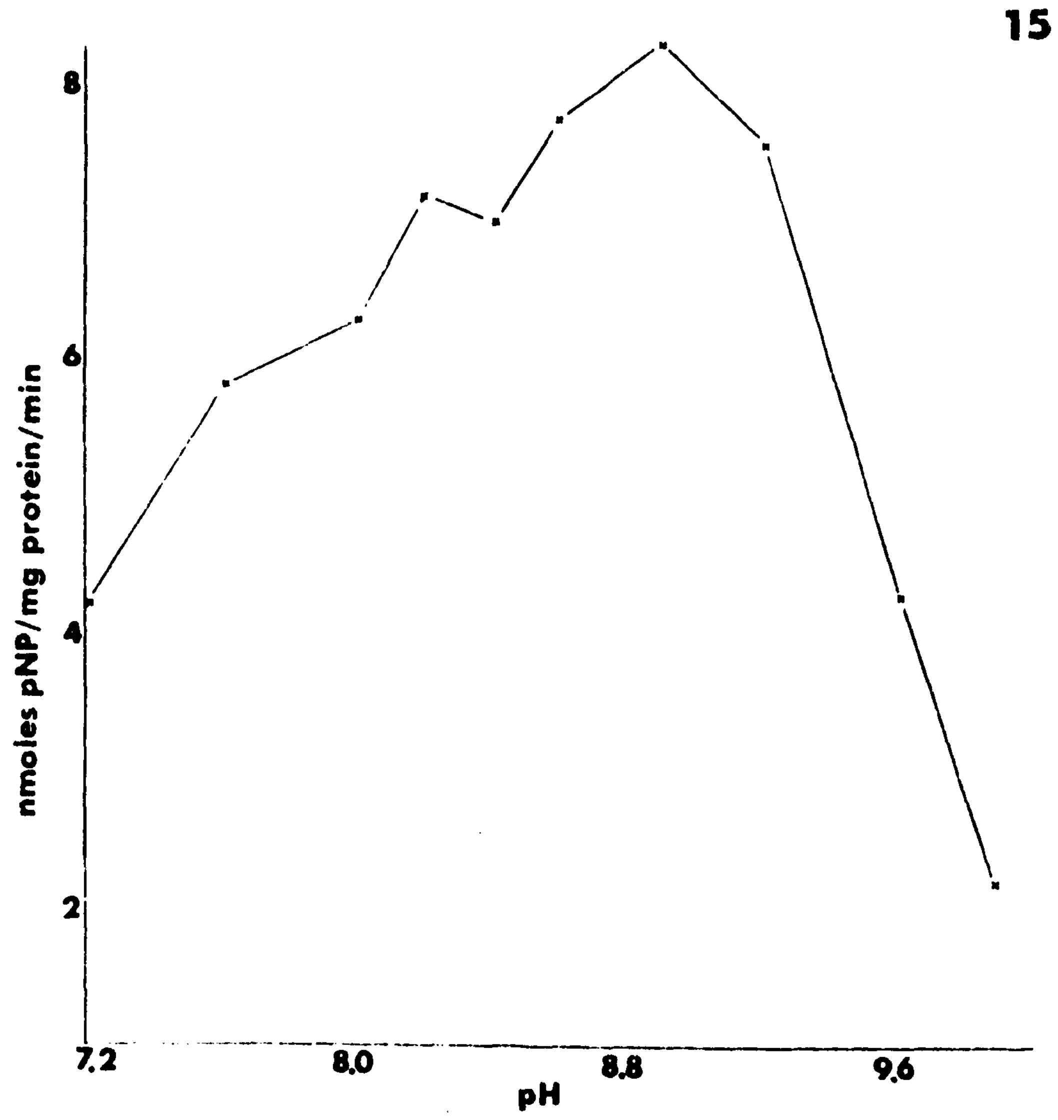

15
nmoles pNP/mg protein/min
8
6
4
2
7.2
8.0
8.8
9.6
pH

Figure 16. pH optimum of cell bound *P. boryanum* alkaline phosphatase activity.

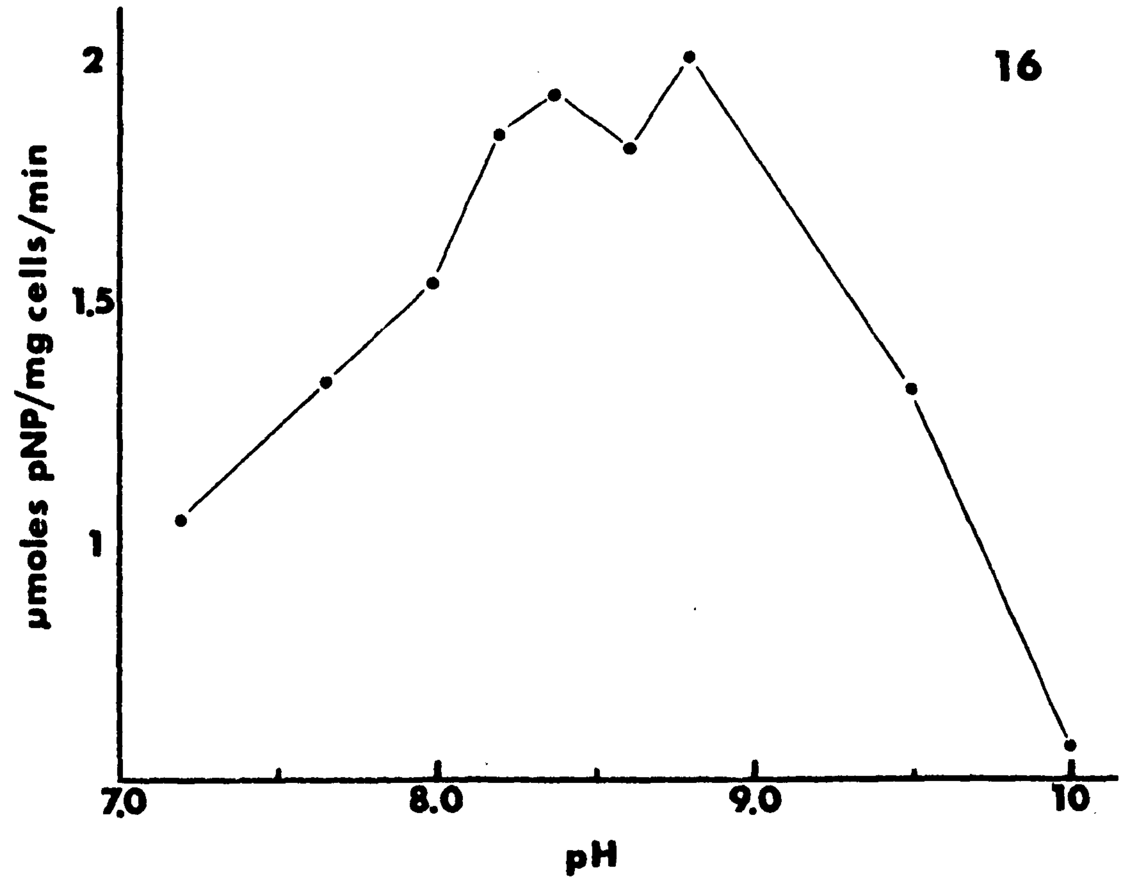

16
µmoles pNP/mg cells/min
2
1.5
1
7.0
8.0
9.0
10
pH

Figure 17. The effect of Ca^{++}, Mg^{++}, and Zn^{++} on cell bound alkaline phosphatase activity in *P. boryanum*. Ion concentration plotted on a logarithmic scale.

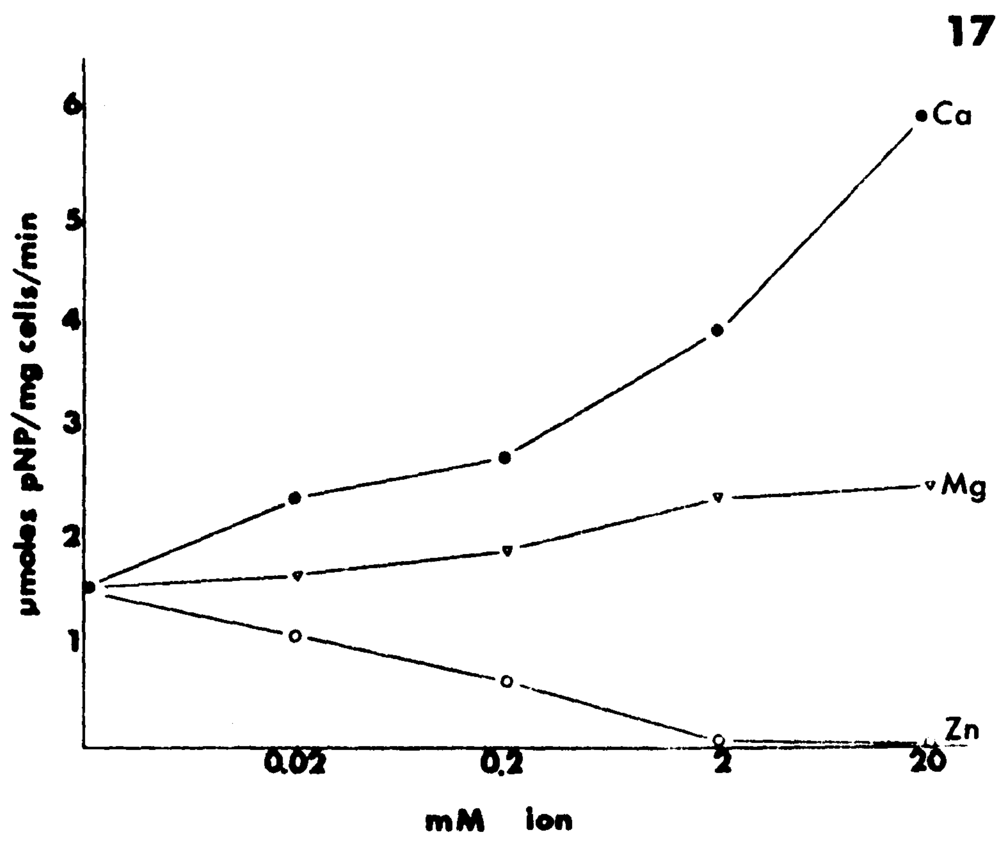
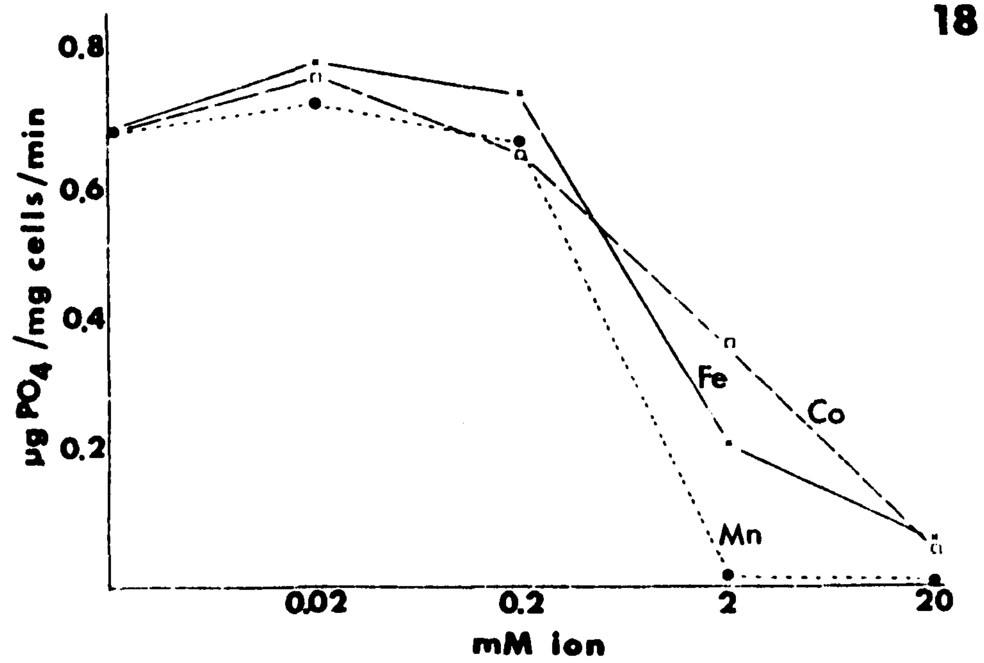
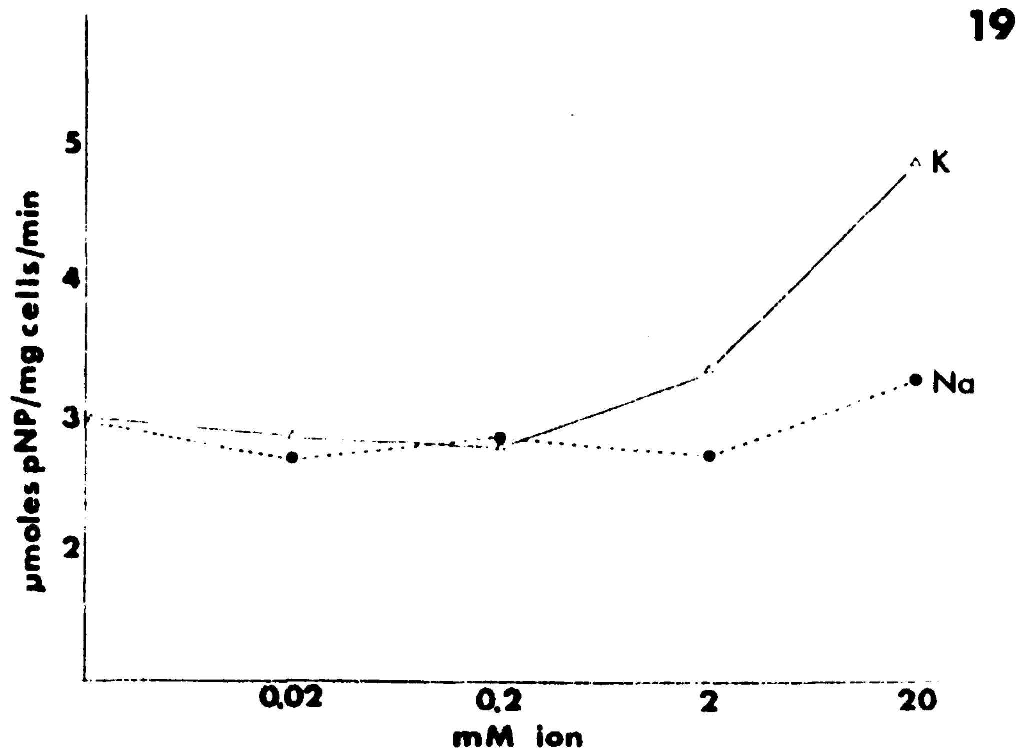

17
μmoles pNP/mg cells/min
6
5
4
3
2
1
Ca
Mg
Zn
0.02
0.2
2
20
mM ion

Figure 18. The effect of Co^{++}, Fe^{++}, and Mn^{++} on _P. boryanum_ cell bound alkaline phosphatase activity. Ion concentration is plotted on a logarithmic scale.

Figure 19. The effect of K^{+} and Na^{+} on _P. boryanum_ cell bound alkaline phosphatase activity. Ion concentration plotted as in Figure 18.


18
μg PO_4 /mg cells/min
0.8
0.6
0.4
0.2
Fe
Co
Mn
0.02
0.2
2
20
mM ion


19
μmoles pNP/mg cells/min
5
4
3
2
K
Na
0.02
0.2
2
20
mM ion

activity fourfold (Fig 17), and both magnesium (Fig 17) and potassium (Fig 19) enhanced activity by one and one-half times at the same concentration of ion. Zinc (Fig 17), manganese, cobalt and iron (Fig 18) were all inhibitory to the cell bound activity of P. boryanum alkaline phosphatase in varying degrees. The zinc ion was inhibitory at all concentrations, completely eliminating all measurable activity at 20 mM.

The successful separation of this periplasmic enzyme from the cells made it possible to compare the effects of those ions tested on cell bound activity with the effects on cell free activity (Fig 20 through 23). As with cell bound activity Ca^{++} was found to have the greatest effect on the enzyme. Activity was nearly doubled with the addition of 20 mM Ca^{++} (Fig 20). The magnesium ion also was stimulatory but to a lesser degree (Fig 20). Zinc and iron ions had little effect on activity at lowest concentration but proved to be inhibitory to a similar level above a concentration of 0.2 mM (Fig 21). Both Co^{++} and Mn^{++} appeared to be slightly inhibitory at a concentration of 20 mM but tended to show some stimulation of activity at lower concentrations (Fig 22). Sodium and potassium ions closely associated with transport across the plasma membrane, had little effect on cell free alkaline phosphatase activity. However, although Na^{+} (Fig 22) also caused no change in activity levels of cell bound alkaline phosphatase; the addition of 20 mM K^{+} (Fig 20)

Figure 20. The effect of Ca^{++}, Mg^{++}, and K^{+} on _P. boryanum_ cell free alkaline phosphatase activity. Ion concentration is plotted on a logarithmic scale.

20

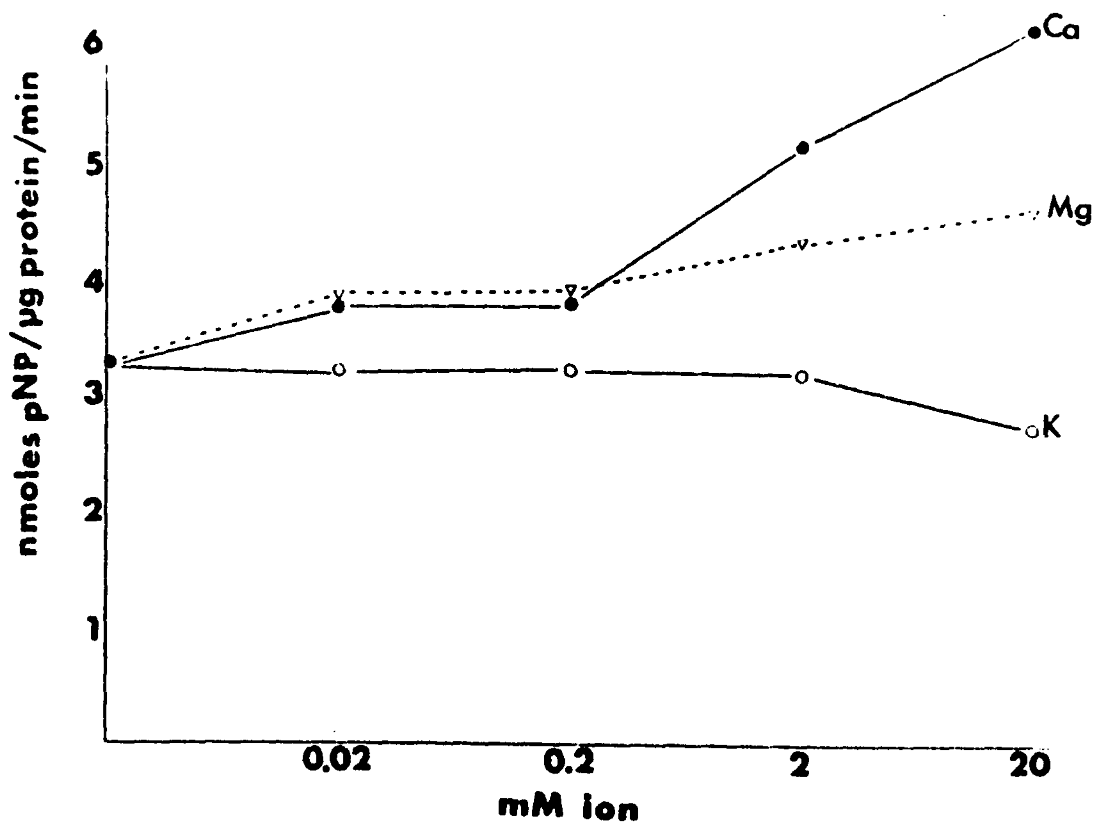
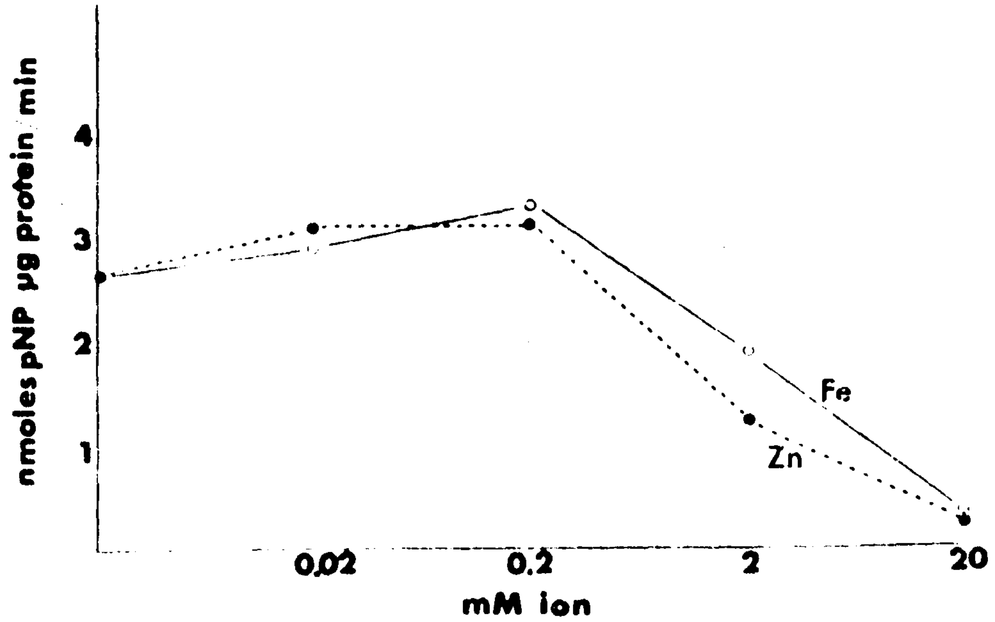


Figure 21. The effect of Fe^{++} and Zn^{++} on _P. boryanum_ cell free alkaline phosphatase activity. Ion concentration plotted on a logarithmic scale.

Figure 22. The effect of Na^{+}, Co^{++}, and Mn^{++} on _P. boryanum_ cell free alkaline phosphatase activity. Ion concentration plotted as in Figure 21.


21
nmoles pNP µg protein min
1
2
3
4
Fe
Zn
0.02
0.2
2
20
mM ion

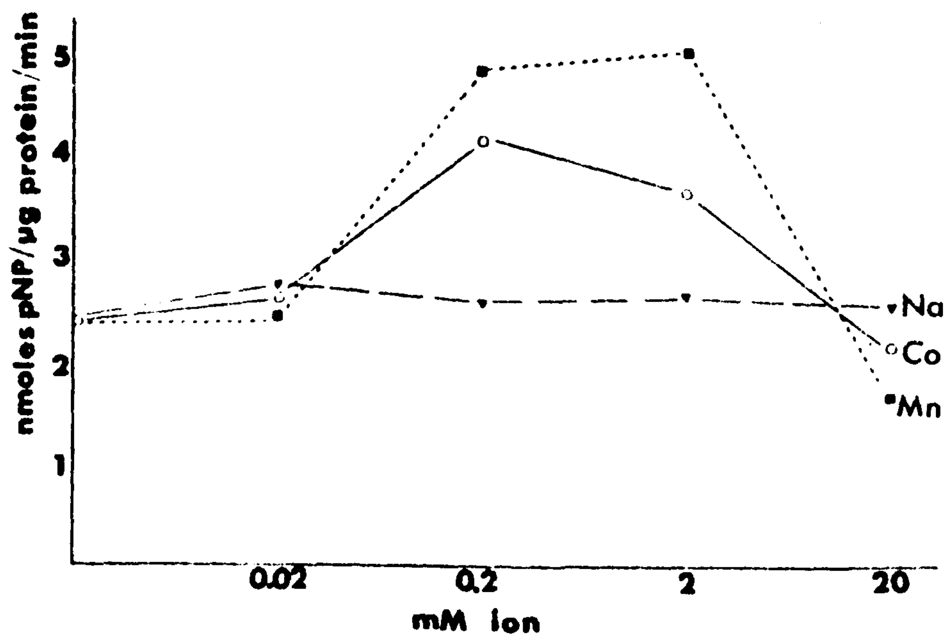
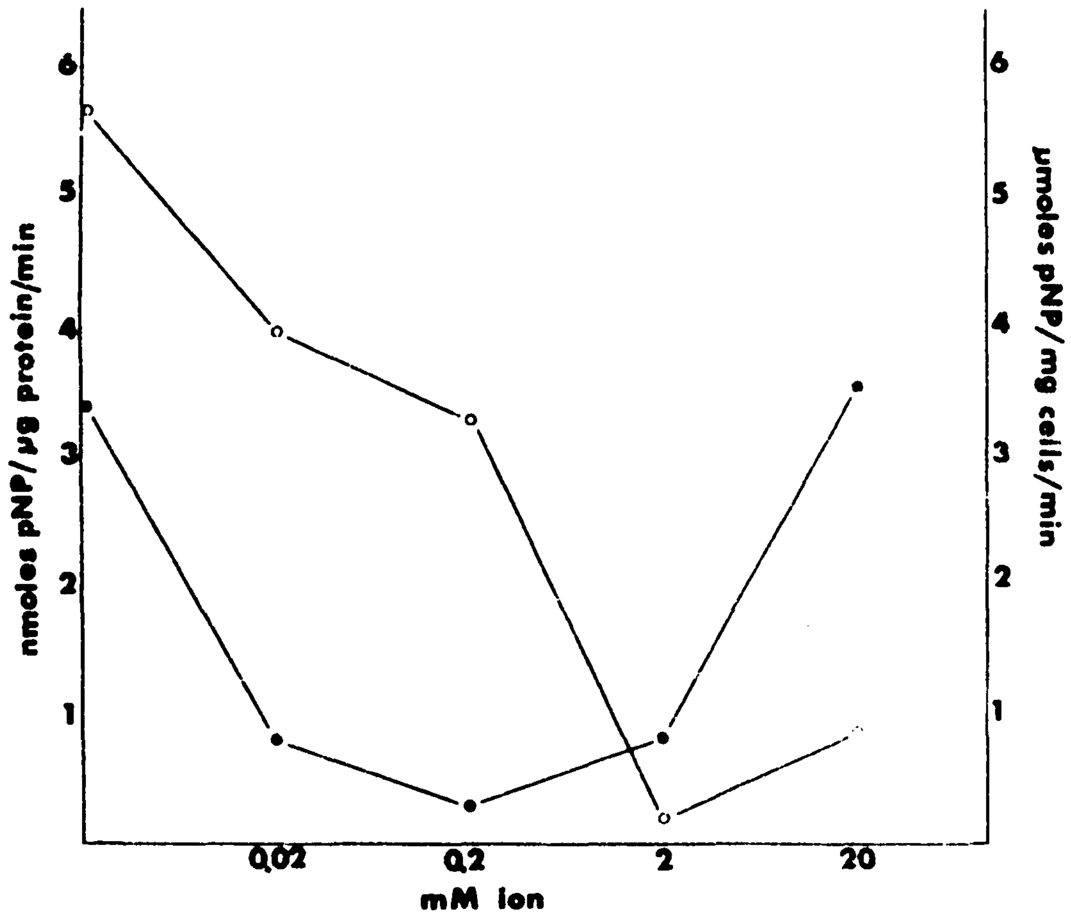

22
nmoles pNP/µg protein/min
1
2
3
4
5
Na
Co
Mn
0.02
0.2
2
20
mM ion

Figure 23. The effect of molybdate on cell bound (open circles O———o) and cell free (closed circles ●———●) *P. boryanum* alkaline phosphatase activity. Ion concentration plotted on a logarithmic scale.

23



stimulated cell bound activity 1.5 fold.

In addition to the above, the effects of molybdate on enzyme activity were tested (Fig 23). With increasing concentration of molybdate, specific activity of cell bound alkaline phosphatase decreased to less than 5% of control activity at a concentration of 2 m*M* molybdate. Increasing concentrations of molybdate cause a decrease in cell free activity to 46% of control levels at a concentration of 0.2 m*M* molybdate. However, as the concentration of molybdate was increased to 2 m*M* and 20 m*M*, the specific activity began to rise again until at 20 m*M*, activity slightly exceeded control levels.

Substrates Hydrolysed by *P. boryanum* Alkaline Phosphatase

The relative rates of hydrolysis of various phosphorylated organic compounds by cell free *P. boryanum* alkaline phosphatase is documented in Table 4. Of the fifteen substrates tested pNPP was clearly the preferred substrate. Although activity of the enzyme was quite low with some substrates, most of those tested were hydrolyzed by the enzyme. As would be anticipated there was no activity detected on preparations of the nucleic acids. The activity of cell bound alkaline phosphatase was tested on several organic phosphate esters, including β glycerophosphate. The specific activity of the cell bound enzyme with β glycerophosphate was found to be 75% of the activity with pNPP. No activity was detected when other substrates were used as listed in Table 4. This

TABLE 4

Relative Hydrolysis of Substrates by Cell Free Enzyme

PHOSPHATE SOURCE	% HYDROLYSIS
p-nitrophenol phosphate	100
Fructose-6-phosphate	43
β glycerophosphate	25
Glucose-6-phosphate	16
Fructose 1, 6 diphosphate	15
Galactose-6-phosphate	10
Glucosamine-6-phosphate	9
Cyclic AMP	5
D L α glycerophosphate	5
2, 3 diphosphoglycerate	5
D (-) 3 - phosphoglycerate	5
DNA (calf thymus - Type I)	0
RNA (yeast-type Xl)	0
bis-nitrophenyl phosphate	0
Cyclic UMP	0

was possibly due to the rapid uptake of released phosphate thus leaving too little P_i in the supernatant to be measurable.

Kinetics of Alkaline Phosphatase

The dependence of both cell free and cell bound enzyme activity on the concentration of pNPP could be described on the basis of Michaelis-Menten kinetics. Use of a Lineweaver-Burk plot, $1/_v$ versus $1/_s$ (Figs 24-25) allowed the caluculation of half saturation values and concentrations of substrate (Km) required to support half the maximum rates. Fig 25 is a plot of cell bound enzyme activity versus concentration of pNPP and Fig 24 shows a plot of inhibited and uninhibited cell free enzyme. Km for the cell free enzyme was calculated to be 1×10^{-3}M, and 3.9×10^{-3}M for the cell bound enzyme. The kinetics of cell free alkaline phosphatase were also plotted when inhibited by inorganic phosphate as shown in Fig 24. The linear plot of inhibited enzyme demonstrates a changed slope but has the same intercept thus indicating competitive inhibition. The calculated dissociation constant for the enzyme inhibitor complex K_i is found to be 4.5×10^{-4}M. This value shows the end product of the alkaline phosphatase reaction to have a high affinity for the enzyme.

Cytochemistry

Control cells as seen in Figs 26-29 contain the usual inclusions found in blue-green bacteria. A few polyphosphate bodies and a number of polyhedral bodies

Figure 24. Lineweaver-Burk plot of *P. boryanum* cell free alkaline phosphatase activity in the presence of varying concentrations of para nitrophenol phosphate. Upper curve shows activity with the addition of 2 m*M* phosphate.

24

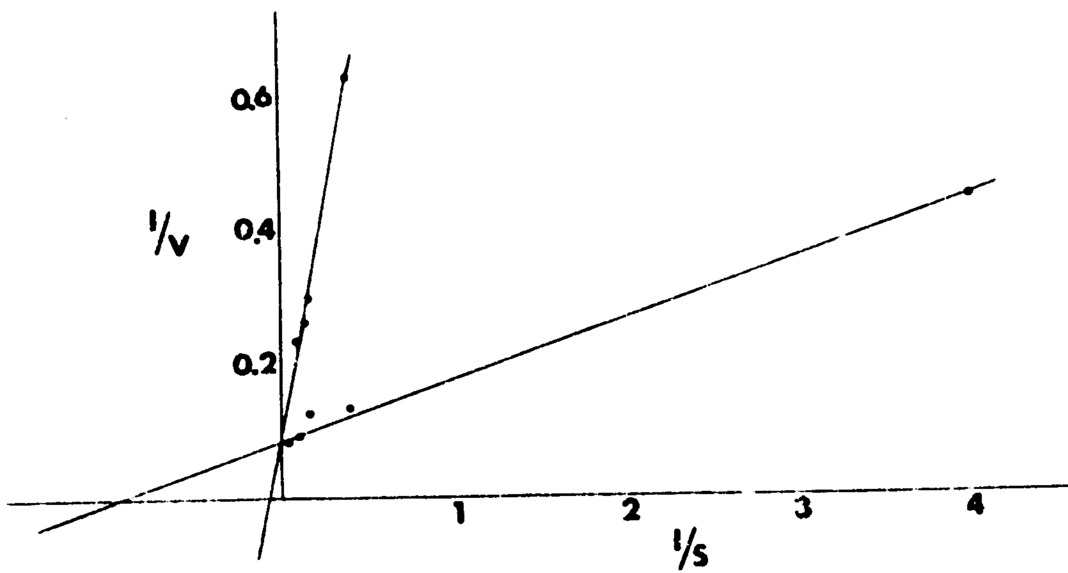
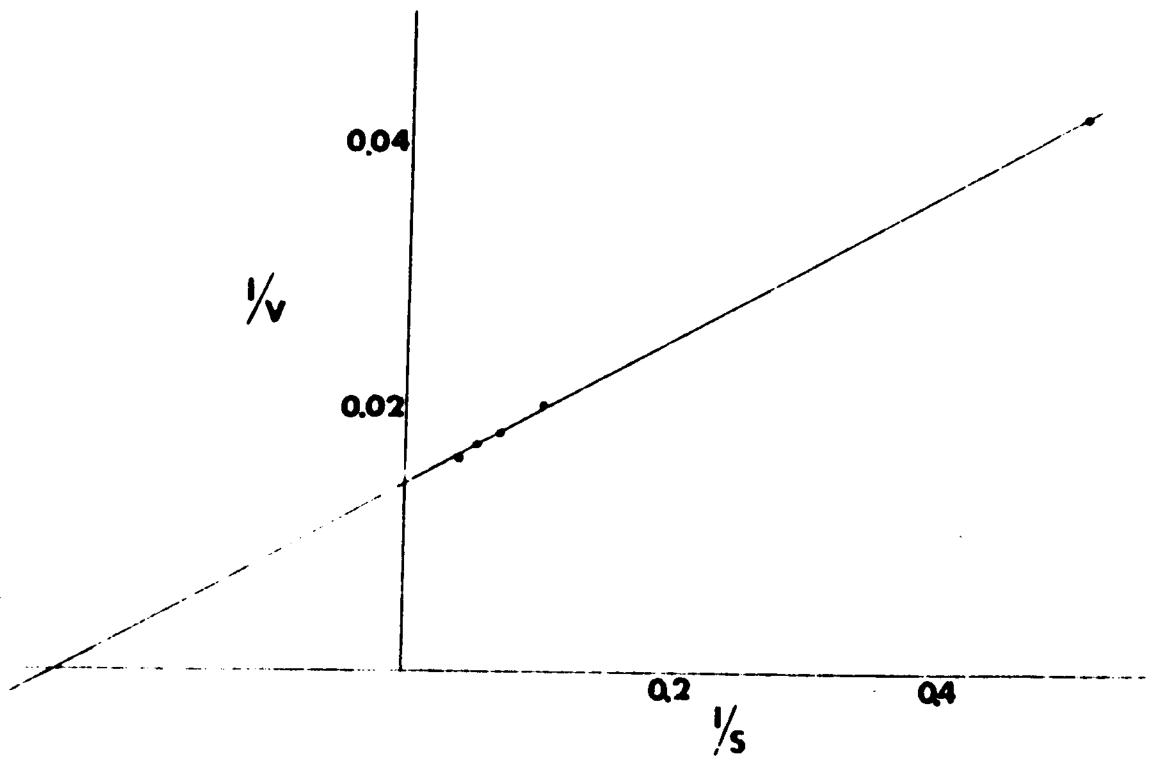


Figure 25. Lineweaver-Burk plot of *P. boryanum* cell bound alkaline phosphatase activity in the presence of varying concentrations of para nitrophenol phosphate.

25

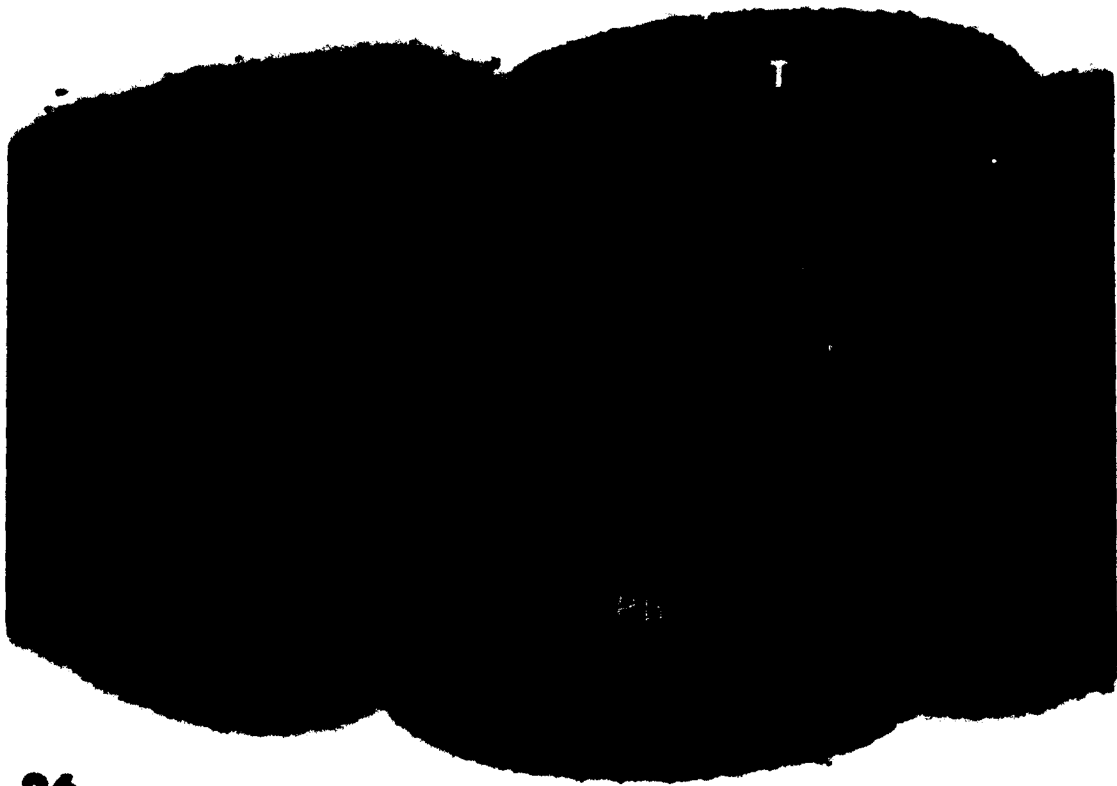


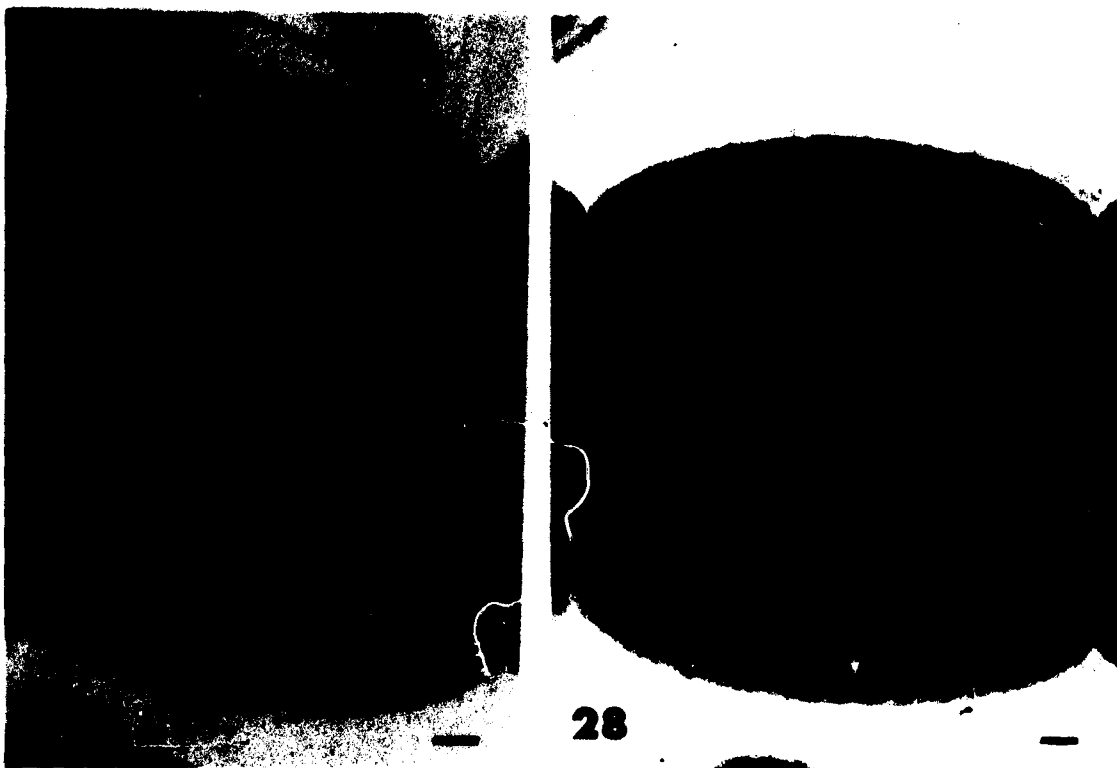
Figures 26-28. *P. boryanum* control cells derepressed and unfixed, treated with no substrate but with the rest of the reaction mixture. Bars represent 0.1 um.

Figure 26. Typical control cell showing no precipitate and normal inclusions. Symbols: T, thylakoids; P, polyphosphate body; Pb, polyhedral body. Postfixed in osmium.

Figure 27. Control cell showing thylakoidal precipitate (arrows). Postfixed in glutaraldehyde-osmium.

Figure 28. Control cell showing clumps of precipitate (arrows) probably due to nonspecific lead salt deposition. Postfixed with glutaraldehyde-osmium.

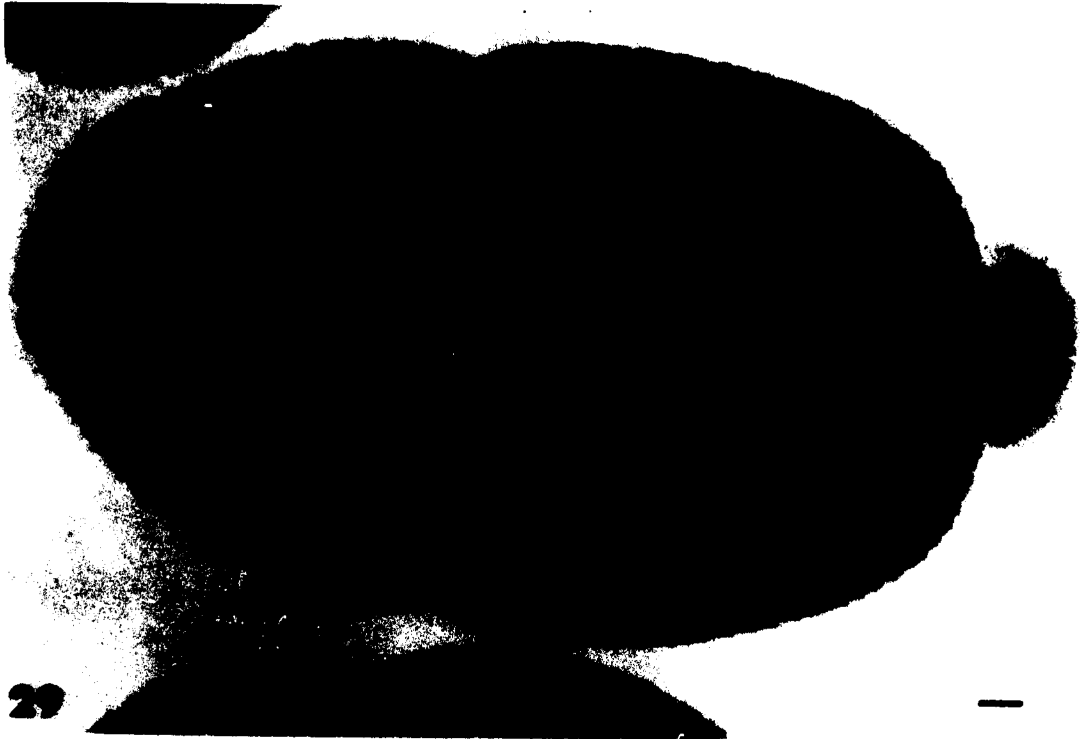

26

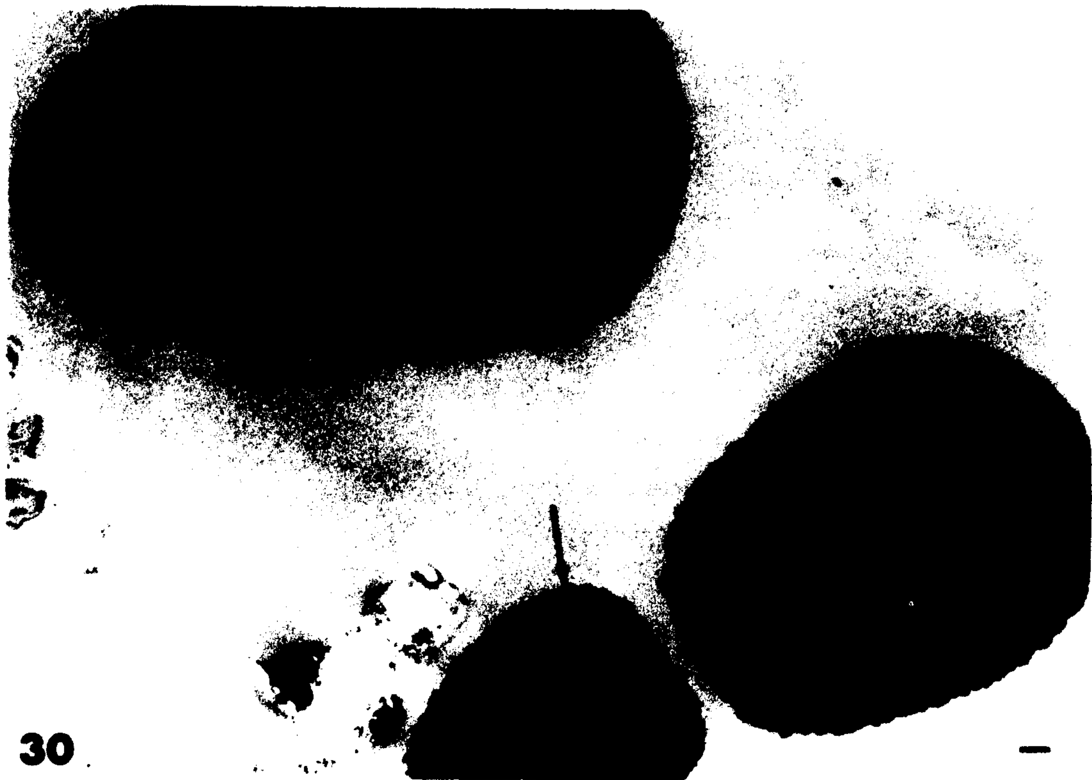

28

Figures 29-30. _P. boryanum_ cells. Bars represent 0.1 um.

Figure 29. Control cell derepressed and unfixed, treated with substrate and no lead. Postfixed in glutaraldehyde-osmium.

Figure 30. Cells derepressed and unfixed, treated with complete reaction mixture. Tangential section showing cell covered with precipitate (arrow). Postfixed in glutaraldehyde-osmium.


29


30

are typically present. After incubation in solution without substrate and treatment with lead nitrate, the majority of the cells lack any precipitate indicative of alkaline phosphatase activity. However, occasional cells treated before fixation and postfixed with glutaraldehyde for 1.5 h and osmium tetroxide for 1.5 h showed light granular precipitates within the thylakoids (Fig 27). A few cells similarly treated contained dense clumps of precipitate near the cell membrane as in Fig 28. When the cells were incubated with substrate not followed by lead nitrate (Fig 29) there was no visible evidence of alkaline phosphatase activity although there was a precipitate of $Ca(NO_3)_2$ present at reactive sites. There was no evidence of precipitate from enzyme activity when any reagent of the complete reaction mixture was lacking. All cells were found to be in excellent morphological appearance following the entire cytochemical procedure.

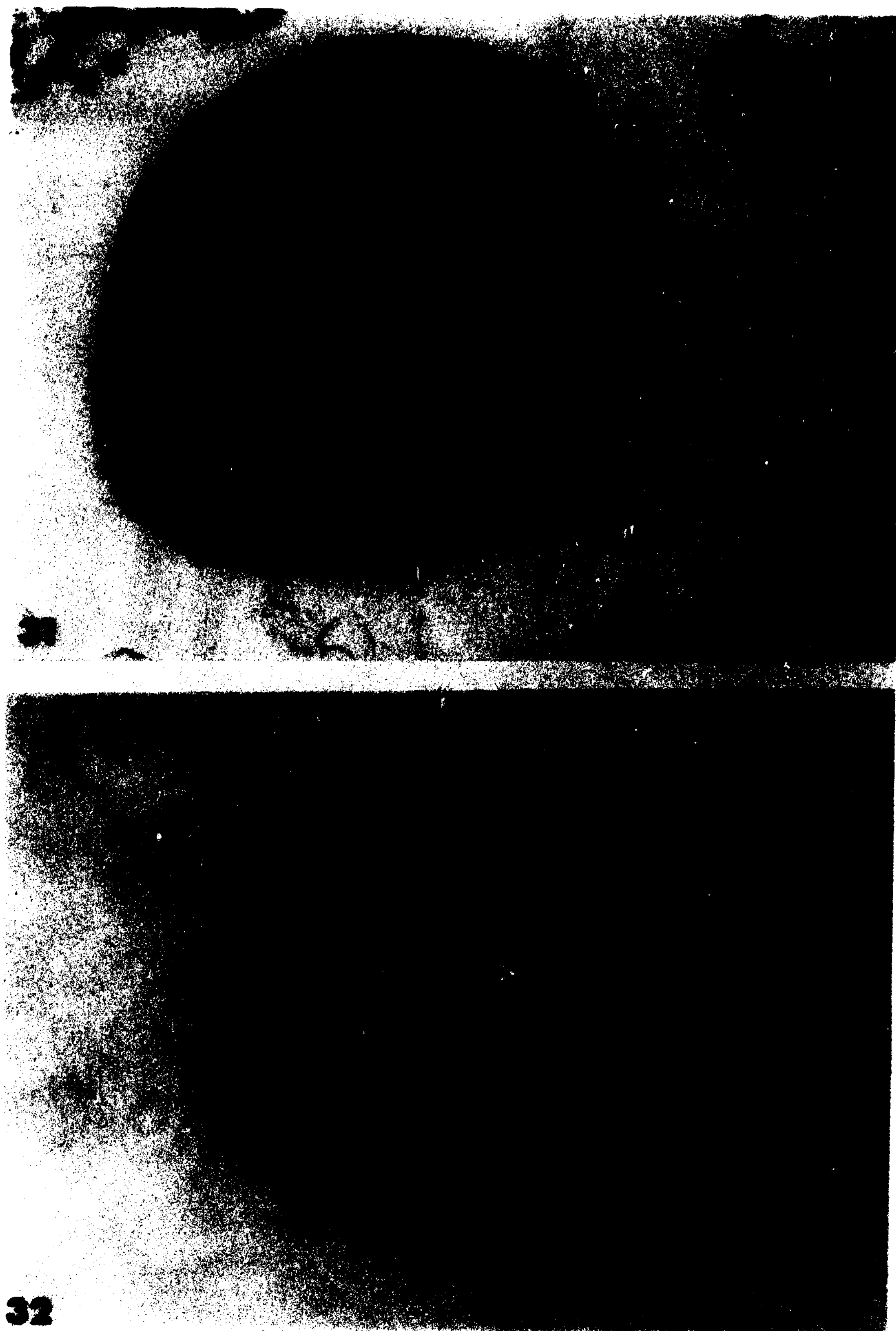
Cells prefixed with para-formaldehyde (Figs 31,32) or pure glutaraldehyde (Figs 33, 35) followed by incubation in solution without substrate and treatment with lead nitrate lacked any precipitate indicative of alkaline phosphatase activity.

Those cells suspended in complete incubation solution followed by lead nitrate showed alkaline phosphatase activity in the cell wall. This was in the form of a granular precipitate surrounding the entire cell, when individual cells are observed as in Fig 38. In filaments,

Figures 31-32 _P. boryanum_ cells derepressed and prefixed in paraformaldehyde. Bars represent 0.1 um.

Figure 31. Control cell treated with reaction mixture lacking only substrate. Postfixed in osmium.

Figure 32. Cell treated with complete reaction mixture. Postfixed in osmium.


31
32

Figures 33-34. *P. boryanum* cells derepressed and prefixed in glutaraldehyde. Bars represent 0.1 um.

Figure 33. Control cell treated with no substrate but with the rest of the reaction mixture. Postfixed in osmium.

Figure 34. Cell treated with complete reaction mixture. In addition to precipitate on the outer surface of the wall, the arrow indicates a clump of precipitate on thylakoid. Postfixed in osmium.

the reaction product was found only in external walls; none was found in cross walls (Figs 39,40). The precipitate often was extremely dense, almost obliterating the entire wall structure. In cells cut tangentially, the granular precipitate appeared to cover the entire surface of the cell (Fig 30). In Figs 40-44 the precipitate can be seen quite clearly within the periplasmic space or more specifically layer 3 of the cell wall. Occasionally a small amount of deposit was observed on the outer surface of layer 4 (Fig 44). In some sections, as shown in Fig 38, a cell showing much reaction product, i.e. continuous precipitate within the cell wall, was found near a cell showing no evidence of alkaline phosphatase activity.

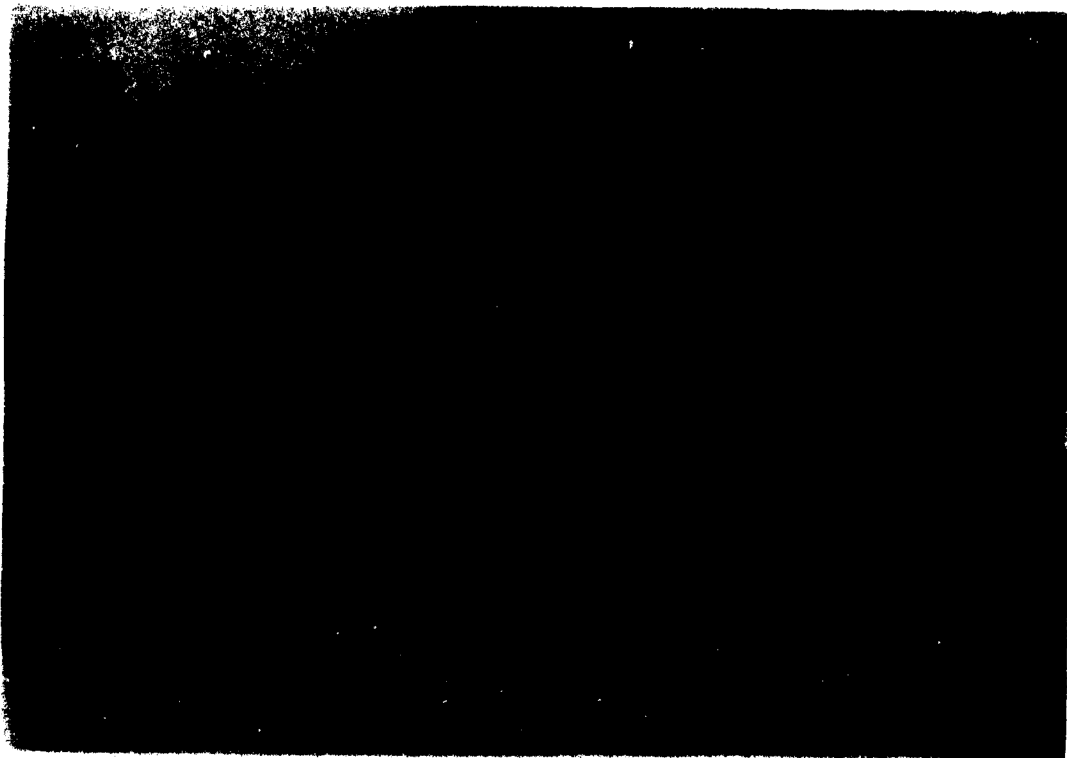
All unfixed cells placed in appropriate incubation solutions and then transferred to fixative solutions after completion of the reaction gave good results. These results were equally good whether the fixative was a combination of glutaraldehyde and osmium or osmium alone. However, when the same reaction was attempted with cells prefixed in glutaraldehyde (Fig 34) or para-formaldehyde (Fig 32) the results were poor, the precipitate being sparse and seemingly nonspecific. This was also true when enhancement of reaction was tried by prefixing the cells in glutaraldehyde in the presence of substrate as seen in Figs 36 and 37.

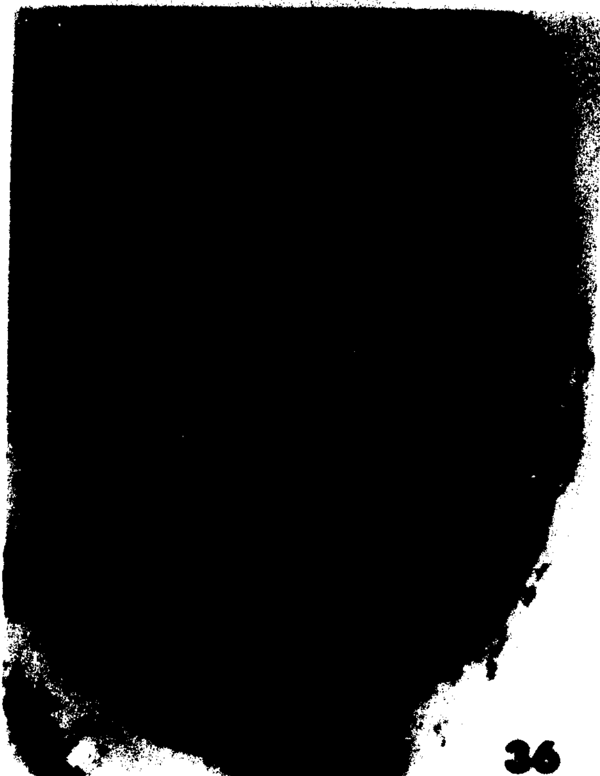
Histochemical techniques were applied at the ultra-structural level to localize alkaline phosphatase activity

Figures 35-37. *P. boryanum* cells derepressed and prefixed in glutaraldehyde. Experimental cells prefixed in the presence of substrate. Bars represent 0.1 um.

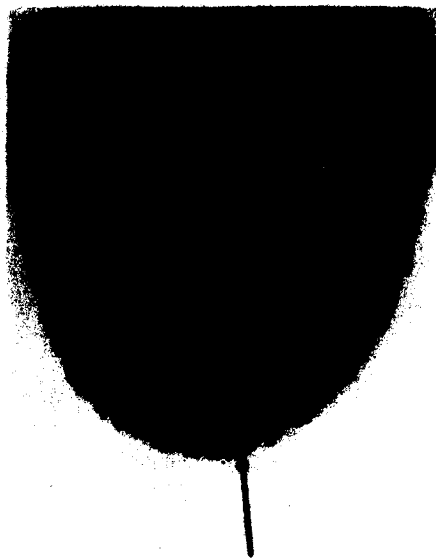
Figure 35. Control cell treated with no substrate but with the rest of the reaction mixture. Postfixed in osmium.

Figures 36-37. Cells treated with complete reaction mixture. Precipitate is shown outside the cell wall (arrows). Postfixed in osmium.





36

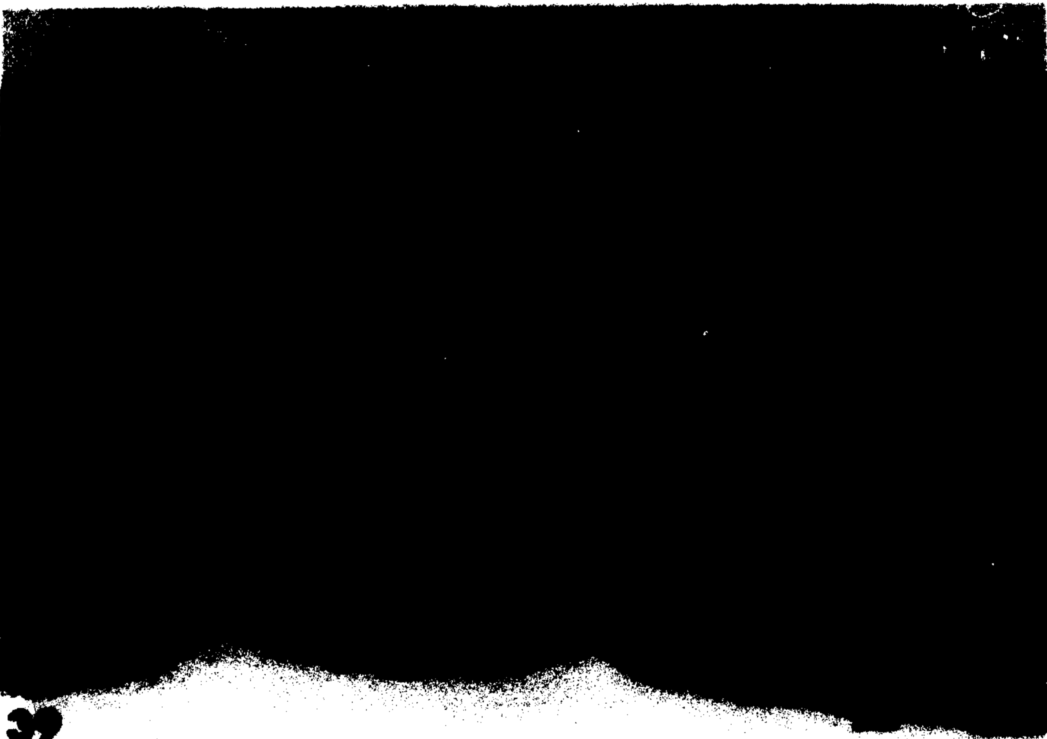


37

Figures 38-39. *P. boryanum* cells derepressed and unfixed, treated with the complete reaction mixture. Bars represent 0.1 um.

Figure 38. Upper cell shows activity in wall; lower cell apparently not derepressed and showing no reaction product. Postfixed in osmium.

Figure 39. Section of a portion of a filament of *P. boryanum* shows enzyme activity in the outer walls (arrows) and none in cross walls. Postfixed in glutaraldehyde-osmium.


39

Figures 40-44. _P. boryanum_ cells derepressed and unfixed, treated with complete reaction mixture. All cells postfixed in glutaraldehyde-osmium. Bars represent 0.04 um.

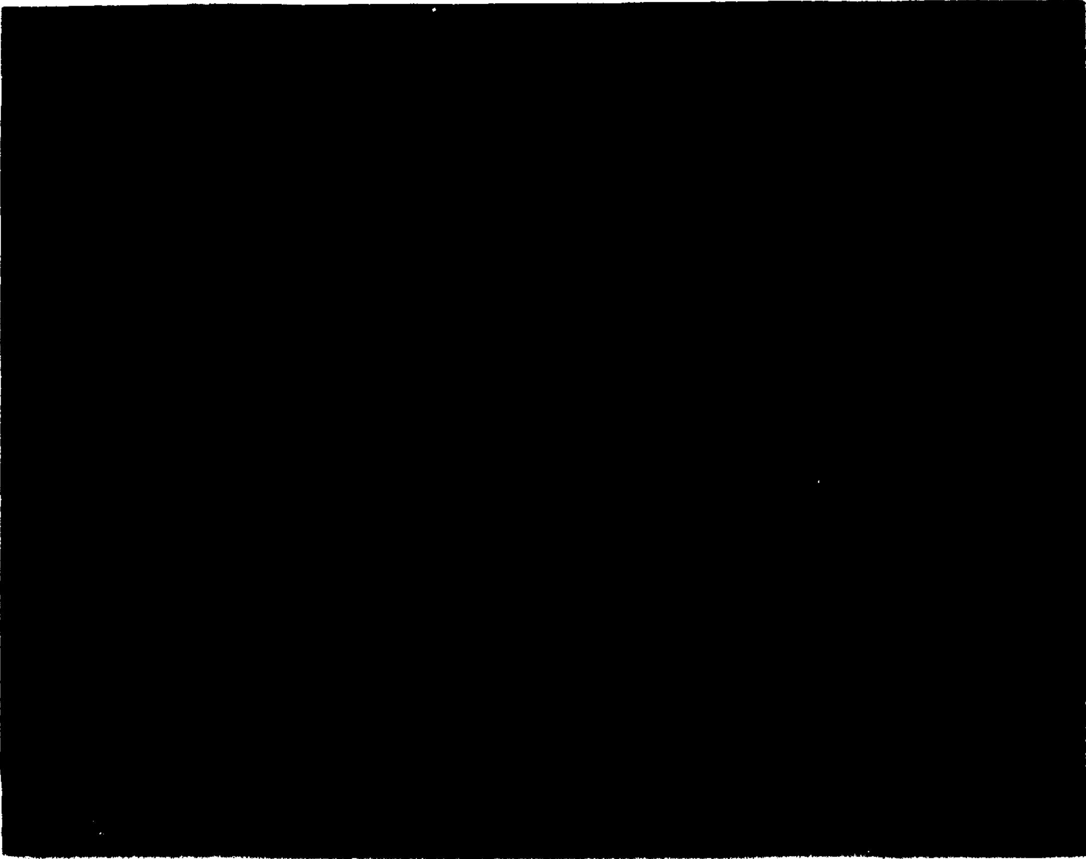
Figures 41-44. Enlarged views of cell wall clearly showing precipitate in periplasmic space (layer 3).

Figure 41. Dense precipitate with an area of no precipitate shows deposit located primarily in periplasmic space; the plasma membrane (cm) and layers 1 through 4 are visible in this section.

Figure 42. Aggregates of precipitate (arrows).

Figure 43. Dense deposit of precipitate (arrow).

Figure 44. More grainlike appearance of precipitate in periplasmic space (small arrow) and apparently a small amount of precipitate on the outer surface of layer 4 (large arrow).



in the blue-green bacterium *Anabaena cylindrica*. In Fig 45 a granular precipitate can be seen surrounding the entire cell, maximally located in layer 3 of the cell wall. Figure 47 shows an enlargement of the wall and reveals that the precipitate is in layer 3. In filaments, the reaction product was found mainly in the external walls with a small amount found in cross walls (Fig 48). In a number of sections granular precipitates of varying density were found in association with the sheath which normally surrounds these cells. Enzyme activity in the medium however, was very low indicating that the active enzyme did not function to any extent in the culture medium.

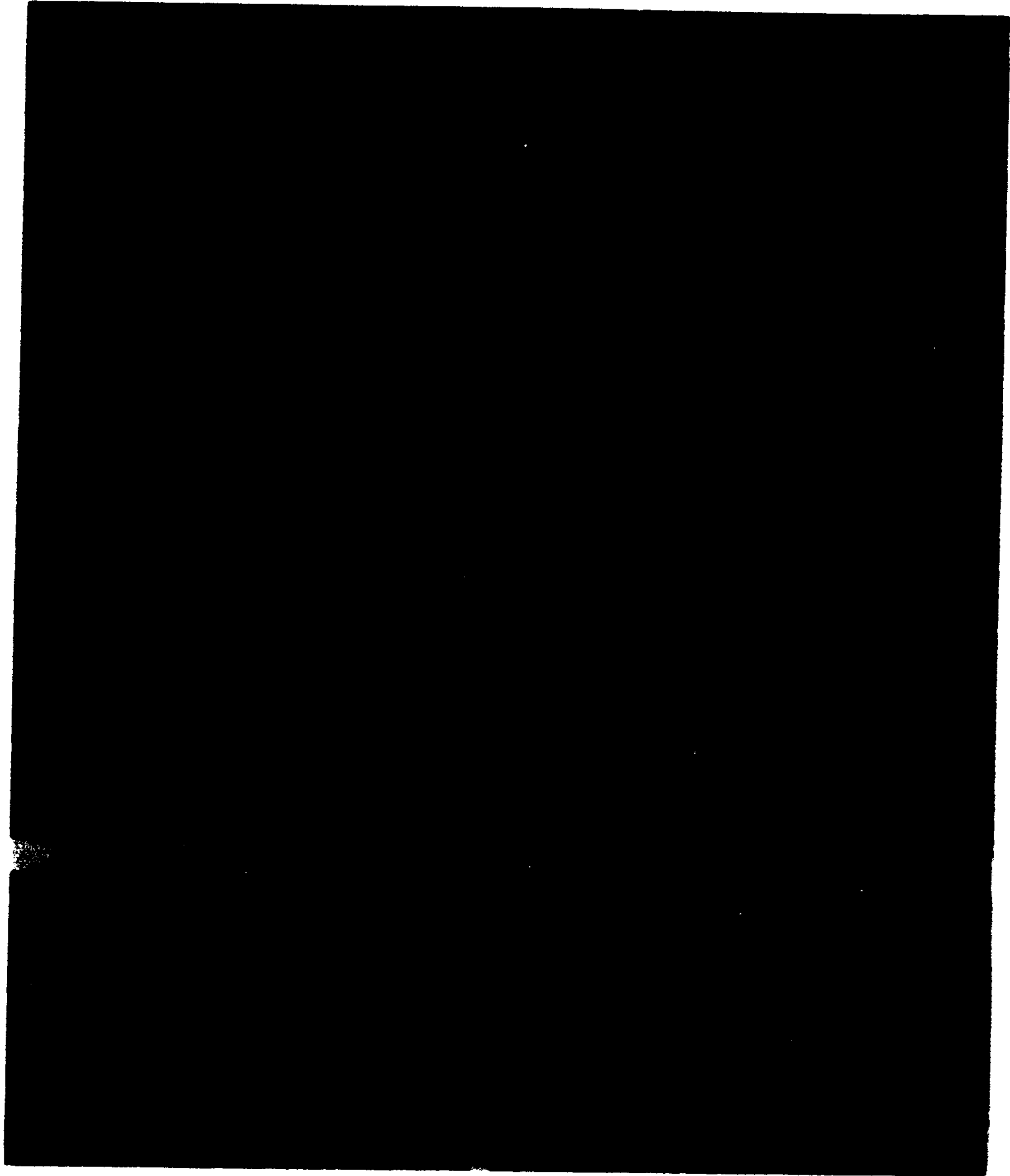
When similar techniques were applied to unfixed cells of the cyanobacterium *Coccochloris peniocytis* the precipitate surrounding these coccoid unicellular blue-greens was quite dense and often seemed to cover the wall completely (Figs 52 and 53). Fig 50 is a control cell and Fig 53 shows a cell with evidence of high alkaline phosphatase activity. Cell bound activity appears to be localized in layer 3 of the wall as seen in Fig 51. The enzyme activity of the medium in which *C. peniocytis* has been cultured was found to be high both in log phase cells and in starved cells. Also this particular cyanobacterium seems to produce the enzyme constitutively. All of these cells treated for localization of alkaline phosphatase activity in this organism are log phase cells.

Figures 45-47. Anabaena cylindrica cells derepressed and unfixed, treated with complete reaction mixture. All cells postfixed in osmium. Bars represent 0.1 um. Symbols: P, polyphosphate body; Pb, polyhedral body; T, thylakoid.

Figure 45. Cell shows activity in the wall and activity in the sheath.

Figure 46. Enlarged view of the wall showing precipitate in the wall and in the sheath.

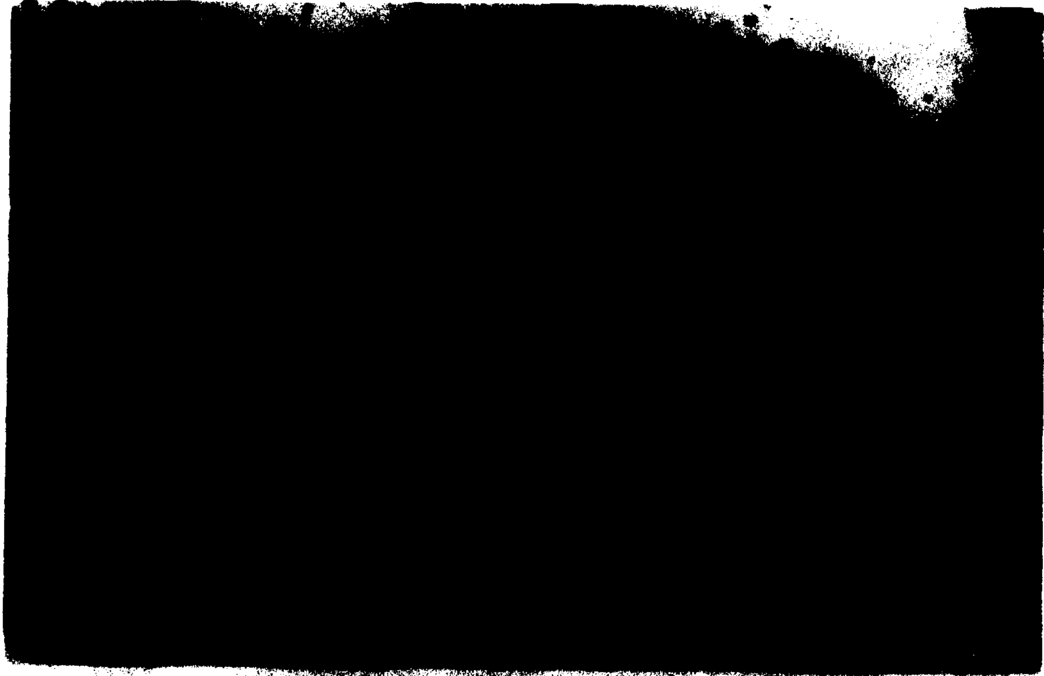
Figure 47. Enlarged view of the wall showing precipitate (arrow) clearly in periplasmic space (layer 3).

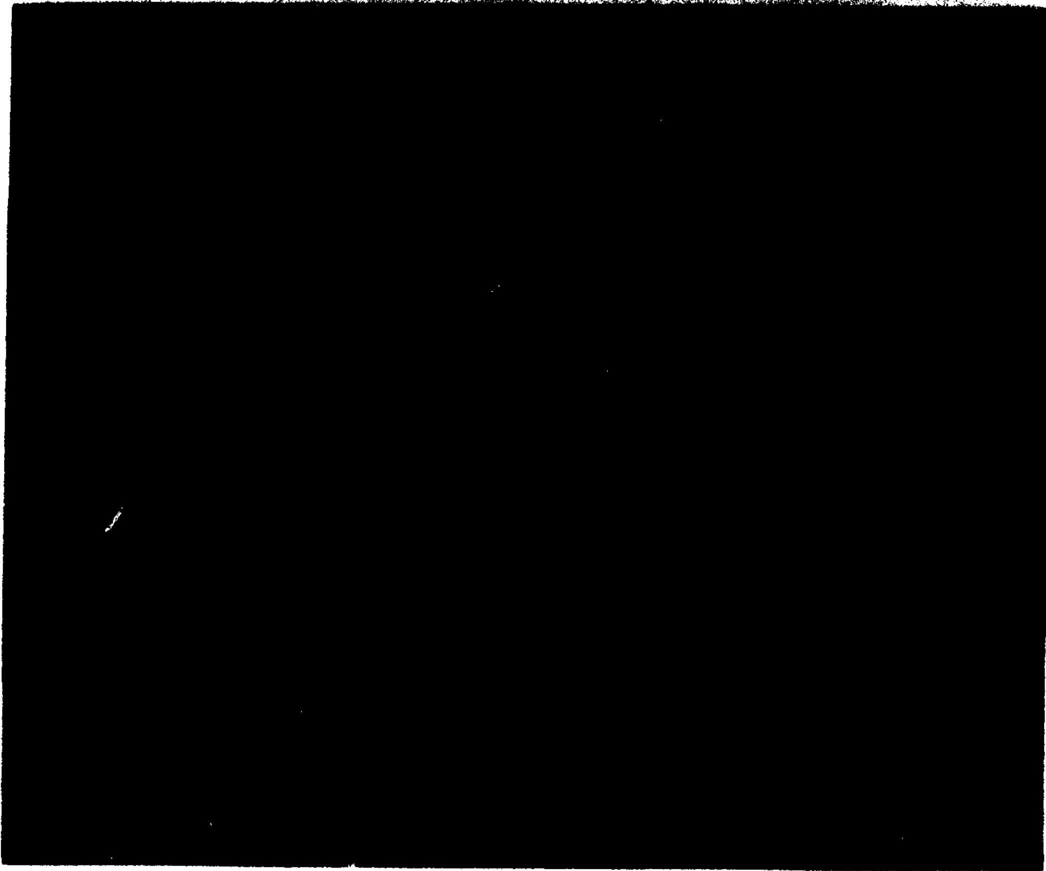


Figures 48-49. *Anabaena cylindrica* cells derepressed and unfixed. Symbols: P, polyphosphate body; Pb, polyhedral body; C, cyanophycin granule; S, sheath. All cells postfixed in osmium. Bars represent 0.1 um.

Figure 48. Cells treated with complete reaction mixture. Precipitate indicative of alkaline phosphatase activity in end walls and sheath. Some activity is also seen in cross walls (arrows).

Figure 49. Control cell treated with no substrate but with the rest of the reaction mixture. Typical control cell with no precipitate and normal inclusions.

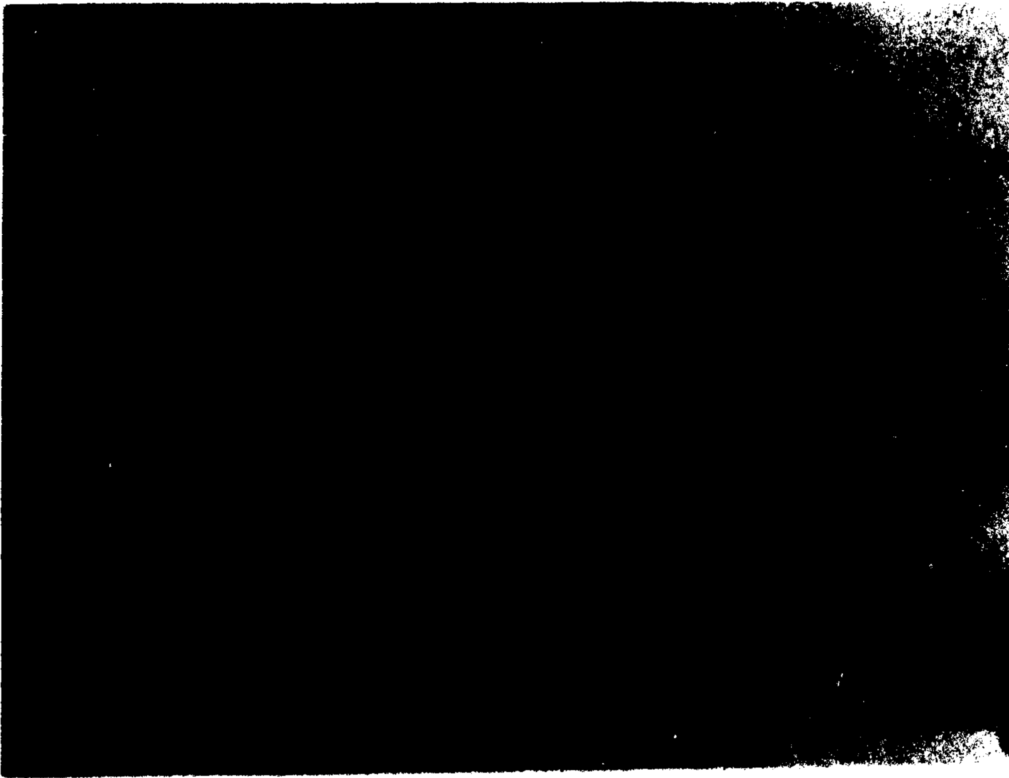




Figures 50-51. Coccochloris peniocytis log phase cells unfixed prior to treatment for alkaline phosphatase localization. Symbols: T, thylakoids; D, DNA. All cells postfixed in osmium. Bars represent 0.05. um.

Figure 50. Control cell treated with no substrate but with the rest of the reaction mixture.

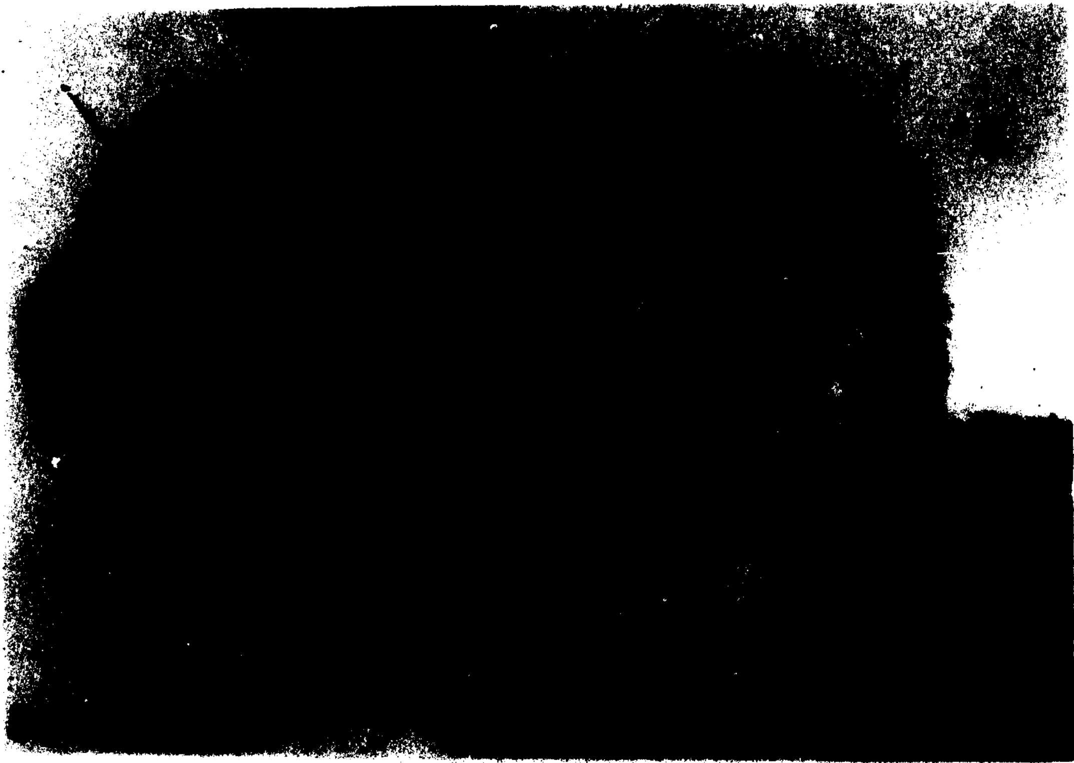
Figure 51. Cell treated with complete reaction mixture. Precipitate indicative of cell bound activity is present in the periplasmic space (layer 3) of wall (arrow).

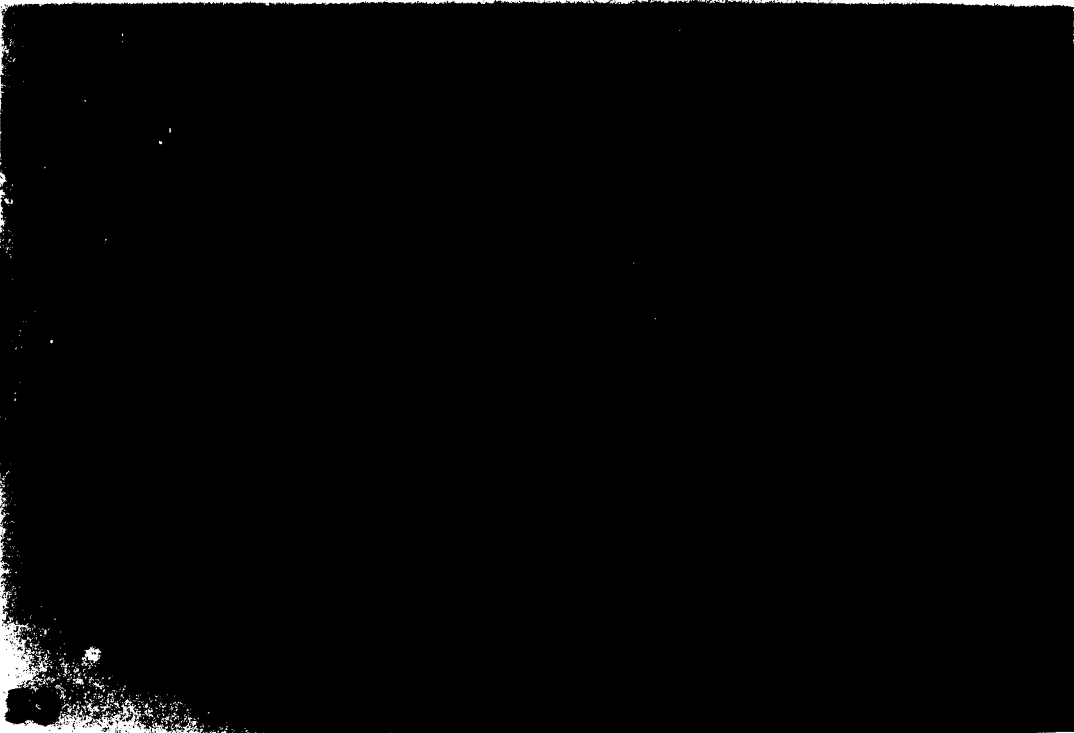


Figures 52-53. *C. peniocytis* log phase cells unfixed and treated with complete reaction mixture. Postfixed in osmium. Bars represent 0.05 um.

Figure 52. Arrows indicate encrustation of reaction product crystals on cell surface probably due to secreted enzyme.

Figure 53. Some reaction product seems to have formed on a particle of cell debris probably due to enzyme free in the menstruum (arrow).





DISCUSSION

Determination of Maximum Alkaline Phosphatase Activity in *Plectonema boryanum*

Growth under phosphate limited conditions manifests an increase in the level of alkaline phosphatase activity of *Plectonema boryanum*. In this study and as observed by Fizgerald and Nelson (1966) in the field, there is a rise from very low levels of enzyme activity in cells grown with just adequate amounts of phosphate ($4x10^{-6}$M phosphate) or surplus phosphate ($1x10^{-4}$*M* phosphate in this study), to much higher levels following a period of hours or days in a phosphate free environment. The cells of *P. boryanum* were grown to the peak of logarithmic phase in a medium containing $1x10^{-4}$*M* phosphate and were then transferred to a phosphate free medium as previously described. Five days of phosphate starvation yielded a maximum amount of enzyme activity in these cells. At that time there was a fourteen fold increase in activity over that seen in log phase cells. This increase was greater than increases shown in *Bacillus subtilis* (Cashel and Freese, 1964) or *Blastocladiella emersonii* (Selitrennikoff and Sonneborn, 1977) and similar to the increase reported for *Anacystis nidulans* (Synechococcus) by Ihlenfeld and Gibson (1975) although somewhat less than that reported in *Escherichia coli* (Torriani, 1960).

It is difficult to make exact comparisons among the specific activities of alkaline phosphatases of various

microorganisms primarily due to differences in assay conditions and means of expressing activity. The highest specific activities of the enzyme found in these studies are greater than those reported in studies by Healey (1973) in *Anabaena variabilis*, by Ihlenfeld and Gibson (1975) in *Anacystis nidulans*, and by Bone (1971) in *Anabaena flos-aquae* grown in continuous culture under phosphate limited conditions. However, specific activity is slightly less than that found by Torriani (1960) in *E. coli*.

All of the alkaline phosphatase activity is firmly bound to the cells in *P. boryanum* which does not normally release any of its enzyme into the surrounding medium. However, when the cultures reached the age of 60 days and no new source of nutrients was made available, some alkaline phosphatase activity could be detected in the medium. This specific activity increased with increasing age from a low of 0.0001 ug/ml/min at 60 days to a high of 0.09 ug/ml/min at 220 days of age. As this enzyme is very firmly bound to these cells, this rise in activity could most likely be due to the presence of dead and dying cells. The alkaline phosphatase activity found in the medium was either free or associated with cell wall debris from the dead cells. These cells were a probable source of organic substrates. The cell free enzyme plus the enzyme associated with living cells could hydrolyze the available organic substrates and thus function in keeping the culture indefinitely viable.

Alkaline Phosphatase Levels in 17 Isolates of Blue-green Bacteria

All of the blue-green bacteria tested to date have been found to be alkaline phosphatase positive with the exception of a marine *Synechococcus* listed by Kuenzler and Perras (1965) as alkaline phosphatase negative. In this study of seventeen isolates of blue-green bacteria, logarithmic phase specific activities were compared with specific activities after five days in a phosphate free environment. Many variations were shown. Table 2 shows isolates which appear to be inducible, others which appear to be constitutive for the enzyme, some which secrete enzyme in log phase and/or under starvation conditions and some which release no enzyme at all as was found in *P. boryanum*.

This is the first study in which any species of blue-green bacteria have been identified as constitutive for alkaline phosphatase. The data show *Oscillatoria sp*. (UTEX 1543), *Plectonema calicothricoides* (UTEX 598) and *Coccochloris peniocytis* (UTEX 1548) to produce the enzyme under both phosphate limited and non limited conditions. There is also great variation in the amount of increase seen in levels of activity when logarithmic activities are compared with phosphate starved activities. Increases ranged from a two-fold increase in *Nostoc commune* (UTEX 584) alkaline phosphatase activity to a twelve-fold increase in *Lyngbya sp*. (UTEX 621). These levels are comparable to those found in other microorganisms (Cashel and Freese, 1964; Okabayashi *et al*, 1974; Ihlenfeld and Gibson, 1975).

In some of the microorganisms reported, due to probable secretion by the cells, varying amounts of enzyme activity were found to be associated with the surrounding medium. *Bacillus subtilis* (Cashel and Freese, 1964) released 93% of its alkaline phosphatase after five hours in phosphate free medium and *E. coli* secreted up to 15% of its active enzyme. Glew and Heath (1971) in *Micrococcus sodenensis* found only 6% of the enzyme to remain cell bound. Of the blue-greens studied Healey (1973) was able to use the secreted enzyme of *A. variabilis* as a readily available source of cell free alkaline phosphatase.

This survey of seventeen isolates of cyanobacteria shows that *P. boryanum*, the major test organism in this study, demonstrates only one of many possible responses to phosphate starvation. The same survival mechanism works in a variety of ways. The reasons for some cells to release enzyme in the normal course of events while others such as *P. boryanum* cells release none are unknown.

Extraction of Enzyme from Plectonema boryanum

The cell walls of gram negative bacteria and blue-green bacteria are very similar (see literature review). However, some of the methods used successfully to disrupt the cell wall of gram negative bacteria are not readily applied to blue-green bacteria. The walls of blue-greens are far more resistant to disruption. Methods using high concentrations of various cations, detergents such as Triton X-100 and EDTA were tried unsuccessfully in an

attempt to release alkaline phosphatase from the cell wall of _P. boryanum_. Finally it was found that lysozyme, the enzyme which attacks the mucopolymer layer (L2), in the presence of EDTA does cause release of _P. boryanum_ alkaline phosphatase. EDTA is necessary as it initiates disruption of the barrier function of the wall, probably through removal of ions such as Mg^{++} necessary for maintenance of cell wall integrity (Leive, 1974). This method results in the total lysis of most of the cells as evidenced by pigment loss and examination with light and electron microscopy. It was not possible to selectively peel off the outermost wall layers by this method. Breakdown of the mucopolymer layer in essence removed the "skeletal" shape retaining and osmotic protective layer of the wall yielding spheroplasts. It is probable that lysis occurred in spite of the high sucrose content of the suspending medium as the cells were agitated throughout the treatment period of 18 hours.

The second method found to yield satisfactory release of _P. boryanum_ alkaline phosphatase made use of polymyxin B sulfate, a membrane specific polypeptide antibiotic (Page and Tsang, 1975). These authors obtained successful release of alkaline phosphatase from whole cells of _Serratia marcesens_ by this treatment. Previously, Cerny and Teuber (1971) obtained release of periplasmic enzymes from _E. coli_ in a similar manner. Extraction of _P. boryanum_ alkaline phosphatase was accomplished in 18 hours

at 25 C and a pH of 8.2, in contrast to the one minute treatment at 37 C and a pH of 7.3 required for release of the _E. coli_ enzyme. Less enzyme was extracted from P. boryanum when a lower pH was tried.

A number of studies suggest that membrane phospholipids are primary receptors for polymyxin (Few, 1955; Newton, 1956; Cerny and Teuber, 1971). A study by Lopes and Innes (1969) demonstrated by electron microscopy that isolated lipopolysaccharide having an appearance similar to the double track layer (L4) was completely disorganized by polymyxin B. Other morphological alterations of the outer double track layer in gram negative bacteria have been observed (Koike _et al_., 1969) following polymyxin B treatment. Whitton (1967) in testing toxicity of polymyxin B to cultures of _A. nidulans_ and _Chloroglea fritischii_ suggested that the antibiotic was either complexed by, or attacked a wall component and the cell membrane. This author also suggested that the presence of a greater amount of extracellular material as found in the walls of blue-green bacteria probably minimized any toxic effect on the plasma membrane.

Successful release of enzyme from _P. boryanum_ by the polymyxin method suggests some association of alkaline phosphatase with lipopolysaccharide in this organism. A most desirable aspect of this method in working with filamentous cyanobacteria is release of enzyme without resultant disruption of most of the cells. Apparently, the antibiotic causes disruptions of all layers of the

P. boryanum cell wall as cells examined by electron microscopy showed sections of the entire outer wall pulled away from the cell(Figs 4,7,8). After loosening of the cell wall layers, release occurred possibly due to interactions of high Na^+ concentration (0.14 *M* NaCl), because the bonds holding the enzyme in place were broken. The high salt concentration may also play an osmotic role, and thus may aid in preventing cell lysis. Specific activity of the cell free enzyme prepared by either method was similar. However, activity did vary from extract to extract as no two batches of cells tended to respond to treatment in exactly the same manner and therefore the amount of alkaline phosphatase varied. In order to compensate for low specific activity the assays of cell free enzyme were continued for one hour at 39 C rather than the fifteen minute room temperature (25 C) assay used in studies of cell bound activity.

Costerton (1973) working with pseudomonad B-16 was able to easily remove the cell wall of this gram negative bacterium layer by layer. Initially treating the cells with 0.5 *M* NaCl followed by 0.5 *M* sucrose, he was able to remove the outer double track layer thus releasing the alkaline phosphatase from both the outer and inner surfaces of this layer. In other reports dealing with removal of wall layers from *E. coli* (Schnaitman, 1971) and *P. aeruginosa* (Ingram *et al.*, 1973) release of lipopolysaccharide seemed to be associated closely with release of alkaline phosphatase. *In vitro*

studies by Day and Ingram (1975) suggested that binding sites for each type of wall-associated enzyme may be composed of lipopolysaccharide molecules. Thus, it seems quite likely that the alkaline phosphatase of P. boryanum will probably be found to have a very specific attachment site of this type within the wall.

Inhibitor Studies

A classical definition of an inducible enzyme is as follows: If there is either a low amount or no enzyme activity present, and a change in environmental factors (e.g. a substrate is introduced) causes the appearance or increase of activity, the system is considered to be inducible (Jacob and Monod, 1961). Therefore, if a particular enzyme system such as the alkaline phosphatase of P. boryanum is to be considered inducible, it must be shown that de novo synthesis of the protein occurs when proper environmental factors favoring enzyme appearance are present. Thus, it was feasible to place the cells of P. boryanum under starvation conditions in the presence of inhibitors acting at the level of the genome. Table 3 lists the inhibitors and their effects on alkaline phosphatase activity in P. boryanum. All of the inhibitors tested were found to cause a decrease of enzyme activity ranging from 30% to 92% of control level. Certain of these well known antibiotics are large molecules, unable to penetrate the barriers of the cell wall and plasma membrane easily (Leive, 1974). Therefore, the cells were pretreated with

10^{-3}M EDTA which interferes with the barrier function of the wall through release of lipopolysaccharide, and of the plasma membrane by causing permeability changes. The inhibitors added following EDTA pretreatment proved to be more effective. Both actinomycin D, which impedes transcription and thus permits no new m-RNA to be made, and puromycin, which stops synthesis of protein at the ribosome, caused substantial inhibition of alkaline phosphatase in the cultures tested. Similar results are reported for *E. coli* K12 (Malamy and Horecker, 1961) and *Micrococcus sodenensis* (Glew and Heath, 1971). On the basis of this data and changes observed in alkaline phosphatase levels after days of starvation conditions, it seems that the alkaline phosphatase of *P. boryanum* is an inducible enzyme.

Studies which make use of mutants of *E. coli* have been carried out which identify the portions of the genome responsible for alkaline phosphatase production (Echols *et al.*, 1961; Garen and Echols, 1962; Willsky *et al*; 1973). Willsky *et al*. (1973) suggest an overall scheme for regulation of alkaline phosphatase in *E. coli*. One of the elements of control would require a protein called R1 for induction which is similar to the C protein required for transcription of the arabinose operon in *E. coli* (Englesberg *et al*; 1965). There is as yet no data of this type concerning cyanobacteria. All evidence indicative of inducible systems in *P. boryanum* and other blue-greens is at present indirect.

With respect to various properties studied, the

alkaline phosphatase of *P. boryanum* seems to resemble the enzyme of other microorganisms in some properties. The cell bound *P. boryanum* enzyme is not sensitive to sodium fluoride, as seen by Torriani (1960) in *E. coli*, although a small amount of inhibition was noted with cell free enzyme. It was of interest to observe an enhancement of activity in the presence of potassium cyanide. According to Mathies (1958) activation of alkaline phosphatase by a compound such as KCN seems to be an indirect effect. This effect is due to the ability of certain compounds to bind metal ions. If these ions are toxic, their removal would result in the enhancement of enzyme activity.

Mercury, a heavy metal, causes total inactivation of cell bound activity. Sicko (1974) reported an inhibition of orthophosphate uptake following treatment of starved *P. boryanum* with $1x10^{-2}$*M* mercuric chloride. Nonspecific protein precipitation causing changes in cell wall proteins could possibly prevent uptake of substrate, or precipitation of portions of the alkaline phosphatase enzyme itself could result in total inactivation.

Another property common to all of the alkaline phosphatase systems considered to be inducible is inhibition by inorganic phosphate (Horiuchi *et al*., 1959; Torriani, 1960; Kuenzler and Perras, 1965; Fitzgerald and Nelson, 1966; Glew and Heath, 1971; Sakaguchi *et al*., 1972; Healey, 1973; Ingram *et al*., 1973; Lopes and Deltour, 1975; Ihlenfeld and Gibson, 1975). The alkaline phosphatase of *P. boryanum* is no

exception. Increasing concentrations of orthophosphate result in decreasing enzyme activity. At the highest concentration tested (20 mM) 32% of the cell free and 43% of cell bound activity remained. Torriani (1960) reported nearly complete inhibition at 100 mM phosphate and approximately 30% activity remaining at a concentration of 10 mM phosphate which is compatible with results in P. boryanum. When cells are placed in a medium containing an excess of orthophosphate, it is possible that the enzyme, found capable of functioning as a phosphotransferase in E. coli (Anderson and Nordlie, 1967), could discontinue acting as a hydrolase and switch over to acting as a transferase. It is likely that further synthesis of the enzyme would not occur. The enzyme itself, however, would still be present as found by Healey (1973) after addition of excess inorganic phosphate to a phosphate deficient culture of Anabaena variabilis. The enzyme simply could have changed its major function for a time.

Temperature Effects, pH Optimum, Substrates Hydrolyzed

The alkaline phosphatase of P. boryanum is a fairly stable enzyme. The extracted enzyme appears to have a much greater degree of stability than the cell bound enzyme as it continues to exhibit activity above control levels when heated at 80 C for 10 minutes. Torriani (1960) who used toluenized suspensions of E. coli to test for alkaline phosphatase stability seems to have been the only one who reported a greater degree of thermal stability in a bacterium

than that reported here. Heating of the _E. coli_ enzyme for 5 min at 100 C caused 50% inactivation. Fifty four percent of cell bound _P. boryanum_ alkaline phosphatase activity remained after 10 min at 70 C, which is similar to the finding by Ihlenfeld and Gibson (1975) of a 50% loss of activity when _A. nidulans_ cells were incubated at 70 C for 11 min. The cell bound enzyme from _Vibrio parahaemolyticus_ (Sakaguchi _et al._, 1972) was reported to have lost 50% of its activity when kept at 55 C for 10 min and the cell free enzyme lost the same amount of activity at 50 C after 10 min.

The pH optimum of the cell bound and cell free alkaline phosphatase of _P. boryanum_ (pH 8.2-9.2) fit well into the range observed in other microorangisms. The range for _A. nidulans_ is pH 8-9 (Ihlenfeld and Gibson, 1975), for _A. variabilis_ pH of about 8.3, for _Mycobacterium smegmatis_ a pH of about 7.8 (David, 1977) and _P. aeruginosa_ a pH of 8-10.5 (Day and Ingram, 1973).

Alkaline phosphatases from a variety of sources have the ability to hydrolyze a large number of phosphate esters (Torriani, 1960; Garen and Levinthal, 1960; Selitrennikoff and Sonneborn, 1977; Ihlenfeld and Gibson, 1975). Table 5 describes the relative rates of hydrolysis of 15 substrates by the cell free P. boryanum enzyme. With the exception of fructose-6-phosphate, generally the rates of hydrolysis were substantially different from those observed by Garen and Levinthal (1960) in _E. coli_ and by Selitrennikoff and Sonneborn (1977) in _B. emersonii_. As Ihlenfeld and Gibson

observed, the substrates hydrolyzed most readily by A. nidulans alkaline phosphatase differ from those hydrolyzed by the E. coli enzyme and P. boryanum differs in this respect from both E. coli and A. nidulans. It is interesting to note that in spite of other differences, most alkaline phosphatases readily hydrolyze pNPP. The preferred in vivo substrates have not been identified other than as probable monoesters.

As with many enzymes, ions appear to play an important role in the functioning of P. boryanum alkaline phosphatase. Wherever possible comparison is made between the enzyme responses of P. boryanum and other microorganisms. However, most reports do not show ion effects on enzyme activity over a range of concentrations as has been done in this study. Therefore, in this study it is possible to report a pattern of effects. Different concentrations of an ion can have effects that are virtually opposite, one concentration having an inhibitory effect and a higher or lower concentration causing stimulation. The cation found to have the greatest stimulatory effect on the alkaline phosphatase activity of P. boryanum is calcium. This particular ion is known to be of key importance in the regulation of living systems. The calcium ion is an essential part of a myosine-adenosine triphosphatase required for skeletal muscle contraction (Davies, 1963), and an ATP-splitting enzyme in hard tissue forming cells seems also to be dependent on Ca^{++} (Linde and Magnussen, 1975) for activity. Mandel

and Higa (1970) observed an increase in the uptake of phage DNA by host bacteria in the presence of calcium ions. In a study of two genera of jellyfish, *Aequorea* and *Halistaurea*, Shimomura *et al*. (1963) demonstrated a bioluminescence mechanism dependent on a specific protein which emits light on exposure to calcium. Glew and Heath (1971) reported the alkaline phosphatase of *M. sodenensis* to require Ca^{++}. These authors determined that there are 8 gram atoms Ca^{++}/mole of enzyme. These firmly bound calcium ions are required for activity and to stabilize the enzyme. Of the cyanobacteria studied, Healey (1973) reported a dependence of the enzyme activity of *A. variabilis* on the presence of Ca^{++}. It is not known how calcium stimulates the enzyme. Perhaps it will prove to be an integral part of the enzyme, or it may simply increase the accessibility of substrate. It may also play a role in stabilizing the enzyme and in this way increase activity.

Magnesium is another ion found to be of importance to certain of the alkaline phosphatase enzymes. This cation had a low stimulatory effect on both the cell bound and cell free enzymes of *P. boryanum* and therefore suggests that it plays a less important role in this organism than in others reported. The *E. coli* enzyme (Schlesinger *et al*., 1968) and *P. aeruginosa* enzyme (Day and Ingram, 1973) both show increased activity in the presence of magnesium salts. Thompson and MacLeod (1974) in pseudomonad B-16 suggest that magnesium both stabilizes and activates alkaline phosphatase,

as the addition of 50 m*M* magnesium doubled activity. In the gram positive bacterium *Micrococcus sodenensis*, Glew and Heath (1971) saw a possible role for magnesium in the synthesis and/or release of the enzyme (most of the alkaline phosphatase of this organism is secreted into the medium).

P. boryanum cell bound alkaline phosphatase is greatly inhibited by zinc at any concentration. This effect is not noticeable with cell free enzyme until a concentration of 2 m*M* zinc is present prior to assay. A similar strong inhibition of this type was found by Ihlenfeld and Gibson (1975) in *A. nidulans*. These are the only blue-green bacteria which have been tested for the effect of zinc on alkaline phosphatase activity. It would be interesting to see if zinc is similarly inhibitory to the alkaline phosphatases of a majority of cyanobacteria. This response to zinc was unexpected as both the purified *E. coli* enzyme (Schlesinger *et al*., 1968) and the *P. aeruginosa* enzyme (Day and Ingram, 1973) contained zinc as an integral part of the protein necessary for activity (see literature review). It has also been reported that Zn^{++} increases the activity of the enzyme in *Vibro parahaemolyticus* (Sakaguchi *et al*., 1972). Walther and Fries (1976) demonstrated Zn^{++} activation of the extracellular alkaline phosphatase in a multicellular marine alga suggesting it to be a zinc dependent enzyme.

Ihlenfeld and Gibson (1975) observed a stimulation of alkaline phosphatase by Mn^{++} and Co^{++}. In *P. boryanum* both ions inhibited the cell bound enzyme at all concentra-

tions. In contrast to this response, the cell free enzyme activity, in the presence of intermediate concentrations of ion (0.2 mM and 2 mM) was stimulated up to 50%, thus being in agreement with observations of A. nidulans (Ihlenfeld and Gibson, 1975). A higher ionic concentration (20 mM) caused a decrease of activity to approximately 40% of control levels. It therefore seems that lower concentrations are more ideal for activity while higher concentrations may interact in some way with the enzyme itself or some component of the extract. Sakaguchi et al., (1972) found both Mn^{++} and Co^{++} to stimulate the activity of V. parahaemolyticus alkaline phosphatase.

A yeast enzyme (Stadtman, 1959) was reported to be dependent on Fe^{++} which was found to inhibit the P. boryanum cell bound and cell free enzyme to approximately the same degree as Zn^{++} inhibition of cell free enzyme. When Na^{+} and K^{+} were tested with both cell bound and cell free enzyme, K^{+} was found to have a slight stimulatory effect on cell bound enzyme. In a marine pseudomonad studied by Unemoto and MacLeod (1975), high concentrations of K^{+} appeared to cause an increase in the penetrability of the outer membrane of this organism. If this were an effect of K^{+} on the outer membrane of P. boryanum, it would be possible for substrate to get to enzyme more easily and result in the observed enhancement of activity.

In the functioning of alkaline phosphatase, an intermediate step involves phosphorylation of a serine in E. coli (Milstein, 1963) and calf intestine (Engstrom, 1964). Gabel

and Thomas, (1976), in a study of inorganic polyphosphates as integral parts of alkaline phosphatases from chicken intestine, suggested that the breakdown of this intermediate phosphorylated serine is enhanced via K^+. Another possible effect of K^+ may be in relation to transport. Gerdes *et al*. (1977) reported a stimulation of inorganic phosphate uptake in *E. coli* in the presence of K^+.

The effect of molybdenum on the activity of *P. boryanum* alkaline phosphatase is somewhat unusual. As seen in Fig 23, molybdate causes inhibition of the cell bound enzyme in *P. boryanum* until the highest concentration is reached. However, the cell free enzyme, which is inhibited by concentrations of molybdate up to 2 m*M*, shows a higher specific activity than control values (0 m*M* molybdate) in the presence of 20 m*M* molybdate. Healey (1973) found Mo^{++} to be inhibitory to the blue-green *A. variabilis* enzyme when assayed with 6 m*M* molybdate. In the presence of 1 m*M* molybdate, *P. boryanum* cell free alkaline phosphatase was inhibited more than 90%. A study of the purified *E. coli* enzyme by Anderson and Vallee (1975) in which various ions were substituted for Zn^{++}, found only Mo^{++} capable of producing a catalytically active enzyme. In the present work, the action of a high concentration is obscure. Perhaps due to a lack of purification, an excess of molybdate may interact in some way with a toxic substance in the preparation and by its removal cause enhancement of activity. Mathies (1958) reported interactions of this type as

discussed earlier in the section on inhibitors.

Kinetics

The alkaline phosphatase activity of *P. boryanum* can be described on the basis of Michaelis-Menten kinetics. Thus, it is possible through use of a Lineweaver-Burk plot to make a direct comparison with the enzyme in other microorganisms. The Km for pNPP is 1.6×10^{-3} *M* for cell free enzyme and 3.9×10^{-3} *M* for cell bound enzyme. These values are toward the upper end of the range for bacteria and algae. Day and Ingram (1973) report a Km of 6.6×10^{-5} *M* for *P. aeruginosa*, 1.2×10^{-5} *M* is reported for the *E. coli* enzyme (Garen and Levinthal, 1960), 3.6×10^{-5} *M* for *B. subtilis* (Thompson and MacLeod, 1974), 7×10^{-4} *M* for *A. variabilis* (Healey, 1973) and 1.16×10^{-4} *M* for *S. marcesens* (Bhatti, 1974). A Lineweaver-Burk plot of enzyme activity in the presence of orthophosphate (2 m*M*) indicated competitive inhibition as found in other derepressible systems studied. The Ki of inhibited enzyme, 4.5×10^{-4} *M*, was compatible with that reported by Bhatti (1974) for the alkaline phosphatase of *S. marcesens*. In *P. boryanum* it appears that the enzyme has a greater affinity for orthophosphate than it has for pNPP.

Cytochemistry of Alkaline Phosphatase in Cyanobacteria

Phosphate starved cells of *Plectonema boryanum* produce a maximum amount of alkaline phosphatase in five days. Studies indicating that the alkaline phosphatases of procaryotic organisms are extracellular enzymes have been

described (see literature review). With the use of histochemical techniques and the electron microscope localization of alkaline phosphatase activity in two filamentous blue-greens, *P. boryanum* and *A. cylindrica*, and in a unicellular coccoid cyanobacterium, *Coccochloris peniocytis*, the localization is clearly shown to be in layer 3 of the periplasmic space.

The modifications of Costerton's (1973) technique developed in this study are well suited for alkaline phosphatase localization in cyanobacteria. It is a two-step procedure, using unfixed cells reacted for a relatively short time in the presence of calcium as the initial capture reagent. Calcium is also used as the stimulating ion, rather than magnesium as used in the Costerton procedure, due to its enhancement of enzyme activity in *P. boryanum* (Fig 17). Substrate is a combination of β glycerophosphate and pNPP rather than β glycerophosphate alone, and the concentration of Tris buffer and barbital in the reaction mixture have been decreased (the former from 0.1 *M* to 0.05 *M* and the latter from 0.024 *M* to 0.005 *M*). The changes in particular of stimulating ion and in substrate makeup gave far better results than had been obtained in previous attempts at localization. Osmium tetroxide, as prepared for the normal fixation of procaryotic organisms (Pankratz and Bowen, 1963), proved to be most satisfactory as a sole fixative. Occasionally, glutaraldehyde fixation followed by osmium postfixation as used by Costerton (1973), had the disadvantage of producing

various intracellular membranous alterations shown when glutaraldehyde cofixation was employed. Very little difference was noted in sectioned material whether post-staining consisted of uranyl acetate and lead citrate or uranyl acetate alone. The effects of these treatments on cell morphology was determined by comparing preparations containing substrate and controls from which substrate was missing with untreated cells from previous work. Very few morphological differences were noted as a result of these treatments.

The problems inherent in any cytochemical technique have been reviewed at length (Hayat, 1973). Extreme care is required to avoid artifacts. A balance must be sought in order to optimize both enzyme activity and ultrastructure. This is rendered even more difficult by the large number of chemical and osmotic changes through which the cells must pass from beginning to end of the procedure. A common cytochemical technique involves the use of aldehyde pre-fixatives which chemically become part of the proteins they stabilize. Glutaraldehyde is generally known for its excellent preservation of ultrastructure, but unfortunately it is also known to inhibit enzyme activity (Barnett _et al._, 1964; Widnell, 1972). Fig 12 shows the degree to which the alkaline phosphatase of _P. boryanum_ is inhibited by glutaraldehyde. The use of 2.5% glutaraldehyde in 0.05 _M_ Tris buffer reduced activity by 90% in 30 min. A minimum fixation time in glutaraldehyde for the preservation of

P. boryanum fine structure would be about one hour. In a recent paper, McNicholas and Hulett (1977) demonstrated the loss of 68% of alkaline phosphatase activity in Bacillis licheniformis caused by use of glutaraldehyde as a pre-reaction fixative. Another aldehyde used in place of glutaraldehyde is paraformaldehyde which has the advantage of being less inhibitory to enzyme activity. Unfortunately, the preservation of ultrastructure is not as good as with glutaraldehyde. It is also likely that possible product diffusion will be more of a problem as prefixed cells require a much greater length of time in the presence of substrate for enzyme activity to take place (i.e. 15-20 min as compared with 1-1½ h). Although P. boryanum ultrastructure was satisfactorily preserved following prefixation in glutaraldehyde or paraformaldehyde, no successful enzyme localization was achieved. When prefixation with glutaraldehyde took place in the presence of substrate, a small percentage of the cells (less than 1%) showed evidence of alkaline phosphatase activity (Figs 36-37) but not with any degree of specificity. These results were most unsatisfactory as compared with unfixed cells prepared by the modification described previously. The deposition was found outside the cell wall (L4) rather than within the wall.

During the course of this study an attempt was made to use a one-step histochemical procedure with lead as the initial capture reagent. This was not successful, as the concentration of lead required, although low (3 mM), did not

remain in solution. Upon coming into contact with the other reagents, a milky precipitate was formed. The problems encountered in keeping the lead in solution have been reported by Hugon (1970). Another undesirable aspect of lead as an initial capture reagant is its inhibitory effect on enzyme activity (Moses and Rosenthal, 1968; McNicholas and Hulett, 1977). Ghosh et al. (1971) found 50-80% inhibition of alkaline phosphatase activity in B. subtilis in the presence of lead salts. Thus, this modified procedure was developed in order to avoid as many of these pitfalls as possible. Coccochloris peniocytis differs from P. boryanum and A. cylindrica not only taxonomically, but also because it appears to be constitutive for the enzyme, and secretes large amounts of enzyme into the surrounding medium. In C. peniocytis the major amount of cell bound activity seems to be located in the periplasmic space. However, probably due to the normal secretion of alkaline phosphatase into the medium, a large number of cells have an encrustation of the precipitate indicative of secreted enzymes beyond the outer portion of L4 (Fig 52). As Costerton (1973) has noted, if a Pb salt is formed by cell free enzyme, the reaction product will form crystals on the closest available surface. This surface may be that of the cell or of cell debris. The first aggregates of reaction product act as "seed crystals" for subsequent deposition.

Filaments of P. boryanum and A. cylindrica are normally surrounded by abundant sheath which might act as a primary

or secondary location for alkaline phosphatase activity. Since P. boryanum loses its sheath rather easily through washing, only a small percentage of cells (1-2%) retain any sheath at the completion of the appropriate cytochemical procedures. There seems to be no noticeable difference in the appearance of the sheath of control cells and experimental cells in P. boryanum. This is in agreement with data (Fig 2) which shows no activity in the supernatants following centrifugation, or in the culture medium.

The sheath of A. cylindrica is usually retained by a majority of the cells following cytochemical reactions. Although only a small amount of activity is found in the supernatants following centrifugation or in the culture medium, many of these experimental cells have a dense precipitate within the sheath (Figs 45-46) which suggests that the sheath in this cyanobacterium may be a secondary location of activity. Any enzyme secreted by the cells must pass through the sheath. Precipitate due to activity here acts as "seed crystals" for further deposition. The cells of C. peniocytis also normally have a small amount of sheath material. It is not readily seen in either control or experimental cells and there is no evidence that it plays any role as a secondary location for alkaline phosphatase activity in this organism.

The activity observed in cross walls of a few A. cylindrica cells was unexpected, as cross walls of filamentous blue-green bacteria do not contain all of the layers found

in end walls. One finds only layers 1 and 2 in duplicate in cross walls. However, it is possible that some enzyme subunits enter the cross wall region of cells which are beginning the process of separation, and therefore have some amount of material (such as lipopolysaccharide) in position which will form layers 3 and 4. As discussed earlier, the lipopolysaccharide of these layers is suggested as the attachment site for alkaline phosphatase (Cheng *et al.*, 1972)

In the present study the observation that some cells have no activity associated with the wall was at first thought to be related to the presence or absence of sheath material in the filamentous blue-greens. However, no correlation was observed and it suggested that some cells are not induced to produce alkaline phosphatase or only produce low levels under the conditions of this study and thus exhibit no activity (Fig 38).

There remains much work to be done on adaptive enzyme systems in Cyanobacteria. This is the initial study of localization of an inducible enzyme in these cells. A number of other enzymes reported to be extracellular in certain gram negative bacteria, such as ribonuclease I, 3' nucleotidase, 5'nucleotidase and acid phosphatase have yet to be observed *in situ* in blue-greens. It would also be of interest to further study the wall makeup in order to determine how these enzymes are positioned in the wall, and to investigate if there are differences between blue-greens

which secrete enzyme into the medium and those which do not.

The extremely diversified effects of ions on the activity of alkaline phosphatase might be clarified if the enzyme were purified. It would then be possible to determine such further parameters as molecular weight and if the major stimulating ion, calcium, were an integral part of the enzyme. The presence of alkaline phosphatase in microorganisms permits usage of organic substrates when preferred orthophosphate is not available. Perhaps a further role such as an effect on some other cellular process may be found to require the participation of alkaline phosphatase. Bhatti *et al*. (1976) have reported the enzyme in *P. aeruginosa* to be of importance in the final stages of cell division under phosphate limited conditions. Further investigation of the phosphatases of many isolates of blue-greens as begun in this study may elucidate other roles for the enzyme and provide information as to the way in which proteins such as alkaline phosphatase are put into functional form in procaryotic cells.

SUMMARY

The alkaline phosphatase of *Plectonema boryanum* and other cyanobacteria was studied. It was found that phosphate starvation results in a substantial increase of alkaline phosphatase activity in *P. boryanum* and certain of the other cyanobacteria tested. All blue-green isolates tested were alkaline phosphatase positive.

Growth of cells in the presence of inhibitors of transcription and translation indicated that enzyme synthesis is "*de novo*". Enzyme was extracted from the cells using lysozyme or polymyxin B making a comparison of the cell bound and cell free form possible. The optimum pH was found to be in the range 8.2 - 9.2 for both forms and, of the ions tested, calcium enhanced activity to the greatest degree. The enzyme appears to be extremely stable, as the cell free form exhibited activity above control levels after heating at 80 C for 10 min.

Sodium fluoride inhibited the cell bound alkaline phosphatase not at all and inhibited cell free enzyme only very slightly. Orthophosphate, the end product of the phosphatase reaction is a potent inhibitor of activity as 20 m*M* orthophosphate caused a decrease in cell free activity to 32% of the control value. Para nitrophenol phosphate was the preferred substrate although the *P. boryanum* enzyme did hydrolyze a number of other phosphomonesters. The Km for cell free enzyme, 1×10^{-3}*M*, and cell bound enzyme,

3.9×10^{-3} $\underline{M}$ were determined through use of a Lineweaver-Burk plot. Orthophosphate was shown to be a competitive inhibitor with a Ki of 4.5×10^{-4} $\underline{M}$.

Cytochemistry at the ultrastructural level has clearly established the periplasmic space as the site of enzyme activity in P. boryanum. Alkaline phosphatase activity is found only in the cellular fraction, none is found in the culture medium. As glutaraldehyde was found to inhibit P. boryanum enzyme activity substantially, a modification of the method of Costerton was used to investigate localization. Unfixed cells were reacted with calcium nitrate, which acted as the initial capture reagant. After this deposition, the cells were suspended in 2% lead nitrate to convert the calcium phosphate to more electron dense lead phosphate. The majority of activity appears associated with layer 3 of the cell wall.

Similar histochemical techniques were applied to Coccochloris peniocytis and Anabaena cylindrica. The cells of C. peniocytis secrete large amounts of enzyme into the surrounding medium and seem to produce alkaline phosphatase constitutively. Cell bound activity in this organism was localized within the periplasmic space (layer 3). A. cylindrica showed enzyme activity to be present in layer 3 of the periplasmic space, with the sheath apparently playing a role as a secondary location for activity.

APPENDICES

Appendix A

Compostion of Culture Medium - Modified Fitzgerald (Fitzgerald *et al.*, 1952). pH 7.3

Concentration is expressed in mg/liter

$NaNo_3$	- 124
$K_2HPO_4 \cdot 3H_2O$	- 13
$MgSO_4 \cdot 7H_2O$	- 25
$CaCl_2 \cdot 2H_2O$	- 36
Na_2CO_3	- 20
$Na_2SiO_3 \cdot 9H_2O$	- 58
Ferric Citrate	- 3
Citric Acid	- 3
Gaffron's minor element solution	- 0.04ml

Gaffron's solution. (in g/liter)

H_3BO_3	- 3.10
$MnSO_4 \cdot 4H_2O$	- 2.23
$ZnSO_4 \cdot 7H_2O$	- 0.287
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	- 0.088
$CuSO_4 \cdot 5H_2O$	- 0.125
$Co(No_3)_2 \cdot 6H_2O$	- 0.146
$Al_2(SO_4)_3 K_2SO_4 \cdot 24H_2O$	- 0.474
$NiSO_4 (NH_4)_2 SO_4 \cdot 61H_2O$	- 0.198
$Cd(NO_3)_2 \cdot 4H_2O$	- 0.154

$Cr\ (NO_3)_2 \cdot 7H_20$	-	0.037
$V_2o_4\ (SO_4)_3 \cdot 16H_20$	-	0.035
$Na_2WO_4 \cdot 2H_20$	-	0.033
KBr	-	0.119
KI	-	0.083

Appendix B

Composition of Culture Medium - Bold's Mineral Medium (Deason and Bold, 1960) pH 7.

A. Six stock solutions, each one 400 ml in volume:

$NaNo_3$	-	10.0 g.
$CaCl_2$	-	1.0 g.
$MgSO_4 \cdot 7H_20$	-	3.0 g.
K_2HPO_4	-	3.0 g.
KH_2PO_4	-	7.0 g.
NaCl	-	1.0 g.

10 ml of each above are added to 936 ml distilled water.

B. Four stock, trace - element solutions are prepared:

1.	H_3BO_3	-	11.42 g/l
2.	$FeSO_4 \cdot 7H_20$	-	4.98 g/l
	$ZnS0_4 \cdot 7H_20$	-	8.82 g/l
	$MnCl_2 \cdot 4H_20$	-	1.44 g/l
3.	$Mo\ 0_3$	-	0.71 g/l
	$CuS0_4 \cdot 5H_20$	-	1.57 g/l
	$Co(NO_3)_2 \cdot 6H_20$	-	0.49 g/l
4.	EDTA	-	50.00 g/l
	KOH	-	31.00 g/l

1.0 ml of each trace - element solution is added to the above Part B solution

Appendix C

Reagents for Murphy - Riley Orthophosphate Determination (Murphy and Riley, 1962)

1. Sulfuric acid (5N). Dilute 70 ml of concentrated reagent grade sulfuric acid to 500 ml.
2. Ammonium molybdate. Dissolve 20 grams of reagent grade ammonium molybdate in water and dilute to 500 ml. Store the solution in a Pyrex glass bottle.
3. Ascorbic acid (0.1M). Dissolve 1.32 grams of ascorbic acid in 75 ml of distilled water. This solution is made on the day it is required.
4. Potassium antimonyl tartrate (1mg Sb/ml). Dissolve 0.2743 grams of potassium antimonyl tartrate in distilled water and dilute to a final volume of 100 ml.
5. Mixed reagent. Mix thoroughly 125 ml of 5N sulfuric acid and 37.5 ml of ammonium molybdate. Add 75 ml of ascorbic acid solution and 12.5 ml of potassium antimonyl tartrate solution. This reagent keeps for 24 hours.

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