STUDIES ON FLUORIDE-INDUCED FILAMENTS OF *ERWINIA CAROTOVORA*

By

BERHANU ABEGAZ

Bachelor of Science in Agriculture
National University
Dire Dawa, Ethiopia
1968

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1970

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1977

Thesis
1977D
A 138s
Cop. 2

OKLAHOMA STATE
UNIVERSITY
LIBRARY

STUDIES ON FLUORIDE-INDUCED FILAMENTS

OF *ERWINIA CAROTOVORA*

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

1003700

ACKNOWLEDGMENTS

I am deeply grateful to Dr. E. A. Grula, my academic adviser, without whose help and guidance my education and training here would have been fruitless. Special appreciation is also extended to Dr. M. Grula for her interest in my study and research.

I am also grateful to my committee members, Dr. L. L. Gee, Dr. R. E. Corstvet and Dr. N. N. Durham for their advice and encouragement in my studies.

In addition, I would like to thank Mrs. Sharon Dunn for typing excellence and advice.

Finally, I would like to express appreciation to my wife, Tezeta, and my son, Daniel, whose understanding, encouragement, patience and sacrifice were instrumental to the completion of this study.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	METHODS AND MATERIALS	22
	Keto Acids	24
	Mucopeptide Isolation and Chromatography	26
	Quantitative Determination of Glucose	27
	Quantitative Determination of Aspartic Acid	28
	Release of CO_2 From U-^{14}C-L-Aspartic Acid	28
	Determination of Total Cellular Phosphorus	29
	Determination of Phosphate Distribution in Cells	30
	Determination of Phosphomonoesterase Activity	32
III.	RESULTS	35
	Studies on Filament Formation	35
	Magnesium Sulfate	42
	Sodium Fluoride	42
	Growth Medium pH	45
	Aspartic Acid	45
	Distribution of Carbon From U-^{14}C-L-Aspartic Acid	51
	Effect of TCA Cycle Intermediates on Filament Formation	52
	Phosphate	58
	Study on Phosphatases (Phosphomonoesterases)	67
	Effect of Membrane-Perturbing Agents	74
	Inhibitors of Filament Formation	77

Chapter Page

Studies on Reversion 80

Effect of Magnesium Chloride. 81
Effect of Magnesium Chloride and Potassium Phosphate 81
Effect of Aspartic Acid 83
Effect of pH. 85
Effect of Phosphorylation Inhibitors. 87
Effect of Mersalyl Acid (Mercurials). 89
Effect of Compounds Which Affect the Cell Membrane 89
Effect of Aerobiosis and Anaerobiosis. 89
Effect of Chloramphenicol (CAP) 90
Effect of Ethylenediaminetetraacetic Acid (EDTA) 91
Effects of Agents Affecting Microtubules and Microfilaments 91
Effect of Cyclic Adenosine Monophosphate (cAMP). 93
Effect of Heavy and Light Inocula 94
Effect of Repeated Washing of Filaments 97
Effects of Pantoyl Laconte (PL) 97

IV. DISCUSSION. 99

V. SUMMARY AND CONCLUSIONS 108

BIBLIOGRAPHY 111

LIST OF TABLES

Table		Page
I.	Percentage Cell Length Distribution (μm) After Growth in GA Medium With and Without NaF.	39
II.	Amino Acid Substituents and Their Effects on Filament Formation.	41
III.	Labelling Patterns of _Erwinia carotovora_ After 17 Hours of Growth in the GA and GAF Media.	53
IV.	The Influence of Inhibitors of Filament Formation on Keto Acid Excretion and CO_2 Evolution.	55
V.	Hydrazone-Forming Compounds Present in Spent Media (GA or GAF).	56
VI.	The Influence of Inhibitors of Filament Formation on the Total Phosphorus Content of _E. carotovora_ When Grown in GA and GAF Media.	62
VII.	Reversion of Filaments Under Various Conditions	82
VIII.	Mass Increase of Filaments Under Various Conditions of Reversion.	84
IX.	Effect of Medium pH on Reversion	86
X.	Mass Increase of Filamentous Cells Inoculated in the Presence of Inhibitors of Substrate or Oxidative Phosphorylation	88
XI.	Mass Increase of Filaments Treated With Anti-Microtubular Agents in Various Reversion Media.	92
XII.	Effect of Theophylline, Caffeine and Dibutryl cAMP on Growth of Reverting Filaments.	95

LIST OF FIGURES

Figure		Page
1.	Growth of Cells in Glucose-Aspartic Medium With and Without NaF.	38
2.	Effect of Various Concentrations of Sodium Fluoride (NaF) in the GA Medium on Filament Formation.	44
3.	Effect of Various Initial pH Values of the GAF Medium on Filament Formation.	47
4.	Effect of Various Concentrations of Aspartic Acid in the GAF Medium on Filament Formation. .	49
5.	Effect of Various Concentrations of Phosphate in the GAF Medium on Filament Formation	60
6.	Chromatographic Separation of Phosphorylated Compounds	66
7.	Activity of Phosphatases in Normal Cells and Filaments at pH 6.8 Determined Using p-Nitrophenyl Phosphate (p-NPP) and the Cells Themselves as Sources of Phosphate Ester Substrates.	70
8.	Effect of pH on Phosphomonoesterase Activities of Normal and Filamentous Cells	72

CHAPTER I

INTRODUCTION

To elucidate the mechanism(s) of cell division various investigators are studying the possible involvement of the major macromolecules and structures within the cell (Marunouchi and Messer, 1973; Suzuki, 1974; Helmstetter, 1974a,b).

Grula (1960a,b) and Grula and Grula (1962a,b; 1964) have studied cell division in this laboratory by first producing filamentous cells using compounds that are capable of inhibiting cell division and then studying the reversion of such cells. Since there is an exaggerated difference in size between filamentous and non-filamentous cells, it is possible that the compound(s) responsible for initiating the cell division process is present or absent in quantities that are sufficient to be detected using conventional techniques.

Various types of compounds such as the D-isomers of serine, methionine, phenylalanine, tryptophan, threonine and histidine, antibiotics such as penicillin, vancomycin, D-cycloserine, mitomycin C, ultraviolet light and S-dichlorovinyl-L-cysteine have been found to inhibit growth and cell division in *Erwinia carotovora* (Grula, 1960b; Grula and Grula, 1962a,b, 1964). By using these cell division

inhibitors substantial information was obtained by Grula and Grula (1964) which implicates the cell wall mucopeptide as an important macromolecule in cell division. Filamentous growth of E. carotovora induced by D-serine could be prevented by incorporating p-aminobenzoic acid, D- or L-alanine, ammonium salts, calcium, zinc, manganese or altering the pH of the growth medium (Grula and Grula, 1962a,b). Pantoyl lactone, a potent stimulator of cell division, also induces reversion of filamentous cells at an accelerated rate to the normal (short) size.

Because pantoyl lactone does not restore mucopeptide content to normal values, the hypothesis was advanced that secondary damage to the cell membrane is responsible for inhibition of cell division (Grula and Grula, 1964). A decrease in the levels of keto acids, proteins and nucleic acids in spent media where filamentous growth had been prevented by osmotic protectors also implicated the cell membrane. In addition to chemical data, electron microscopic studies by Grula and Smith (1965) showed that both the cell membrane and the cell wall invaginate together at the time of division with the cell membrane initiating and sustaining the process. It has also been shown that filamentous cells of *E. carotovora* contain normal levels of DNA and RNA (Grula, Smith, Parham and Grula, 1968); however, the nuclear body of filamentous cells also fails to undergo separation (Grula and Smith, 1965).

The requirement of a rigid mucopeptide or solid media for protoplast reversion and division has been reported (Bazill, 1967; Landman, Ryter and Frehel, 1968). Inouye and Pardee (1970) and Shapiro, Siccardi, Hirota and Jacob (1970) have implicated the cell membrane in chromosome replication. Helmstetter (1974a,b) has suggested that the cell envelope controls the timing of chromosome replication because the DNA is attached to the cell membrane.

Recently it was observed that sodium fluoride (NaF) is a potent inhibitor of cell division in E. carotovora (M. Grula, unpublished data). Even though a voluminous literature has accumulated relating to the activity of NaF on enzymes such as the phosphatases (Belfanti, Contardi and Ercoli, 1935), enolase (Taitzer and Himoe, 1974) pyruvate dehydrogenase, succinate dehydrogenase (Terner, 1955a,b) and adenylate cyclase (Rahmaman and Jarett, 1974; Neer, 1976), no information has been published suggesting how free fluoride ions might inhibit the cell division process. Therefore one phase of my research has focused on trying to find a structure or metabolic difference between normal and NaF-induced non-dividing (filamentous) cells of E. carotovota.

As mentioned above, cell division in this organism is a constrictive process. Hence, this process may involve a structure which is capable of exerting a physical force to pull in the cell envelope (purse string hypothesis). This physical force could be equated to a microtubule-microfilament type assembly present in eukaryotic cells. A

search for this type of molecule was made in both the dividing and non-dividing cells of this organism.

Metabolic lesions which lead to deficiencies or faulty synthesis of wall, membrane, nucleic acids or proteins, be they structural or enzymatic, could also be responsible in creating non-dividing condition(s).

Because cell division is an extremely complex process, I have restricted the above literature review only to some of the major findings of Grula and Grula pertaining to this process. A review of the roles played by NaF, microtubules and microfilaments in biological systems follows.

Sodium fluoride is well known for displaying great versatility among metabolic inhibitors. Apart from its well-defined action on enolase, it is an inhibitor of the phosphatases, some transphosphorylases and of reactions of the Kreb's cycle (Terner, 1955a,b; Warburg and Christian, 1942). Its inhibitory effect on the pyruvate, α-ketoglutarate and succinic dehydrogenases is also known (Krebs, 1943; Leibecq and Peters, 1949; Slater and Bonner, 1952; Terner, 1955a,b). Krebs (1943) classified fluoride as a selective inhibitor whose inhibitory action could be taken to indicate that phosphorylated intermediates are involved. Also fluoride ions form complexes with several metal enzyme systems including those dependent on iron, calcium and magnesium (Krebs, 1943; Slater and Bonner, 1952; Terner, 1955a,b).

Warburg and Christian (1942) elucidated the fluoride inhibition of enolase, which catalyzes the formation of

phosphoenolpyruvic acid from 2-phosphoglyceric acid. They showed that magnesium is required for the enzymatic reaction. The weak inhibition by fluoride which is dependent on magnesium concentration was also observed by Taitzer and Himoe (1974) in the absence of phosphate. Inhibition by mixtures of fluoride and phosphate was found to be much stronger than by either anion alone and was principally due to the cooperative binding of these two anions by the enzyme enolase to form an enzyme-magnesium ion-fluoride ion-phosphate complex. They reasoned that the fluoride ion probably inhibits the reaction by acting as an analog of the hydroxide ion generated during the normal enolase-catalyzed reaction.

Hamilton (1969) found that low concentrations of sodium fluoride inhibited use of exogenous glucose by washed cells of *Streptococcus salivarius* whether added before or after the substrate. Complete inhibition of exogenous glucose was achieved by sodium fluoride concentrations above 0.96 mM. Inhibition of glucose uptake caused an immediate reduction in the lactic acid formed, although the percentage conversion of glucose to lactic acid was not altered by sodium fluoride at levels below 0.24 mM. Above this concentration, short periods of non-stoichiometric lactate formation were observed, but continued incubation in the presence of sodium fluoride always resulted in production of normal quantities of lactic acid. Sufficient fluoride was absorbed by cells at fluoride concentrations above 1.2 mM in a 30-minute period to significantly reduce subsequent glycolytic activity by

the cells in a medium free of the inhibitor. A comparison of the fluoride sensitivity of exogenous glucose metabolism and endogenous polyglucose degradation indicated that the prime site of fluoride action in these cells was at some point before glucose-6-phosphate formation. The inhibition could possibly involve glucose transport, the phosphorylation of glucose by hexokinase or some associated reaction. Addition of low concentrations of fluoride to cells incubated in the presence of glucose resulted in an immediate reduction in the intracellular level of glucose-6-phosphate.

Najjar (1948), studying the properties of phosphoglucomutase, found that the crystalline enzyme has a pH optimum of 7.5 for activity. In the absence of cysteine, the enzyme is practically inactive. Magnesium at an optimum concentration of 2.5 to 5.0 X 10^{-3}M increased the activity fourfold. The enzyme was inhibited by fluoride in the absence of inorganic phosphate. By varying the concentration of fluoride, magnesium and organic phosphate, it was shown that a complex of magnesium-fluoride-organic phosphate was formed which presumably competed with magnesium for the enzyme.

Glycogen phosphorylase of Streptococcus salivarius was activated by AMP and sodium fluoride when assayed in the direction of phosphorolysis. Activation by sodium fluoride plus AMP was greater than the sum of their individual effects. The effects of AMP and fluoride were reversible, since preincubation with these compounds followed by dialysis restored activity almost to the control values; however, some

inhibition of enzyme activity was noted with the samples preincubated with fluoride (Spearman, Khandelwal and Hamilton, 1973). When assayed in the absence of AMP and fluoride in the direction of synthesis, the enzyme was slightly inhibited by glucose and glucose-6-phosphate and activated by phosphoenolpyruvate and ADP-glucose. In the presence of AMP and fluoride, the enzyme was inhibited by glucose, glucose-6-phosphate and ADP-glucose but was activated by phosphoenolpyruvate.

Polynucleotide phosphorylase which catalyses the synthesis of highly polymerized polynucleotides from 5' -nucleoside diphosphates with the release of orthophosphate is relatively insensitive to fluoride and low concentrations of arsenate (Manago, Ortiz and Ochoa, 1956).

Slater and Bonner (1952), studying the succinic dehyrogenase enzyme complex, found that fluoride and phosphate separately had very slight effects on the enzyme complex; however, they found the inhibition to be of much greater magnitude when both fluoride and phosphate are present. They also found that manganese and magnesium had no effect on the inhibition by fluoride and phosphate.

Terner (1955a,b) has shown that in the presence of 0.01 M fluoride both the rate of respiration and substrate disappearance of pyruvate, citrate and α-ketoglutarate was inhibited. The inhibition was reversed by the addition of p-nitrophenol (pNP) which, despite the presence of fluoride, produced its stimulatory effects. The inhibitory effect of

fluoride on respiration in the presence of succinate, fumarate and malate was not reversed by pNP. Sodium fluoride inhibits the activity of the pyruvate dehydrogenase enzyme complex (Terner, 1955a,b); however, pNP overcomes the inhibitory effect. Fluoride also strongly inhibits the formation of citrate from acetate.

Acid and alkaline phosphatases from various organs are subject to inactivation by fluoride. The alkaline phosphatases when treated at lower pH ranges with fluoride are strongly inhibited. The inactivation is not reversed by the addition of calcium chloride (Belfanti, Contardi and Ercoli, 1935).

In Escherichia coli, a phosphatase enzyme capable of hydrolyzing α-methyl-D-glucoside-6-phosphate, was inhibited by fluoride. Fluoride inhibition exhibited a sharp pH dependence, being strong at acid pH but very weak at alkaline pH ranges. Addition of the uncoupling agent, sodium azide, restored the pH dependence. Intracellular pH of glycerol-grown E. coli under aerobic conditions was 0.7 pH units more alkaline than the surrounding medium, but sodium azide caused equilibration of hydrogen ion concentration between the medium and the cytoplasm.

Rothman and Cabib (1966), studying the effect of pH on inhibition of yeast growth by fluoride, found that reversion of inhibition occurs at a higher pH than the pH of the medium (5.2 to 5.4) used to grow the yeast cells. Adjustment of the pH to 5.2 - 5.4 or addition of sugar phosphates or inorganic

phosphate did not modify the effect of fluoride. The pH effect probably resulted, they argue, from the impermeability of the yeast cells to fluoride at more alkaline pH ranges; however, the site(s) of action remains unknown (Rothman and Cabib, 1966; Haguenauer and Kepes, 1972). Fluoride inhibition of glucose metabolism in S. salivarius was found to be pH dependent with complete inhibition by 0.12 mM sodium fluoride occurring at pH values below 6.0 (Hamilton, 1969).

Adenylate cyclase, the enzyme responsible for the conversion of ATP to cAMP, is stimulated by low concentrations of fluoride (Neer, 1976). The activation is not readily reversed by dilution, extensive washing of the membrane-bound enzyme or dialysis. Activation occurs in the absence of added magnesium ions, but the rate of activation is increased twofold by 5 mM magnesium ions. The enzyme is also activated by Triton X-100 and to a lesser extent by sodium dodecyl sulfate. The activity of the fluoride-activated enzyme can be increased by Triton X-100. Thus NaF alone does not cause full expression of adenylate cyclase activity. the authors believe that adenylate cyclase exists in its membraneous environment in a state of inhibition. Treatment with detergents or freezing of tissues leads to non-specific changes in membrane structure, which causes a reduction of the inhibition and thus increased catalytic activity. Unlike hormones, NaF does not stimulate adenylate cyclase activity of intact cells, having an effect only on broken cell preparations (Perkins and Moore, 1971).

Studies on the effect of azide and fluoride on adenylate cyclase from rat adipocyte plasma membrane indicate that different mechanisms of stimulation of the enzyme occur in the presence of these ions. Even though 10 mM NaF stimulates adenylate cyclase activity, higher concentrations of fluoride, unlike azide, cause inhibition of the enzyme. Sodium fluoride activated adenylate cyclase is further activated by sodium azide (Rahmaman and Jarett, 1974).

Cell-free extracts and acetone powder preparations from *Agave americana* phosphorylated mevalonic acid through reactions catalysed by mevalonate kinase and pyrophosphomevalonic acid through reactions catalysed by mevalonate kinase and phosphomevalonate kinase. The addition of fluoride prevents the dephosphorylation of the compounds formed (Gracia, Suarez, Aragon and Mayor, 1972).

In cell-free systems from rabbit reticulocytes, NaF and cycloheximide inhibited protein synthesis. NaF was found to have no apparent effect on the rate of incorporation of previously initiated nascent chains but to cause an apparent breakdown of polysomes to single ribosomes in intact cells. The 80S ribosomes isolated from NaF-treated reticulocytes actively incorporated amino acids in the cell-free system and exhibited other properties that indicate they are similar or identical to monomeric ribosomes isolated from untreated cells. The observations were interpreted to indicate a NaF-sensitive reaction occurs which is related to initiation of new peptide chains on ribosomes (Lin, Mosteller and Hardesty, 1966).

Bleiberg, Zauderer and Baglioni (1972) reported that approximately one-fourth of the ribosomes of P-3 mouse myeloma cells in tissue culture are bound to membranes of the endoplasmic reticulum. Incubation with NaF caused polyribosome disaggregation. At the same time approximately one-fourth of the membrane-bound ribosomes were released from the membranes. After reversing the inhibition of NaF, free ribosomes became associated with membranes until approximately the same proportion of free and membrane-bound ribosomes as found in untreated cells is restored.

Lee and Wang (1968) studied the nature of the inhibitory action of inorganic phosphate, fluoride ion and some detergents on 5' -adenylic acid deaminase activity of rat brain and liver and showed that inorganic phosphate as well as ATP is a negative effector on the deaminase. Inorganic phosphate was found to be a competitive inhibitor in the absence of ATP, but in the presence of ATP, the nature of the inhibition was uncompetitive. It appears that inorganic phospate competes with the substrate AMP and with the activator ATP simultaneously at the active site and the effector site, respectively. Fluoride inhibited the deaminase noncompetitively in the absence of ATP, whereas an uncompetitive effect was obtained in the presence of ATP.

A study made on the levels of pyruvate, α-ketoglutarate and acetoacetate in the blood of rats poisoned with different inhibitors of carbohydrate metabolism, namely arsenite, maleate, iodoacetate, alloxan, malonate and fluoride showed

that each of the inhibitors increase the blood level of pyruvate and α-ketoglutarate. Alloxan and malonate cause a large rise in the level of acetoacetate. The relatively large accumulation of acetoacetate in malonate-poisoned animals is supportive evidence that pyruvic acid is involved in two major degradative pathways, one through the citric acid cycle and the other resulting in the synthesis of acetoacetate when the cycle is interrupted by malonate. Since fluoride inhibits both succinic and α-ketoglutarate dehydrogenases, a concommitant increase of acetoacetate should have occurred; however, in fluoride poisoning, acetoacetate does not accumulate (Hawary, 1955).

Chicken erythrocytes incubated in radioactive inorganic phosphate in the presence of glucose showed a rapid increase in internal ATP. Upon addition of sodium fluoride and potassium cyanide, the ATP level of the cells was reduced to 2% of the initial content within a few hours. At the same time, however, specific radioactivity of the residual ATP increased dramatically. Associated with the depletion of ATP the erythrocytes became sensitive to membrane-perturbing drugs (Gazitt, Ohad and Loyter, 1976).

The fact that cell division is a constrictive process in _E. carotovora_ (Grula and Smith, 1965) suggests that a microtubule-microfilament system is involved in the process. Such proteins could contract during division to initiate and sustain the process and remain in the relaxed state during the rest of the growth cycle (a "purse-string" hypothesis).

Microtubule is a general name given to a class of subcellular components found in a wide variety of eukaryotic cells. They are considered as straight cylinders with or without a central core (Adelman, Borisy, Shelanski, Weisenberg and Taylor, 1968). Microtubules are approximately 240 ± 20 A° in diameter (Goldman, 1971; Olmstead and Borisy, 1973). Other terms used to describe these cylindrical components are filaments and microfilaments. Filaments are approximately 100 to 120 A° in diameter while microfilaments are 40 to 60 A° in diameter (Goldman, 1971).

Microtubules occur adjacent to the wall of plant cells, in various elongated cells including neurons, in the sperm manchette and in melanocytes. They are associated with microscopic projections of cytoplasm (microspikes) and with spiny axopodia of Actinospherium (Malawista, 1965; McIntosh and Porter, 1967; Newcomb and Bonnett, 1965; Taylor, 1966; Tileny, Hiramota and Marshland, 1966).

The ubiquitous nature of microtubules in diverse eukaryotic cells suggests a wide variety of functions (Dehm and Prockop, 1972; Diegelman and Peterkofsky, 1972; Holmes and Choppin, 1968; Olmstead and Borisy, 1973). It is unlikely that a single explanation will be given to cover the function of microtubules which have been implicated in chromosome movements during cell division (McIntosh and Porter, 1967; Olmstead and Borisy, 1973), intracellular transport of material (Adelman et al., 1968; Brown and Bouk, 1974), the development and maintenance of cell form, cellular motility

(Goldman, 1971; Holmes and Choppin, 1968) and sensory transduction (Olmstead and Borisy, 1973). In some cases, such as in flagella and the axostyle of protozoa, they are part of a system for movement of cells. In other instances, such as in the mitotic spindle of melanocytes, they are associated with movement, but a different mechanism is probably involved (Adelman et al., 1968).

Colchicine and vineblastine have been known to bind to mitotic spindle protein and arrest mitosis (Zacheus et al., 1974). More recently it has been shown that these alkaloids can dissociate the microtubule structure into its subunits (Dehm and Prockop, 1972; Goldman, 1971) by binding to different sites on the microtubule protein.

Treatment of pancreas β cells with vinecristine results in a scattering of microtubules. The scattering effect of vinecristine on the microtubules decreases the release of insulin (Malaisse, Greider, Malaisse and Lacy, 1971). Vinecristine inhibits insulin release by virtue of its specific action upon the microtubule system in the β cells of the pancreas.

The study on reversible darkening of frog skin under the influence of melanocyte-stimulating hormone was compared to treatment of the frog skin with colchicine. Such treatment prevents the melanin granules from being redistributed by inactivating microtubule activity (Malawista, 1965). Other investigators using colchicine, vineblastine or vinecristine demonstrated that microtubules are involved in the

secretion of insulin by islets of Langerhans from rat, histamine by rat mast cells and amine granules by nerve cells (Diegelman and Peterkofsky, 1972; Gillespie, Levine and Malawista, 1968; Malaisse, Greider, Malaisse and Lacy, 1971; Malawista, 1965).

According to studies performed in some animal systems the cytoplasmic microtubules consist of an actin-like material and are thought to play an important role in controlled cellular movements (Brinkley, Barham, Barranco and Fuller, 1974).

Colchicine-resistant mutants of *Chlamydomonas reinhardii* were found to be resistant to vineblastine. Inclusion of colchicine or vineblastine into the medium improved the growth of the organism. The mutation was thought to be due to a change in microtubule structure. This change resulted in abnormal cell shape (Warr and Gibbins, 1974).

Colcimid, like colchicine and vineblastine, reversibly breaks down microtubules. Cells treated with colchicine generally show morphological alterations. All the mentioned drugs cause loss of birefringence, and spindles fade and shrink as if there were no tubules present (Brinkley, Barham, Barranco and Fuller, 1974). Treatment of nerve microtubules with these drugs results in a reversible conversion of microtubules into nerve filaments (Goldman, 1971; Margulis, 1973).

The secretion by frontal bone of collagen and non-collagen protein from chick embryo tendons was studied using colchicine and vineblastine, and it was observed that both

drugs decreased the activity. The binding of the drugs occurred at very low concentrations (Dehm and Prockop, 1972; Diegelman and Peterkofsky, 1972). In order to examine the kinds of intracellular processes which might be involved in the secretion of collagen, isolated tendon cells were incubated with colchicine and vineblastine. Addition of these agents to the medium markedly inhibited the secretion of ^{14}C-collagen. With all concentrations of colchicine or vineblastine, levels of intracellular ^{14}C-collagen were significantly greater than in control cells. The intracellular collagen concentrated by the treatment of colchicine or vineblastine was no different from the collagen secreted by the control cells. In further experiments they also found that dibutryl cAMP produced a small increase in the total fraction of ^{14}C-collagen which was secreted into the medium (Dehm and Prockop, 1972; Diegelman and Peterkofsky, 1972; Goodman, Rasmussen, DiBella and Guthrow, 1970).

Rotenone, a potent inhibitor of mitochondrial respiration, is also an effective antimitotic drug. It was found to inhibit the binding of colchicine to isolated brain bovine tubulin. It has been suggested that it binds directly to tubulin, and thus further prevents microtubule assembly (Brinkley, Barham, Barranco and Fuller, 1974).

Griseofulvin inhibits mitosis without binding directly to tubulin subunits. It is thought that it exerts its activity by binding to the membrane (Gillespie, Levine and Malawista, 1968; Gillespie and Lichtenstein, 1972; Brinkley,

Barham, Barranco and Fuller, 1974; Malaisse, Greider, Malaisse and Lacy, 1971).

Although mammalian cells do not require exogenous nucleosides for growth, cells will take up nucleosides and utilize them for the synthesis of nucleic acids if they are present in the extracellular environment. Colchicine can inhibit the transport of nucleosides in several types of mammalian cells. The action of colchicine on transport is reversible, although not completely, upon its removal from the medium. Inhibition of adenosine, guanosine, uridine and thymidine transport by colchicine appears to be competitive. Since colchicine does not prevent the uptake of 2-deoxyglucose or α-aminoisobutyric acid, it appears to be specific for the nucleosides. Its effect on nucleoside uptake appears to be unrelated to its action on microtubules. Luminocolchicine, an analog of colchicine, which neither disrupts nor binds to microtubules, inhibits thymidine and uridine transport; however, podophyllotoxin and several colchicine analogs which do interact with microtubules are also effective inhibitors of thymidine transport. It was further observed that colchicine had no effect on the synthesis of DNA or RNA, nor did it inhibit the _in vitro_ phosphorylation of thymidine or uridine. Podophyllotoxin, which appears to interact at the same binding site on microtubule protein as does colchicine, was more effective than colchicine in preventing transport. Colcimid which is equivalent to colchicine as an antimitotic

agent was as effective as colchicine as an inhibitor of transport (Margulis, 1973; Mizel and Wilson, 1972).

The distribution of surface-bound concanavalin A on membranes of 3T3 and simian virus 40-transformed 3T3-cultured mouse fibroblast cells was studied using the shadow cast replica technique utilizing hemocyanin as a marker (Ukena, Borysenko, Kanovsky and Berlin, 1974). The investigators found that prefixing with paraformaldehyde makes the binding site distribution random on both types of cells. Labelling of transformed cells, if carried out at 37° C, results in clustering of concanavalin A binding sites. Treatment of the transformed cells with colchicine, cytochalasin B or 2-deoxy-glucose did not alter the inherent random distribution of binding sites as determined by fixation before labelling. Colchicine treatment (37° C) of transformed cells resulted in the formation of cap-like aggregation of binding site clusters near the center of the cell, whereas cytochalasin B and 2-deoxyglucose led to the formation of patches of concanavalin A binding sites over the entire membrane and eliminated the inward displacement of patches observed on untreated cells. All the drugs utilized affected the topographical distribution of the induced clusters on transformed cells. This has led to the belief that microtubules regulate the mobility of surface receptors. There is a suggestion that an energy requirement could be mediated through microtubules (Ukena, Borysenko, Kanovsky and Berlin, 1974).

Cytochalasin is an alkaloid metabolite obtained from Helminthosporium dematioidium. It can inhibit cytokinesis, cell locomotion, cytoplasmic streaming, blood clot refraction and movement associated with some developmental processes. It also inhibits transport of hexoses across the cell membrane and there is evidence that this effect is independent of the effects described above (Lin, Santi and Spudich, 1974). Cytochalasin B has been found to be a competitive inhibitor of glucose transport and may therefore be considered a membrane-perturbing agent. The results suggest that the effects on microfilaments and glucose transport may be dissociable if cytochalasin A is used instead of cytochalasin B (Loten and Jeanrenaud, 1974). Cytochalasin B binds rapidly to Hela cells and bovine blood platelets at both high and low concentrations. Low affinity sites were found in Dictyostelium amoebae. Bacterial cells (Enterobacter aerogenes) bind relatively low levels of cytochalasin B regardless of concentration because of low levels or the lack of affinity sites (Lin, Santi and Spudich, 1974; Loten and Jeanrenaud, 1974; Ukena, Borysenko, Kanovsky and Berlin, 1974).

Cytochalasin B causes separation of mammalian cells to nucleate and enucleate components. The enucleate component (cytoplasts) remains intact one to three days and synthesis of RNA occurs in them. They are, however, deficient of centrioles, microtubules and the Golgi apparatus. Reconstruction of the separated enucleate and nucleate components

could be achieved under certain conditions (Veomett, Prescott, Shay and Porter, 1974).

Studies using BHK-21-F cells to initiate cell fusion by parainfluenza virus SV5 lead to the conclusion that nuclei in eukaryotes move by a specific mechanism and microtubules are involved. Microtubules are also responsible for the movement of cytoplasmic particles. Colchicine treatment does not affect the 80 A° diamater filaments; in fact, the concentration of these filaments is increased (Goldman, 1971; Holmes and Choppin, 1968; Olmstead and Borisy, 1973). On contact, BHK-21-F cells lose their ruffled movement, and this immobilization is abolished by colcimid, colchicine or vineblastine. These drugs also inhibit the aggregation of polymorphonuclear leucocytes by concanavalin A. Low temperatures cause dissolution of concanavalin A binding sites on polymorphonuclear leucocytes (microtubules) and in addition decrease the agglutinability of transformed cells by concanavalin A (Berlin and Ukena, 1972; Goldman, 1971).

Yin, Ukena and Berlin (1972) also showed that colchicine, colcimid and vineblastine reduce the sensitivity of SV3T3 cells to agglutination by concanavalin A. It has been suggested that microtubules either stabilize the arrangement of elements within the fluid matrix that may otherwise provide a medium for their randomization, or they are essential for the induction of various molecular arrangements by exogenous multivalent substances such as concanavalin A. Colchine and vineblastine abolish the topographical localization of

membrane transport systems as determined by functional analysis of polymorphonuclear leucocytes, while phagocytosis by polymorphonuclear leucocytes, which depends on stable surface-to-particle contact, is only slightly inhibited by colchicine (Yin, Ukena and Berlin, 1972).

Goodman, Rasmussen, DiBella and Guthrow (1970) found cAMP to simultaneously increase cellular uptake of calcium while stimulating protein kinase activities. They also found that the microtubular protein subunit was a natural substrate for a cAMP dependent protein kinase in brain tissue. This led them to conclude that there is some kind of relationship between secretory vesicles, microtubules and the plasma membrane.

Dynein, a protein component of microtubules, forms arms between adjacent tubulins on microtubules of cilia and flagella. In the presence of magnesium and calcium ions dynein catalyses the hydrolysis of ATP (Margulis, 1973; Olmstead and Borisy, 1973). The microtubules from brain cells are also thought to be responsible in axoplasmic transport (Pollard and Burns, 1974).

An actin-like protein has been reported to be present in E. coli strain CBH by Minkoff and Damadian (1975). The polymerization of the actin-like compound was found to be dependent on the presence of KCl but not magnesium ions (Minkoff, Abramowitz and Damadian, 1976). The constrictive process of division in our test organism and the data presented by these investigators compelled us to investigate

the presence or absence of a similar component in the test organism using conventional drugs whose mode of action is directed toward tubular macromolecules in the cell.

CHAPTER II

METHODS AND MATERIALS

The organism used throughout this research was Erwinia carotovora (Grula and Grula, 1976), formerly known as Erwinia sp. (Grula, 1960a). Stock cultures of the organism were maintained on nutrient agar slants containing 0.5% sodium chloride (NaCl) with or without 1.0% D-glucose. Transfer of stock cultures was made alternately in 24-hour intervals to any one of the two types of slants. The medium used to grow the organism contained the following per 100 ml: D-glucose, 150 mg; aspartic acid, 280 mg; K_2HPO_4, 174 mg; KH_2PO_4, 136 mg; $MgSO_4 \cdot 7H_2O$, 3.0 mg; and trace minerals (Grula, 1960b).

The trace minerals were constituted separately as a stock solution and added into the medium such that the following amounts were present (micrograms per 100 ml of medium): HBO_3, 0.5; $CaCO_3$, 10; $CuSO_4 \cdot 5H_2O$, 1; $FeSO_4(NH_4)SO_4 \cdot 6H_2O$, 50; KI, 1; $MnSO_4 \cdot H_2O$, 2; MoO_3, 1; $ZnSO_4 \cdot 7H_2O$, 5. Unless otherwise specified, the pH of the medium before sterilization was always 6.8.

In order to obtain filaments, 67.2 mg of sodium fluoride (NaF) dissolved in 8.0 ml of sterile deionized water was added to 92 ml of the above medium at the time of inoculation

to make a final volume of 100 ml or else an equal amount of deionized water was added to obtain the final volume.

Glucose was prepared as a stock solution, autoclaved separately for 10 minutes at 15 pounds pressure at 121° C and added to the medium aseptically. All other ingredients added to this chemically-defined medium were adjusted to a pH of 6.8 and either sterilized in the autoclave or filter sterilized as dictated by the heat lability of the ingredient.

Media with and without NaF were inoculated with cells grown for 24 hours at room temperature on nutrient agar plus 0.5% NaCl slants. The cells were washed twice in 0.85% NaCl, suspended in the salt solution and adjusted to an optical density (OD) of 0.1 (540 nm, Spectronic 20, Bausch and Lomb) in 18 x 150 mm Kimax test tubes. Medium (100 ml in 250 ml Erlenmeyer flasks) was inoculated using 1.0 ml of this suspension. The cultures were incubated in a 25° C room on a rotary shaker. Incubation for 17-18 hours in the presence of NaF gives rise to fairly long filaments in the range of 20-100 μm, with the majority of the filaments between 40-60 μm in length.

The media used to study reversion fall into two categories, namely: non-growth-supporting and growth-supporting. Each of these two categories have two types of media, and each contained the following components per 100 ml:

Non-growth-supporting media:

1) $MgCl_2 \cdot 6H_2O$, 203 mg

2) $MgCl_2 \cdot 6H_2O$, 203 mg; K_2HPO_4, 281.8 mg;
KH_2PO_4, 51.7 mg

Growth-supporting media:

3) $MgCl_2 \cdot 6H_2O$, 203 mg; aspartic acid, 280 mg

4) $MgCl_2 \cdot 6H_2O$, 203 mg; K_2HPO_4, 281.8 mg;
KH_2PO_4, 51.7 mg; aspartic acid, 280 mg

The pH of the media containing phosphate was 7.2-7.4.

To study reversion of filaments, cells were harvested between 17 and 18 hours. The filaments were then washed once in sterile trace mineral solution followed by a second washing with sterile deionized water. The sedimented cells were then made into a thick slurry by adding a small quantity of sterile deionized water and inoculated into the reversion media aseptically (drop-wise) until the desired OD reading at 540 nm was achieved. When high cell densities were desired, the initial OD reading just after inoculation was between 0.30 and 0.45; for low cell densities it was between 0.10 and 0.20.

Keto Acids

When keto acids were to be analyzed, a known aliquot of medium containing cells that had been growing for a known period of time was spun at approximately 6000 RPM for 15 minutes. The supernatant was then acidified either with 0.2 ml of 2/3 N H_2SO_4 and 2.0 ml of 4.0% tungstic acid per ml of supernatant or 10% perchloric acid and allowed to stand at

4° C for 15 minutes. After the necessary deproteinization process had taken place the acidified supernatant was centrifuged as described above and the sedimented cells discarded (Sherma and Zweig, 1971).

The method of extraction was essentially that of Sherma and Zweig (1971). Two ml of 0.5% 2,4-dinitrophenylhydrazine (DNPH) in 6 N HCl was added to 50 ml of deproteinized supernatant and the mixture allowed to react for 30 minutes at room temperature. The supernatant was then extracted three times with 15 ml portions of chloroform containing 20% ethyl acetate. The combined chloroform-ethyl acetate fraction was extracted three times with 5 ml portions of 1 N Na_2CO_3. The combined Na_2CO_3 extract was acidified at 4° C with 5 ml of 6 N HCl and shaken gently until no more gas evolved. The hydrazones and derivatives were extracted from the acidified Na_2CO_3 fraction three times with 5 ml portions of chloroform-ethyl acetate fraction, dried under a gentle stream of air, and the dried keto acid derivatives stored in the freezer until used.

Identification of the keto acid derivatives was carried out using ascending chromatography on Whatman #1 paper in the presence of various types of solvents (El Hawary and Thompson, 1953; Isherwood and Cruickshank, 1954a,b; Cavallini and Frontali, 1954; Isherwood and Navis, 1956).

To aid in the identification of the keto acids, some were also converted to their respective amino acids (Kun and Hernandez, 1956). To bring about this conversion, 5 ml of

glacial acetic acid was added into test tubes containing the dried phenylhydrazone derivatives of the keto acids, shaken vigorously, and then decanted into Pyrex Sorvall centrifuge tubes (1 x 4 in). About 100 mg of platinum oxide (platinum black) was added and hydrogen gas gently bubbled into the mixture for 2 hours, after which the converted keto acids (now amino acids) were dried under a hot stream of air. The amino acids were chromatographed and identified using the system of Heathcote and Jones (1965).

Keto acid quantitation was performed using the system of Haidle and Knight (1960). To cell free sample, 0.5 ml of 0.1% DNPH in 2 N HCl was added and the volume adjusted to 4.5 ml by addition of deionized water in 18 x 150 mm Kimax test tubes. After shaking the tube very vigorously it was incubated at room temperature for 20 minutes, at which time 3.0 ml of 95% ethanol was added. Vigorous shaking was once again performed and the mixture was further incubated at room temperature for 20 minutes, after which 1.0 ml of 6 N HCl was added, the tube again shaken vigorously and incubation continued for an additional 10 minutes, after which OD readings (540 nm) were made. Quantitation of the keto acids was accomplished by comparing the OD values obtained to a standard graph made using known concentrations of sodium pyruvate.

Mucopeptide Isolation and Chromatography

Mucopeptide was isolated using the system of Grula, Smith and Grula (1965). Hydrolysis of the mucopeptide was

done *in vacuo* in tubes using 6 N HCl for 24 hours at 100-105° C. Hydrolysed samples were chromatographed on Whatman #1 paper or Cellulose MN300 thin-layer plates using the solvent system of Heathcote and Jones (1965). Amino acids were detected by spraying the chromatograms with a solution of 0.5% ninhydrin in 95% acetone, followed by heating at 100° C for approximately 5 minutes.

Quantitative Determination of Glucose

The quantity of glucose present in spent media at various times after incubation was determined using the Glucostat reagent (Worthington). Preparation of the reagent was done according to the following procedure: The contents of the chromogen and the glucostat vials were dissolved separately in 20 ml portions of deionized water, and combined together in a graduated cylinder where the final volume was adjusted to 90 ml using deionized water. To 1 ml sample (spent medium) and 1 ml of glucose standard (0.2 mg) 9 ml of the above reagent was added. After incubation for 10 minutes at room temperature 1 drop of 4 N HCl was added and the incubation continued for an additional 5 minutes. During each step of the procedure, when reagents were added, the tube contents were thoroughly mixed. At the end of the 5-minute incubation period, OD readings were taken at 400 nm using the Spectronic 20 spectrophotometer. Amount of glucose present in the sample was determined using the following formula:

$$\frac{\text{Absorbance of unknown sample}}{\text{Absorbance of glucose standard}} \times \text{Conc. of Glucose Std.}$$

$$= \text{Concentration of Glucose in Unknown Sample.}$$

Quantitative Determination of Aspartic Acid

To 1.0 ml portions of spent medium 0.5 ml of 3.0% ninhydrin in methylcellosolve was added. After thorough mixing of the samples the tubes were stoppered with glass marbles and placed in a boiling water bath for 15 minutes. The tubes were then removed, cooled to room temperature and 5.0 ml of 50% isopropanol added. Absorbance was measured at 570 nm using a Spectronic 20 spectrophotometer and the reading obtained compared to values on a standard curve obtained using known amounts of aspartic acid.

Release of CO_2 from U-^{14}C-L-Aspartic Acid

Cells were allowed to grow in glucose-aspartic acid (GA) and glucose-aspartic acid-NaF (GAF) media containing various inhibitors of filament formation until an OD of 0.10-0.20 was achieved. As soon as the desired growth was obtained, the cultures were transferred to a hood where labelled aspartic acid (0.01 μc/ml in medium) was added and air bubbled through the medium at a constant rate. Air from the culture medium was channeled through an exit tube into test tubes containing 10 ml portions of concentrated Hyamine hydroxide 10-X (Packard) to trap the $^{14}CO_2$ released by the cells. At the end of one hour the air flow was stopped, and the increase in

mass of the cells was obtained using spectrophotometric measurements. The total labelled $^{14}CO_2$ in the Hyamine was determined by adding 1 ml of the Hyamine to scintillation vials containing 10 ml PCS Solubilizer (Amersham/Searle). After thorough mixing, the vials were placed in a scintillation counter (Nuclear Chicago), and the actual counts present were obtained 1.5 hours later.

Determination of Total Cellular Phosphorus

Digestion of normal (short) and filamentous cells for total phosphate estimations were carried out using the procedures of Bennet and Williams (1957). Aliquots of cell suspension were washed twice with cold trace mineral solutions containing 1×10^{-4} M $MgSO_4$ and 0.02 M HEPES, pH 6.8. The sedimented cells were then digested with 0.7 ml of 10 N H_2SO_4 for 3 hours in an oven at 150° C. At the end of the 3 hours, the tubes containing the digested cells were partially cooled and 1-2 drops of 30% hydrogen peroxide added to each tube, and the mixture was placed in an oven (150° C) until all bubbling had stopped. Hydrogen peroxide treatment was continued until the contents in the tubes were clear. Once the clear material was obtained, 1.0 ml of deionized water was added to each tube and the tube heated to 100° C for 10 minutes before phosphate quantitation was performed.

The procedure used to determine the quantity of phosphate available in the digested samples was essentially that of

Norris and Ribbons (1971). A volume of unknown sample containing phosphate (5-20 μg) was added into 18 x 150 mm Kimax test tubes and diluted to 5.4 ml using deionized water. To each of the tubes were then added 0.2 ml of 0.02% ascorbic acid followed by 0.4 ml of 5.0% ammonium molybdate. Thorough mixing was accomplished between each addition. The color was allowed to develop for 20 minutes at room temperature and OD readings taken at 730 nm using a Spectronic 20 spectrophotometer. A blank containing 5.4 ml of deionized water acidified with 0.4 ml of 60% perchloric acid or 0.36 N H_2SO_4 and treated in a similar manner to the test was used to adjust the spectrophotometer to give 100% transmittance readings. Even though sulfuric acid-digested cells do not require further acidification prior to addition of the above reagents, 0.4 ml of 60% perchloric acid was added to acidify the samples whenever free phosphate was determined from the different cellular fractions. Quantity of phosphate in the unknown samples was obtained by comparing OD readings with a standard curve made using KH_2PO_4. To ensure repeatability of the analysis, a standard sample of KH_2PO_4 (10 μg) was always prepared and run at the same time unknown samples were run.

Determination of Phosphate Distribution in Cells

The Park and Hancock system (1960) with slight modifications was used to determine phosphate distribution in the

different cellular fractions. In place of trichloroacetic acid we used 10% perchloric acid for both the cold (10 minutes in an ice bath with occasional mixing) and hot (incubation in a 90° C water bath for 6 minutes) extraction steps. The trypsinization process was also omitted. Aliquots (0.5 ml) from each of the fractions were digested using the procedure of Bennet and Williams (1957) and their phosphate contents determined according to Norris and Ribbons (1971).

In order to perform chromatography of phosphorylated compounds, 1 ml aliquot of the ice-cold extract (initial extract obtained using ice-cold 10% perchloric acid) was treated with 1 ml of 10% KOH and kept at 4° C for 24 hours to precipitate the potassium perchlorate. Chromatography of phosphorylated compounds (present in the supernatant fraction) was then performed on Whatman #1 paper using the following solvents: 1) isopropyl ether - n-butanol - 80% (w/v) formic acid (30:30:20). This solvent system is especially useful in resolving 3-carbon phosphates. 2) n-butanol - n-propanol - acetone - 80% (w/v) formic acid - 30% (w/v) trichloroacetic acid (40:20:25:25:15). The chromatographs were then sprayed with a mixture of 60% (w/v) perchloric acid, 1 N HCl, 4% ammonium molybdate and water (5:10:25:60). Inorganic phosphate yields a yellow color. Readily labile sugar phosphates like glucose-1-phosphate yield yellow spots after heating for 1 minute at 85° C. Exposure of the sprayed paper to ultraviolet (uv) light for 3-5 minutes yields bluish-green spots (all sugar phosphates including those that were not detected

during the previous steps appear as bluish-green spots). Since non-phosphorylated compounds (such as citrate, lactones, ribose, polyols) can give false positive spots, the uv irradiated chromatographs were placed in an ammonia chamber to bleach them out. Phosphorylated compounds will retain their original color under such conditions (Bandrunski and Axelrod, 1951). Whenever the quantity of phosphorylated compounds spotted on chromatography paper was small we used the spray reagent of Wade and Morgan (1953). In such cases the chromatographs were first sprayed with 0.1% ferric chloride in 80% ethanol followed by 1.0% sulfosalicylic acid in 80% ethanol. Phosphate esters give white spots on a pale mauve background immediately after adding the sulfasalicylic acid.

Determination of Phosphomonoesterase Activity

Phosphomonoesterase activities of normal and filamentous cells were determined using the system of Garihle and Laskowski (1955). Cells (17-hour) were harvested and washed twice with cold trace mineral solution supplemented with 1×10^{-4} M $MgSO_4$ and 0.02 M HEPES, pH 6.8. The sedimented cells were then suspended in deionized water such that 6-9 mg/ml of cells were present in the suspension. Into 18 x 150 mm Kimax test tubes were then added 0.3 ml of the cell suspension, 3.0 ml of 0.3 M glycine-NaOH buffer, pH 9.0, 2.0 ml of 1×10^{-3} M _p_-nitrophenylphosphate, 0.9 ml of 0.3 M $MgSO_4$

and enough deionized water to make a final volume of 9.0 ml. The test tubes were then incubated at room temperature for 2-6 hours at which time the cells were removed by centrifugation, and the yellow color (*p*-nitrophenol at alkaline pH values) read at an OD of 400 nm using the Spectronic 20 spectrophotometer. Whenever phosphomonoesterase activity at pH 6.8 was to be determined, 1.2 ml of 0.1 M HEPES, pH 6.8 was added instead of the glycine buffer. At the termination of experiments of this type, i.e., at pH 6.8, glycine buffer at pH 9.0 was added to increase the color intensity of the *p*-nitrophenol released. The quantity of *p*-nitrophenol released was then determined from a standard curve made using the same compound.

CHAPTER III

RESULTS

Studies on Filament Formation

Different types of physical and chemical agents such as ultraviolet light, various antibiotics, D-amino acids, high temperature have been found to inhibit growth and induce filament formation in gram-negative rod-shaped bacteria when used at appropriate doses or concentrations (Iyehara and Otsuji, 1975; Grula and Grula, 1962a, b, 1964; Walker and Kovarik, 1975; Walker, Kovarik, Allen and Gustafson, 1975).

Studies made on filamentous cells of Erwinia carotovora obtained by growth in the presence of D-serine or antibiotics such as penicillin in glucose-ammonia and/or glucose-aspartic acid (GA) media have been reported from this laboratory (Grula and Grula, 1962a,b, 1964; Grula and Hopfer, 1972). Besides being sensitive to the above-mentioned compounds, this organism also grows into the filamentous state in the presence of NaF when it is grown in GA or aspartic acid (same as GA except that glucose is absent) medium.

To determine if NaF-induced filamentation occurs only in GA and aspartic acid media or in other types of medium formulations as well, we grew cells in glucose-ammonia, nutrient broth (single and double strength), GA and aspartic

acid media. Even though sufficient quantities of NaF were added (capable of inducing filamentation in aspartic acid medium), normal (short) cells were obtained in both glucose-ammonia and nutrient broth media. The only significant observation made from these studies was the increased lag period in both media (lag period in GA is about 8-10 hours). The glucose-ammonia medium was of special interest in that it resembles GA medium except for the substitution of ammonium chloride (3.2 mg/ml in medium) for aspartic acid. If glucose and/or ammonium ions are involved in filament formation in conjunction with NaF, filamentous cells should have been present; however, cells growing in glucose-ammonia medium were always short. The presence of NaF (1.6×10^{-2} M) in the GA medium decreased the rate of growth substantially (Figure 1). Under these conditions of growth, cells are short for the first 10-12 hours. Filamentation begins at the end of this incubation period and proceeds for an additional 12 hours or more. Cell size determination for cells grown in GA and GA containing NaF (1.6×10^{-2} M) for 17 hours are shown in Table I.

The studies made on filament formation using the various media, especially the glucose-ammonia medium, led us to believe that aspartic acid plays an essential role in causing the cells to be filamentous in the presence of NaF. We also studied the effects of other amino acids (at equimolar concentrations to aspartic acid) on filament formation. Results pertaining to growth and cell size determinations using these

Figure 1. Growth of cells in glucose-aspartic acid medium with and without NaF. Solid line indicates growth in the absence of NaF (control cells); dashed line indicates growth in the presence of 1.6×10^{-2} M NaF (filaments).

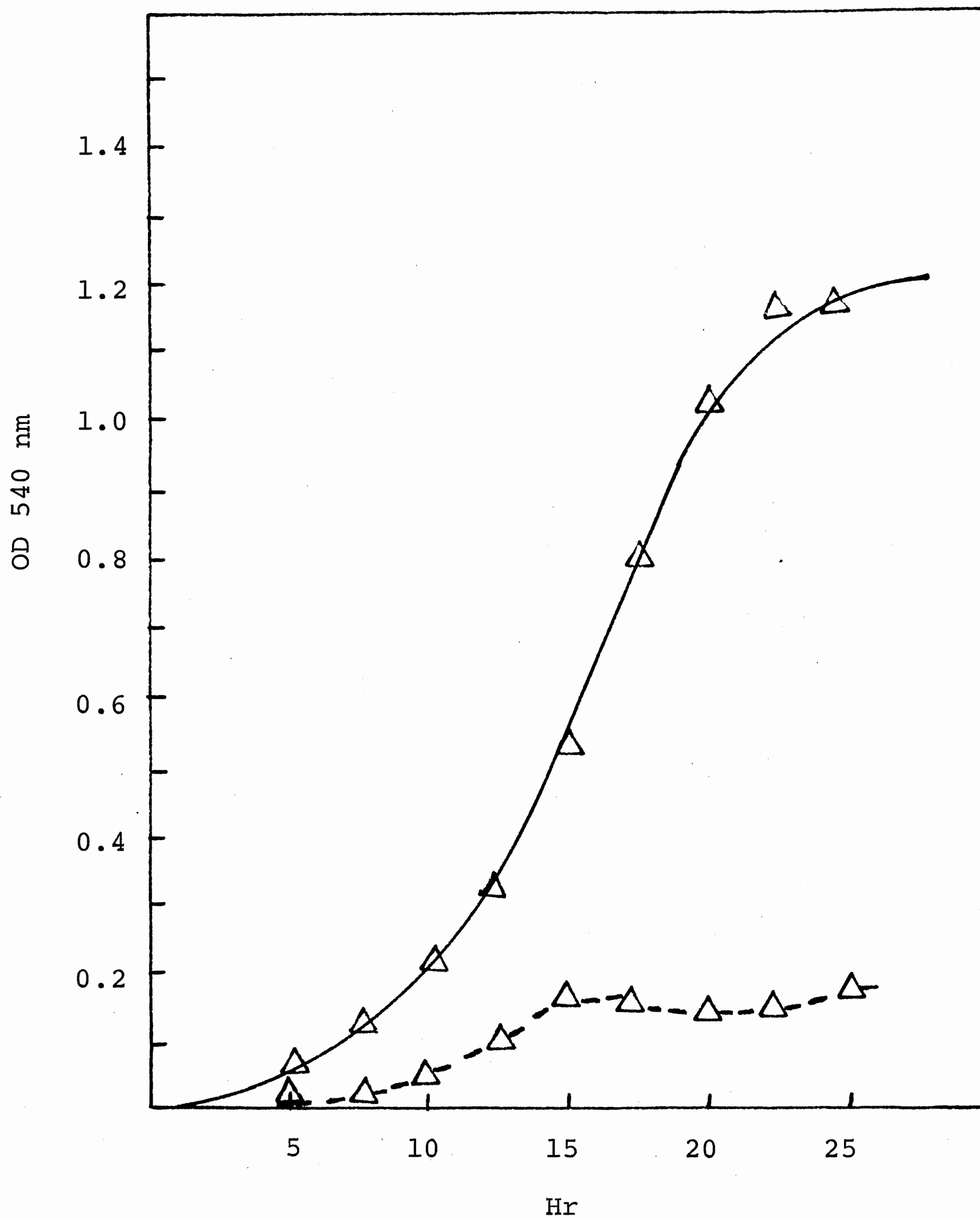

1.4
1.2
1.0
0.8
0.6
0.4
0.2
OD 540 nm
5
10
15
20
25
Hr

TABLE I

PERCENTAGE CELL LENGTH DISTRIBUTION (µm) AFTER GROWTH IN GA MEDIUM WITH AND WITHOUT NaF

Cell Length (µm)	Percentage Cell Lengths* GA Medium	GAF Medium†
0 - 10	80	5
11 - 20	15	10
21 - 30	5	10
31 - 40	-	13
41 - 50	-	30
51 - 60	-	15
61 - 70	-	8
71 - 80	-	5
81+	-	5

*Incubation time was 17 hours

†GAF = glucose-aspartic acid-sodium fluoride

amino acids are summarized in Table II. Only the D-isomer of aspartic acid in conjunction with NaF induced significant filamentous growth. Cells allowed to grow in the presence of L-asparagine were no more than 15 μm in length after 17 hours; all other amino acids were ineffective in inducing filament formation.

In addition to these studies, we also investigated the effects other amino acids may have on filament formation when added at various concentrations into the GA medium in the presence of NaF. Incorporation of any amino acid (2.8 mg/ml or more in medium) in the GA medium completely prevented filament formation. Lower concentrations of amino acid (less than 2.8 mg/ml in medium) did not prevent filament formation, while higher concentrations completely stopped filamentation. Even though individual amino acids prevent filamentation when their concentration is 2.8 mg/ml or more, combinations of amino acids, as long as their total concentration is equal to or greater than 2.8 mg/ml, can also prevent filamentation. During these types of studies, amino acids from the aspartic acid family, especially L-lysine or a combination of L-lysine and L-threonine, were found to delay filament formation when used at lower concentrations, but never prevented its occurrence. Acid or enzymatically hydrolyzed casamino acids (2.5 mg/ml or more in medium) also prevented filament formation.

The results obtained from studies made on filament formation in the GA medium formulation containing amino acids

TABLE II

ABILITY OF AMINO ACIDS OTHER THAN ASPARTIC ACID TO INDUCE FILAMENTOUS GROWTH IN THE PRESENCE OF NaF*

Amino Acid	Growth†	Cell Length¶
L-aspartic acid	++++	F
D-aspartic acid	++++	F
L-asparagine	++++	Intermediate F (15 μm)
L-lysine	+	S
L-threonine	+	S
L-tryptophan	+	S
L-cysteine	+	S
L-alanine	++	S
glycine	++	S
L-proline	++	S
L-glutamic acid	±	S
L-methionine	++	S
L-tyrosine	++	S

*Medium used was the glucose-mineral salts medium normally added to aspartic acid. Concentration of each amino acid was 2.25×10^{-2} M.

†++++ indicates excellent growth; 0 indicates no growth.

¶F indicates filamentous cells (>30 μm); S indicates short cells (<5 μm).

(other than aspartic acid) and the results of growth from the glucose-ammonia medium convinced us of the need to re-evaluate the role played by each component of the GA medium in filament formation. By omitting each component of the GA medium one at a time we were able to find the nutrients essential for filament formation. These are: L-aspartic acid, 2.25×10^{-2} M, pH 6.8; potassium phosphate, 2×10^{-2} M, pH 6.8; magnesium sulfate, 1×10^{-4} M; NaF, 1.6×10^{-2} M. Detailed studies on the role played by each of the essential medium components were then carried out.

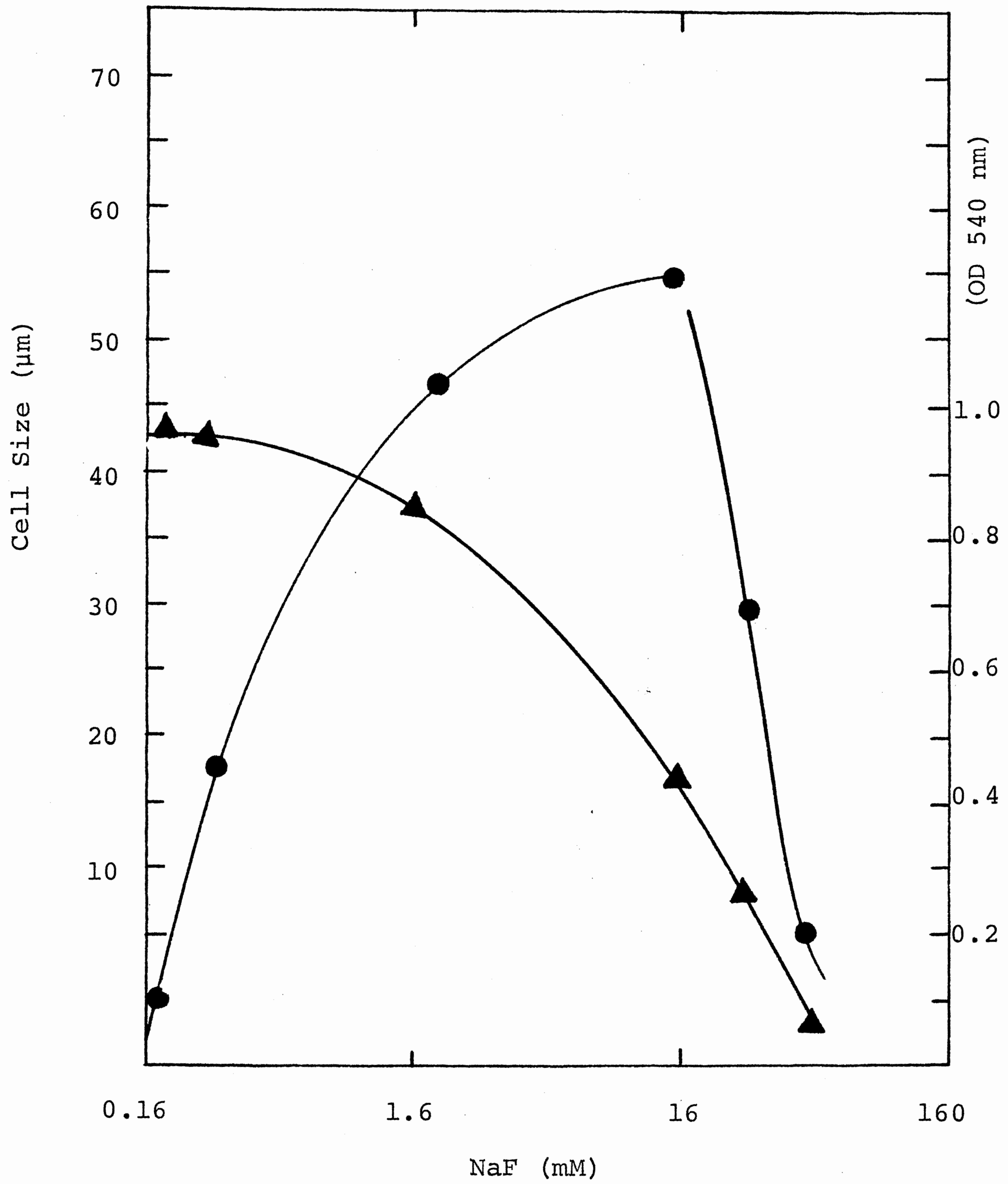
Magnesium Sulfate

Magnesium sulfate (1×10^{-4} M) serves as a source of both magnesium and sulfur for the growing cells. Omission of this compound completely prevents growth. Since filament formation requires growth, the compound has to be present.

Sodium Fluoride

The quantity of NaF present in the medium dictates whether there will be growth of filamentous or normal (short) cells. When the concentration of NaF is between the ranges of 4×10^{-4} to 7×10^{-2} M, filament formation will occur (Figure 2). Any concentration less than 4×10^{-4} M or more than 7×10^{-2} M causes production of cells that are short (less than 10 μm) (Figure 2).

Figure 2. Effect of various concentrations of sodium fluoride (NaF) in the GA medium on filament formation. Cell size determinations (●, closed circles) were performed at 17 hours of growth (▲, closed triangles).



Cell Size (μm)
70
60
50
40
30
20
10
(OD 540 nm)
1.0
0.8
0.6
0.4
0.2
0.16
1.6
16
160
NaF (mM)

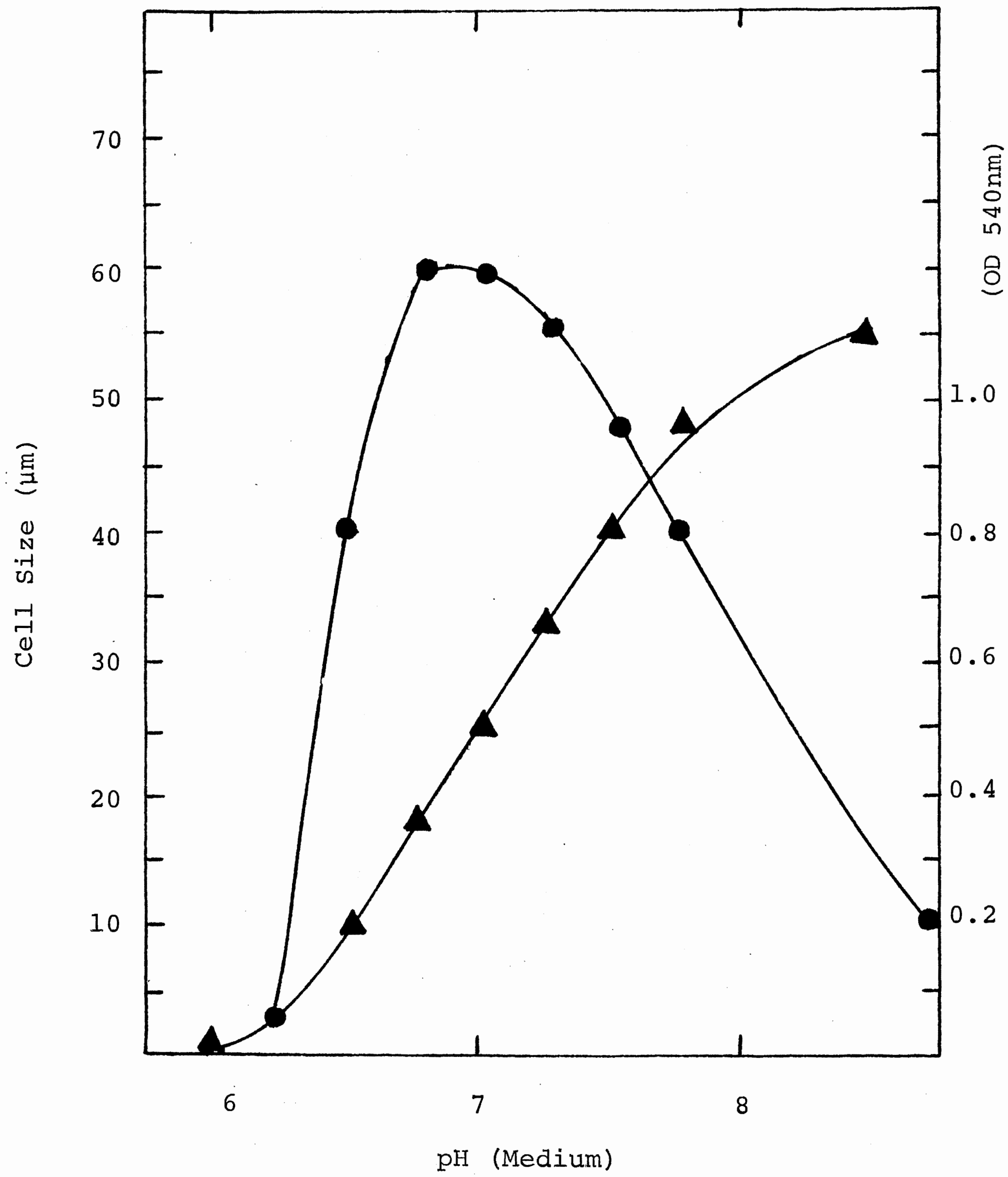
Growth Medium pH

As explained in the Methods and Materials section, the organism is grown in a medium wherein the initial pH is 6.8. To study possible correlations of pH with cell size, initial pH of the medium was adjusted from 5.8 to 8.7. Even though the organism does not grow at pH values less than 5.8, growth at 6.0-6.2 gives rise to cells that are no more than 10-15 μm in length 17 hours after inoculation. With a shift of pH towards neutrality a concomitant increase in cell size was observed. Cells growing in media where the initial pH varied from 6.5 to 7.4 showed predominantly filamentous cells (over 40 μm in length). Even though an increase of pH from the acidic side towards the alkaline side enhances filament formation, pH values more alkaline than 7.4 cause production of cells that are fairly short. Growth in media having an initial pH value of 8.7 were approximately 10 μm in length at 17 hours in the presence of NaF. Data pertaining to the effects of pH on cell size are shown in Figure 3.

Aspartic Acid

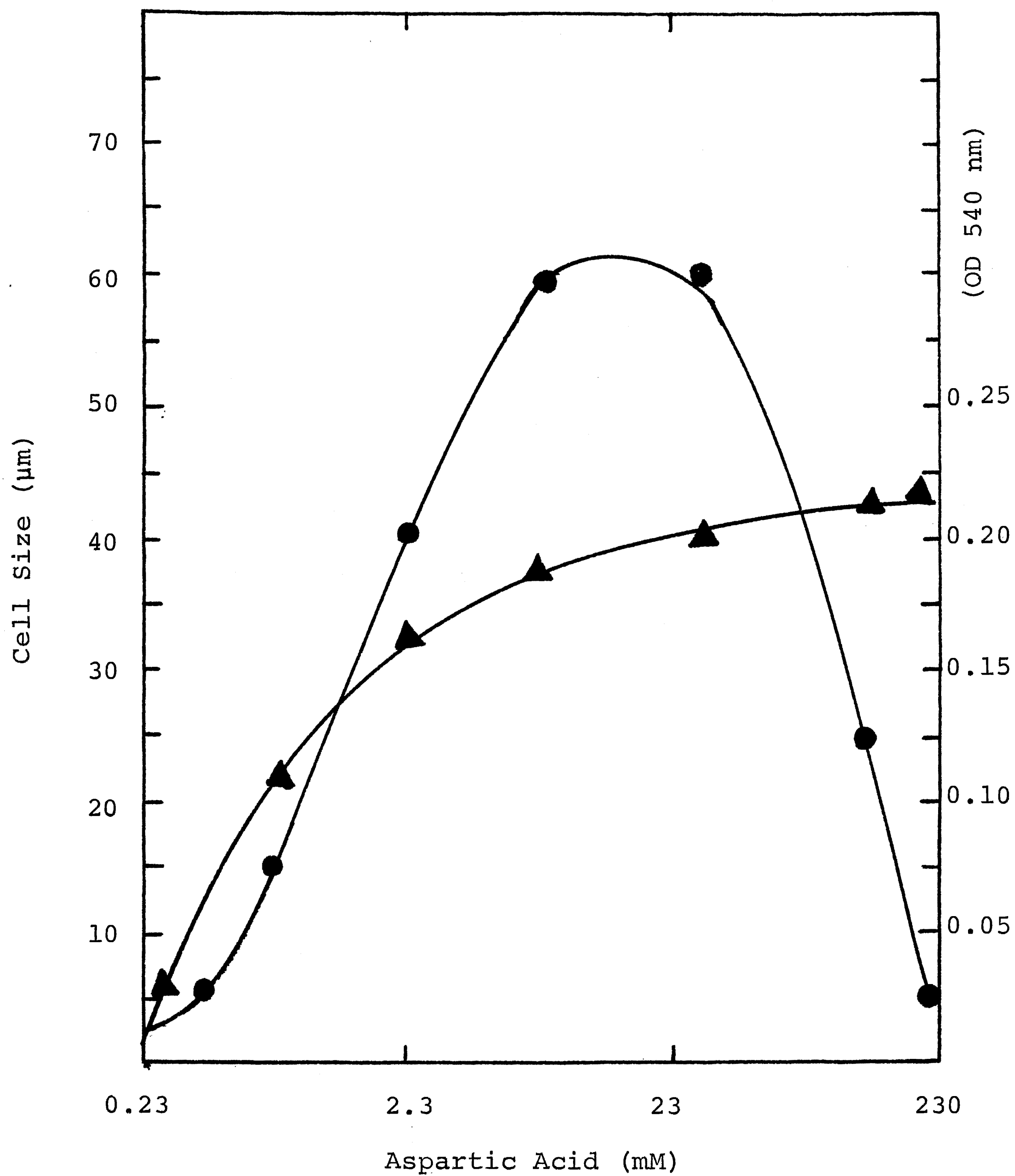
Aspartic acid is one of the essential nutrients for filament formation. Studies made using various concentrations of aspartic acid in the GA medium formulation in the presence of NaF (Figure 4) clearly show that a range of concentration of this compound must exist in the medium before filament formation is possible. Any concentration of

Figure 3. Effect of various initial pH values of the GAF medium on filament formation. Cell size determinations (●, closed circles) were performed at 17 hours of growth (▲, closed triangles). Concentration of NaF was 1.6×10^{-2} M.



70
60
50
40
30
20
10
Cell Size (μm)
(OD 540nm)
1.0
0.8
0.6
0.4
0.2
6
7
8
pH (Medium)

Figure 4. Effect of various concentrations of aspartic acid in the GAF medium on filament formation. Cell size determinations (●, closed circles) were performed at 17 hours of growth (▲, closed triangles) in the presence of NaF (1.6×10^{-2} M).



70
60
50
40
30
20
10
Cell Size (µm)
(OD 540 nm)
0.25
0.20
0.15
0.10
0.05
0.23
2.3
23
230
Aspartic Acid (mM)

aspartic acid less than 1 x 10^{-3} M or greater than 0.1 M gives rise to cells that are always short (10 μm or less). The existence of a lower limit (1 x 10^{-3} M) in which the cells are short helped us to investigate if filament formation was attributable to either the amino or dicarboxylic groups of the aspartic acid. Cells growing in GA medium containing 1 x 10^{-2} M aspartic acid are filamentous after 12 hours of growth. Using the GA medium containing only 1 x 10^{-3} M aspartic acid we added 9 x 10^{-3} M concentrations of either L-glycyl-L-aspartic acid (to check if the free dicarboxylic groups are needed for filament formation) or L-aspartyldiethyl ester (to determine if the free amino group is needed for filament formation) or α-methylaspartic acid (an analog that contains free amino and carboxylic groups). In these experiments, where the total concentration of each analog and aspartic acid was 1 x 10^{-2} M, growth and cell size determinations were made over a 24-hour period of growth. Filament formation did not occur under conditions where glycylaspartic acid and aspartyldiethyl ester were used; however, excellent filamentous growth occurs in the presence of α-methylaspartate. It appears therefore that both the amino and carboxylic groups have to be available and in the free form to permit inhibition of cell division in the presence of NaF.

Aspartic acid enters the tricarboxylic acid (TCA) cycle either in the form of oxaloacetate (OAA) after undergoing transamination with α-ketoglutarate or in the form of

fumarate after a direct elimination of ammonia (NH_3) by the enzyme aspartase (according to Dr. M. Grula, the enzyme is present and active in both normal and NaF-grown filamentous cells). The presence of 4-carbon dicarboxylic acids (particularly malic, OAA or fumaric acids) in our medium formulations always increases the growth rate of the test organism. It appears therefore the carbon skeleton of aspartic acid is used extensively for synthesis of micro- and macromolecules by the cells. Since induction of filamentous growth is directly related to the metabolism of aspartic acid it appeared necessary to investigate further the metabolism of this amino acid in _E. carotovora_.

Distribution of Carbon from U-^{14}C-L-Aspartic Acid

Depletion of glucose and aspartic acid from the GA and GAF media was determined at various time intervals during growth. The rate of disappearance of these two compounds was found to be much lower in GAF than in GA medium. Since aspartic acid is responsible for the induction and sustenance of filamentous growth in GAF medium, further investigation of the distribution of its carbon into cellular materials was conducted. To determine if differences exist in the distribution of carbon from aspartic acid in the test organism when it is allowed to grow in GA medium in the presence and absence of NaF, U-^{14} C-L-aspartic acid was incorporated into the growth medium. Distribution of carbon from aspartic acid,

i.e., the labelling pattern in the different cellular micro- and macromolecules was determined using the fractionation procedure of Park and Hancock (1960). There appears to be little or no significant difference in labelling patterns in normal versus filamentous cells (Table III).

Effect of TCA Cycle Intermediates on Filament Formation

Sodium fluoride is known to inhibit pyruvate, α-keto-glutarate, and succinate dehydrogenase enzyme complexes (Slater and Bonner, 1952; Terner, 1955a,b). To determine if filament formation is a direct consequence of the block of any or all of these enzyme complexes, we supplemented the glucose-aspartic-NaF (GAF) medium with various concentrations of the TCA cycle intermediates (citrate, malate, fumarate or succinate). Each of these intermediates was used at concentrations of from 0.5 to 1×10^{-7} M. Concentrations higher than 0.5 M prevented filament formation, while lower concentrations allowed filamentous growth to proceed. Even though filament formation was not inhibited at these concentrations ($0.5\text{-}1 \times 10^{-7}$ M), the rate of growth was drastically increased in the presence of these TCA intermediates.

p-Nitrophenol (1×10^{-3} to 1×10^{-5} M) is known to overcome NaF-induced inactivation of the pyruvate and α-keto-glutarate dehydrogenases; therefore, this compound (1×10^{-2} to 1×10^{-5} M) was studied for its possible effects on filament formation. In spite of its toxicity at concentrations

TABLE III

LABELLING PATTERNS OF *ERWINIA CAROTOVORA* AFTER 17 HOURS OF GROWTH IN THE GA AND GAF MEDIA*

Fraction	Short Cells†	Filaments†
Cold TCA (amino acids and acid soluble components)	780	700
75% Ethanol (lipids and some proteins)	950	1,050
Hot TCA (nucleic acids and breakdown products)	770	620
Trypsin Soluble (proteins)	3,980	3,360
Residue (mucopeptide and trypsin insoluble proteins)	1,740	2,210
Total Counts Obtained	8,220	7,940

*U-^{14}C-L-aspartic acid 0.02 μc/ml of medium was added at 12 hours of growth

†CPM/mg dry weight of cells

greater than 1 x 10^{-2} M, lower concentrations allowed the growth of filamentous cells. The growth rate of these filamentous cells was similar to that obtained in the GAF medium containing TCA intermediates, i.e., high.

In addition to the above studies, the types of keto acids excreted into the medium during the process of normal and filamentous growth were also studied. Since various inhibitors of filament formation (low phosphate, manganese ion, pantoyl lactone, high pH, n-propanol and low aspartate) overcome the effect of NaF they were also employed in this study to determine if a correlation exists between cell division and type and/or quantity of keto acid excretion.

As shown in Table IV, there is no direct relationship between inhibition of filament formation and either the quantity or type of keto acid excreted. Moreover, the inhibitors of filament formation do not display a common trend in affecting excretion of keto acids by the cells. Even if there was a disparity in the amount of keto acid excreted per unit time per mg dry weight of cells, the types of keto acids isolated were the same in all cases. Also, although approximately 5% of the total keto acids excreted into the medium at pH 8.7 was α-ketoglutarate, it was not present in detectable quantities at the pH of 6.8. On the other hand, pyruvate and oxaloacetate accounted for 70-75% and 20-25% of the total keto acids excreted into the medium, respectively. The types of keto acid and the equivalent converted amino acids are shown in Table V.

TABLE IV

THE INFLUENCE OF INHIBITORS OF FILAMENT FORMATION ON KETO ACID EXCRETION AND CO_2 EVOLUTION

Medium Additions	mg/keto acid*	CPM of $^{14}CO_2$ †
GA (pH 6.8)	1.3	95,000
GAF (pH 6.8)	0.8	80,000
GA (pH 8.7)	1.5	41,000
GAF (pH 8.7)	1.5	50,000
GA + $MnCl_2$ (1×10^{-4} M)	1.0	101,000
GAF + $MnCl_2$ (1×10^{-4} M)	1.0	120,000
GA + PL (6.8×10^{-2} M)	0.4	70,000
GAF + PL (6.8×10^{-2} M)	0.4	79,000
GA + n-propanol (0.7%)	3.0	107,000
GAF + n-propanol (0.7%)	3.0	144,000
GA with low PO_4 (1×10^{-4} M)	5.3	66,000
GAF with low PO_4 (1×10^{-4} M)	4.5	72,000

*mg keto acid/mg dry weight of cells/hr. Quantitated by comparison to known amounts of pyruvic acid.

† $^{14}CO_2$ counts/min released/mg dry weight of cells/hr

TABLE V

HYDRAZONE-FORMING COMPOUNDS PRESENT IN SPENT MEDIA (GA AND GAF)

Compounds	Rf Values*	Amino Acid Formed†
Pyruvate hydrazone	0.27	Alanine
α-ketoglutarate hydrazone	0.06	Glutamic acid
Oxaloacetate hydrazone	0.09	Aspartic acid
GA medium:		
Unknown-1	0.26	Alanine
-2	0.06	Glutamic acid¶
-3	0.09	Aspartic Acid
GAF medium:		
Unknown-1	0.27	Alanine
-2	0.05	Glutamic acid¶
-3	0.085	Aspartic acid

*Solvent system was: n-butanol-ethanol-0.5 N NH_4OH (70:10:20)

†Solvent system was that of Heathcote and Jones (1960). The amino acid was formed from the corresponding fatty acid hydrazone using platinum black as a catalyst in a reducing environment (bubbling hydrogen gas). The procedure was that of Kun and Hernandez (1956) and is used to prove the identity of keto acid hydrazones.

¶α-ketoglutarate hydrazone was found in measurable quantity only in media where the pH was 8.7.

The large quantity of pyruvate (approximately 70-75% of all keto acids) in the spent medium suggested a possible involvement of the reverse reaction of the anaplerotic reactions to provide the cells with 3-carbon intermediates of glycolysis. Since this reaction is accompanied by the evolution of carbon dioxide, we trapped $^{14}CO_2$ released during the metabolism of U-^{14}C-L-aspartic acid in the presence and absence of NaF. Even though the CO_2 that will be released during the metabolism of aspartic acid cannot be exclusively due to the decarboxylation of OAA or fumarate to yield phosphoenolpyruvate or pyruvate, it was hoped that a significant difference might be detected between normal and filamentous cells. Data pertaining to CO_2 evolution in GAF medium containing various inhibitors of filamentous growth are also shown in Table IV. As can be seen, there is no direct and consistent positive or negative relationship between CO_2 evolution and inhibition of division by NaF or its reversal by various compounds or conditions. Moreover, as shown in Table IV, there was no correlation between CO_2 production and keto acid excretion. Also, incorporation of pyruvate (1×10^{-2} M to 1×10^{-8} M) into the GAF medium does not prevent filament formation, although the growth rate is increased.

Because aspartic acid can be converted to β-alanine by decarboxylation accompanied with CO_2 evolution, β-alanine (1×10^{-2} to 1×10^{-8} M) was also incorporated into the GAF

medium. It was observed that induction of filamentous growth was not prevented.

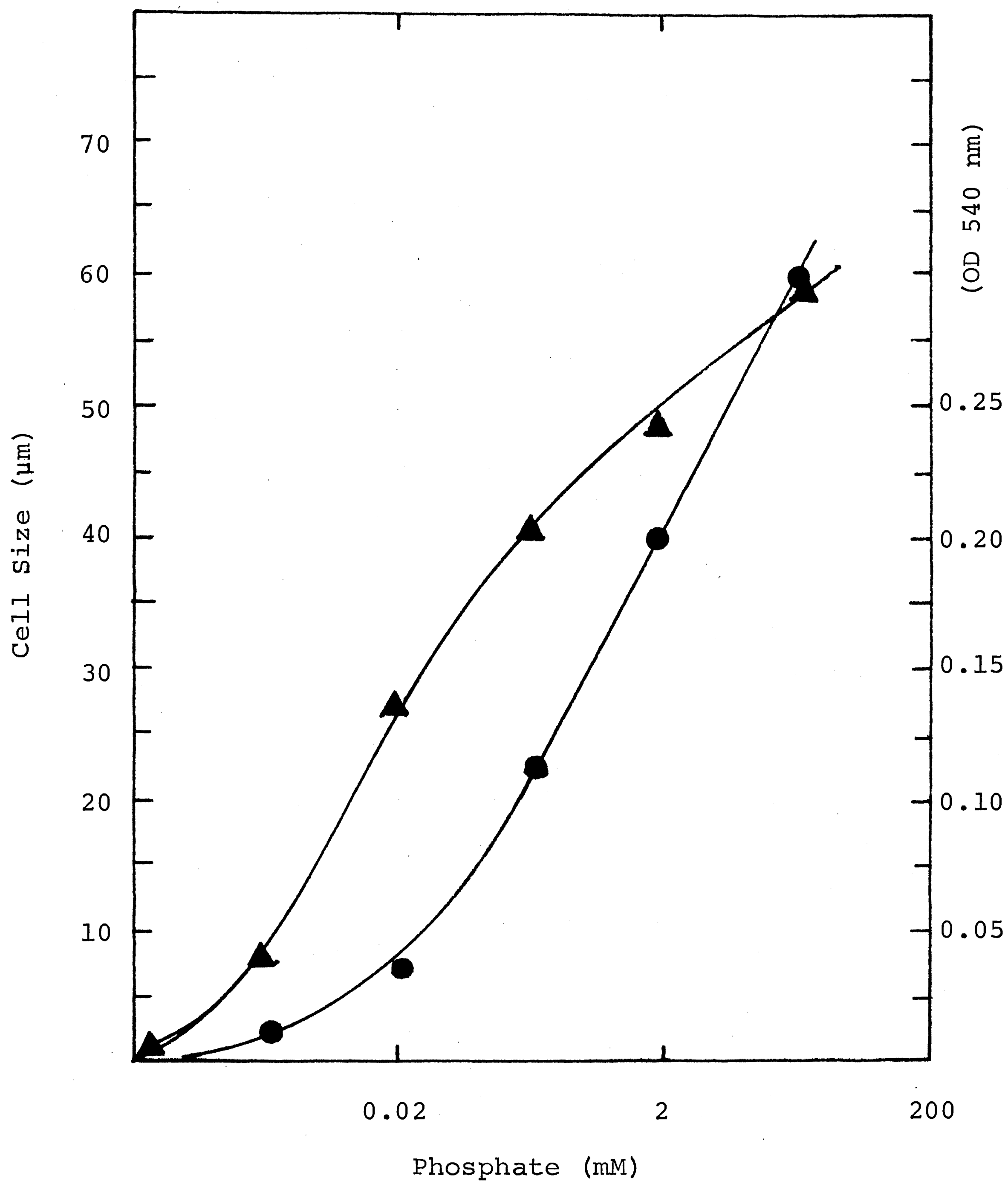
Aspartic acid metabolism was not investigated further since these rather "broad" types of metabolic studies did not point the way to any particular area that could be profitably explored.

Phosphate

Phosphate (potassium salt) in the GA medium serves as an inorganic nutrient as well as buffer. To investigate its role as a buffer in filament formation we substituted N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) at the same molar concentration and same pH value. Unlike aspartic acid and NaF, phosphate has only a lower limit for induction of filamentous growth. Growth of cells occurs in a medium containing phosphate at less than 1.4×10^{-4} M concentrations (Figure 5); however, even in the presence of NaF, the cells are always short. An increase in the concentration of phosphate (greater than 1.4×10^{-4} M) brings about an increase in cell size until the majority of the cells are over 40 μm in length when the concentration approaches 2×10^{-4} M. Any additional increase in phosphate concentration (beyond 2×10^{-4} M) does not cause a further increase in cell size.

The direct correlation between increase in phosphate concentration and filamentation suggests formation of a possible fluorophosphate complex similar to the type Warburg and Christian (1942) found when studying the effects of NaF on

Figure 5. Effect of various concentrations of phosphate in the GAF medium on filament formation. Cell size determinations (●, closed circles) were performed at 17 hours of growth (▲, closed triangles) in the presence of NaF (1.6×10^{-2} M).



Cell Size (μm)
70
60
50
40
30
20
10
(OD 540 nm)
0.25
0.20
0.15
0.10
0.05
0.02
2
200
Phosphate (mM)

the enzyme enolase. If complex formation occurs in Erwinia, it is most likely very complex and not subject to direct analysis at this time since we are working with a growing cell system. In addition, several compounds are involved (phosphate, NaF, divalent cations and aspartic acid), one of which is extensively metabolized.

Because filamentous growth occurs only when phosphate is present at a concentration greater than 1.4×10^{-4} M, we determined the amount of total phosphate (as an inorganic form after ashing) and the types of phosphorylated compounds (using acid extraction procedures) in normal and filamentous cells. We also studied the effects inhibitors of filamentous growth (manganese ion, high pH, n-propanol, low phosphate, low aspartate) have on total phosphate concentration in cells grown in GA and GAF media. Since the exogenous phosphate present in the medium has to be removed to permit good quantitation, all cell preparations were washed three times in cold trace mineral solution supplemented with 1×10^{-4} M $MgSO_4$ and 0.02 M HEPES, pH 6.8. The sedimented cells were treated as explained in the Methods and Materials section, either for total phosphate determinations (Bennet and Williams, 1957) or fractionated (Park and Hancock, 1960) to determine the distribution of phosphate-containing compounds in different fractions of the cell.

The total phosphate concentrations of short cells (except those that have grown into filamentous forms in GAF medium) was always found to be at least 30-40% less (Table VI)

TABLE VI

THE INFLUENCE OF INHIBITORS OF FILAMENT FORMATION ON THE TOTAL PHOSPHORUS CONTENT OF E. CAROTOVORA WHEN GROWN IN GA AND GAF MEDIA

Medium Formulations	Cell Size*	Total Phosphorus (μg/mg Dry Weight of Cells)
Aspartic acid alone (pH 6.8)	S	35
Aspartic acid-NaF (pH 6.8)	F	60
GA (pH 6.8)	S	40
GAF (pH 6.8)	F	40 - 70
GA (pH 8.7)	S	25
GAF (pH 8.7)	S	30
GA + $MnCl_2$ (1×10^{-4} M)	S	20
GAF + $MnCl_2$ (1×10^{-4} M)	S	25
GA + PL (6.8×10^{-2} M)	S	40
GAF + PL (6.8×10^{-2} M)	S	60
GA + n-propanol (0.7%)	S	36
GAF + n-propanol (0.7%)	S	60
GA with low PO_4 (1×10^{-4} M)	S	35
GAF with low PO_4 (1×10^{-4} M)	S	40

*Cell size determinations were made after 17 hours of growth: S indicates short; F indicates filaments.

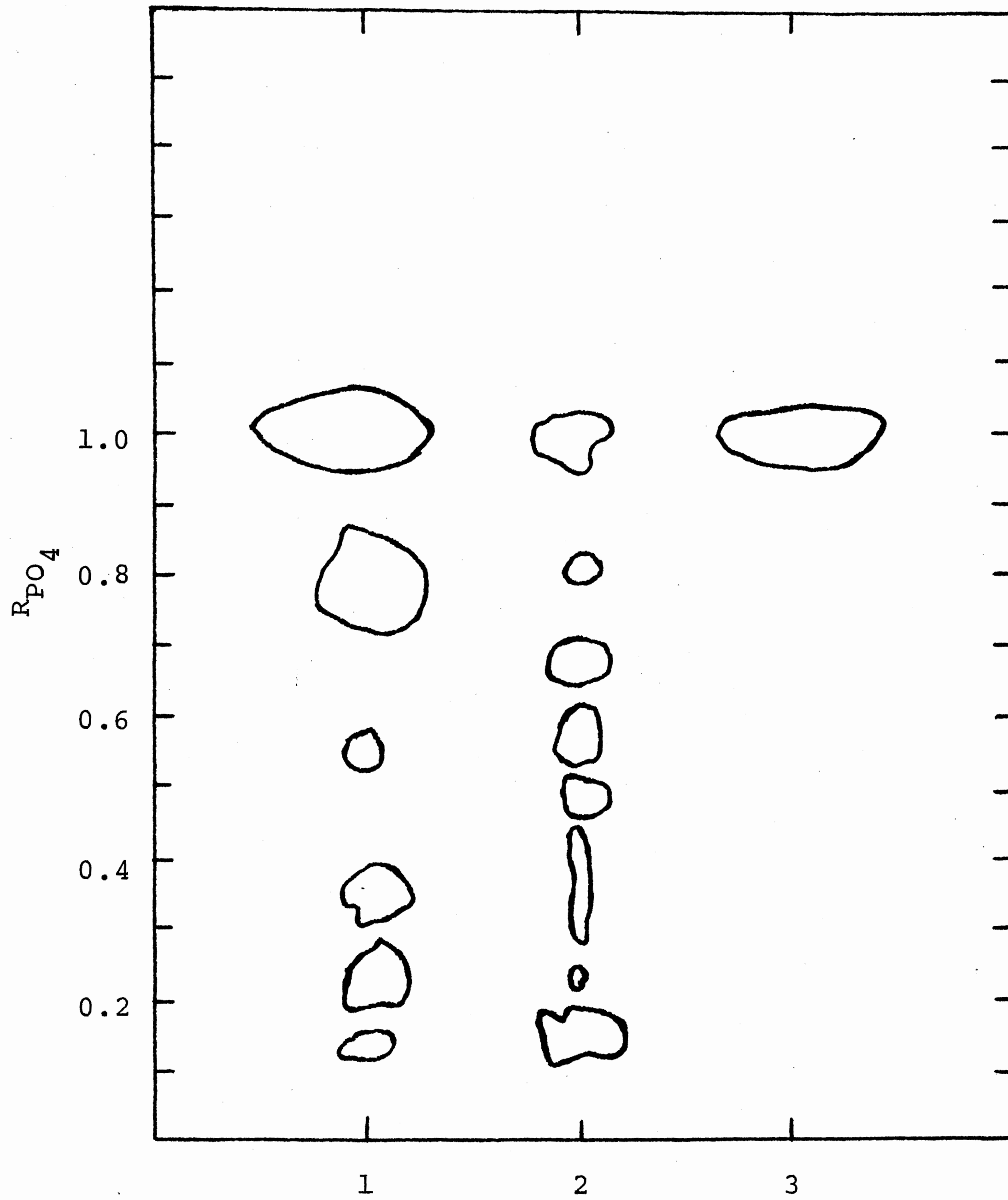
than that found in filaments. One common factor (Table VI) that all inhibitors of filament formation have (exceptions are pantoyl lactone and n-propanol, which are excellent inhibitors of filament formation but showed no effect on the phosphate content whatever) is the inhibition in the increase of total phosphate (concentration of phosphate in all cases was less than or equal to the content of phosphate in normal cells) in cells grown in GAF medium.

The apparent increase in total phosphate of filamentous cells convinced us of the need to determine where, i.e., in which macromolecule of the cell the increase occurred. Even though combined absolute values were not repeatable in the various fractions obtained using the Park and Hancock (1960) procedure, we always found more phosphate (after ashing) in the cold 10% perchloric acid extract and more organic phospate in the 75% ethanol extract of filaments. On the other hand, less phosphate was found in the protein-mucopeptide fraction of filamentous cells. The hot perchloric acid extract which comprises mainly the nucleic acids were about the same in both types of cells. To be sure that there was no difference in total quantity of nucleic acids and types of nucleotides, extraction of nucleotides were performed on normal and filamentous cells using hot 5% TCA (Park and Hancock, 1960). Two-dimensional chromatography (Dorough and Seaton, 1954) was performed using n-butanol, acetic acid, water (4:1:1) in the first direction. After drying, the chromatographs were again run in the first direction using

acetone, n-butanol and water (8:1:1). The second direction utilized saturated ammonium sulfate, isopropanol, water (79: 1:10). Spots were detected using ultraviolet light. Because there appeared to be no qualitative or quantitative differences in those nucleotides that are readily resolved by this system (ADP and ATP are poorly resolved), such compounds do not appear to be directly involved in the cell division process in E. carotovora.

The high content of inorganic and organic phosphates in the first two extracts were chromatographed on Whatman paper #1 to see if there existed differences only in the quantities available or if a qualitative difference also existed. Under these chromatographic conditions, we observed gross differences in both quality and quantity of phosphorylated compounds, especially sugar phosphates in normal and filamentous cells. The most predominant phosphorylated compounds as well as their appropriate location and amount observed to be present either in excess or in extremely low quantities are presented in Figure 6. Values (R_{PO_4}) were computed relative to the inorganic phosphate spot. Tentative surveys made on phosphorylated compounds indicate that differences in 6-carbon, 3-carbon and 5-carbon phosphorylated sugars exist between filamentous and normal cells. These tentative identifications are as follows: R_{PO_4} 0.22 found in large quantities in filamentous cells has not yet been identified; R_{PO_4} 0.38 found in large amounts in filaments could be F-1,6-PP, G-1-P or G-6-P; R_{PO_4} 0.56 found in both types of cells but in

Figure 6. Chromatographic separation of phosphorylated compounds. Symbols are as follows: 1, filaments; 2, normal cells; 3, inorganic phosphate. The R_{PO_4} values were obtained using inorganic phosphate as the spot for reference. The solvent used was n-butanol, n-propanol, acetone, 88% formic acid, 30% TCA (40:20:25:25:15) run twice. The spray reagent was that of Wade and Morgan (1953).


1.0
0.8
0.6
0.4
0.2
R_{PO_4}
1
2
3

larger amounts in short cells could be ribose-5-phosphate, ribulose-5-phosphate, xylulose-5-phosphate; R_{PO_4} 0.75 found in filaments in very large quantity could be any of the 3-carbon phosphorylated sugars such as glycerol phosphates. None of these identifications are intended to be final. Further work is progressing relative to identification of these compounds.

Study on Phosphatases (Phosphomonoesterases)

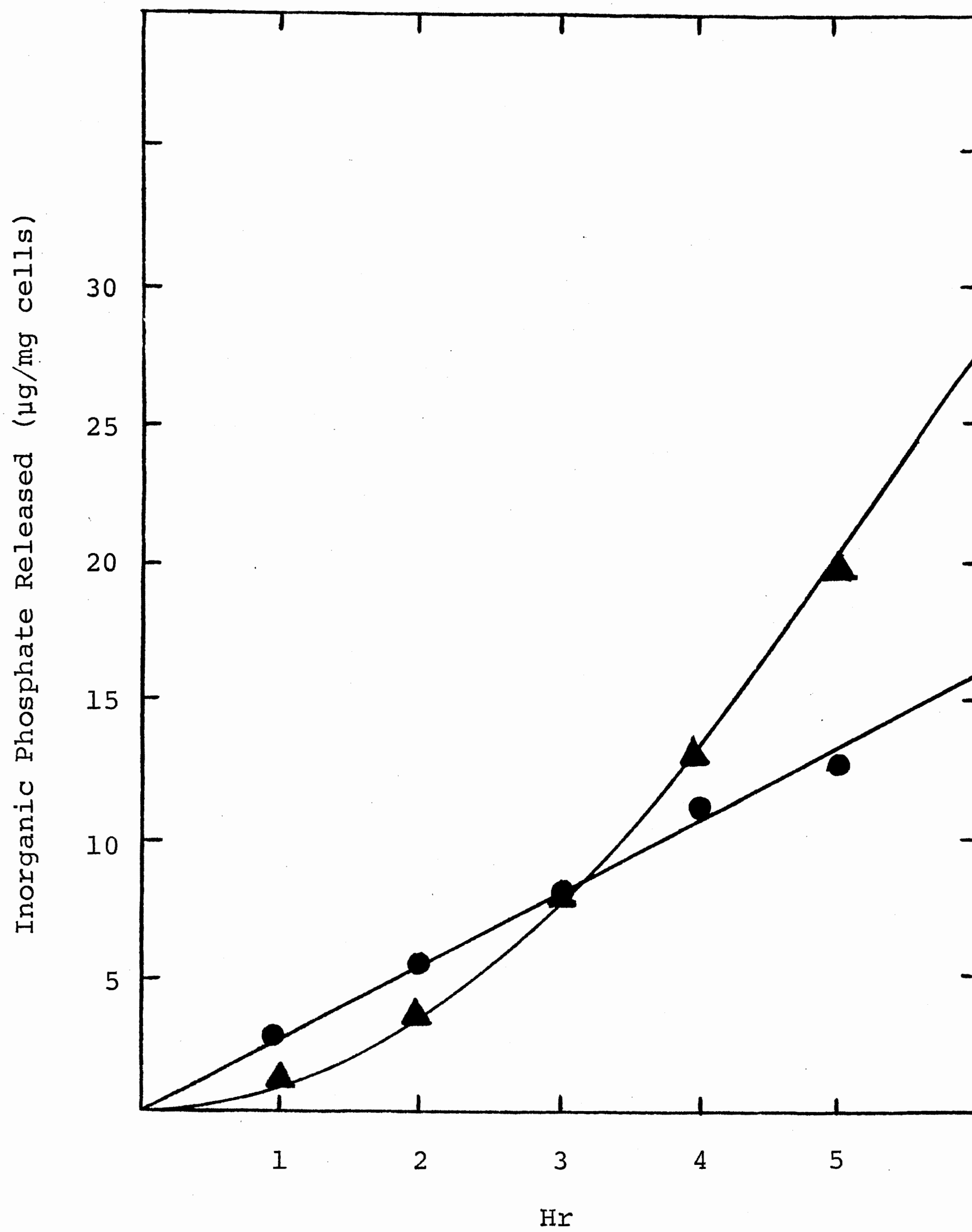
The fact that more total phosphate is available in filaments suggests that either accumulation of phosphorylated compounds has occurred or the rate of dephosphorylation of certain molecules is hampered in the presence of NaF. To resolve which of these possibilities was occurring, it was decided that a study of the activities of phosphatases (phosphomonoesterases) in normal cells and filaments should be done.

One approach that was utilized to study the activities of the phosphomonoesterases was through the use of the cells themselves as source for both the phosphatases and the phosphate ester substrates. Cells, grown for 17 hours (short cells and filaments), were washed three times with cold trace mineral solution supplemented with 1×10^{-4} M $MgSO_4$ and 0.02 M HEPES, pH 6.8 in a 4° C room. The sediments were then dispersed with fresh trace mineral solution such that 5-10 mg/ml dry weight of cells was achieved and these were then placed

on a rotary shaker in a 25° C room to allow cellular phosphomonoesterase activity to occur. Whenever supernatant samples for inorganic phosphate determinations were taken, OD measurements were performed on the cell culture to monitor the presence of inorganic phosphate from lysing cells (especially from filaments). As shown in Figure 7, the amount of inorganic phosphate released by the filaments is much lower than control cells for the first three hours of incubation; however, upon continued incubation the total inorganic phosphate released by the filaments increased until it surpassed that of the control cells. This increased release of inorganic phosphate from filaments with time was not associated with any apparent lysis.

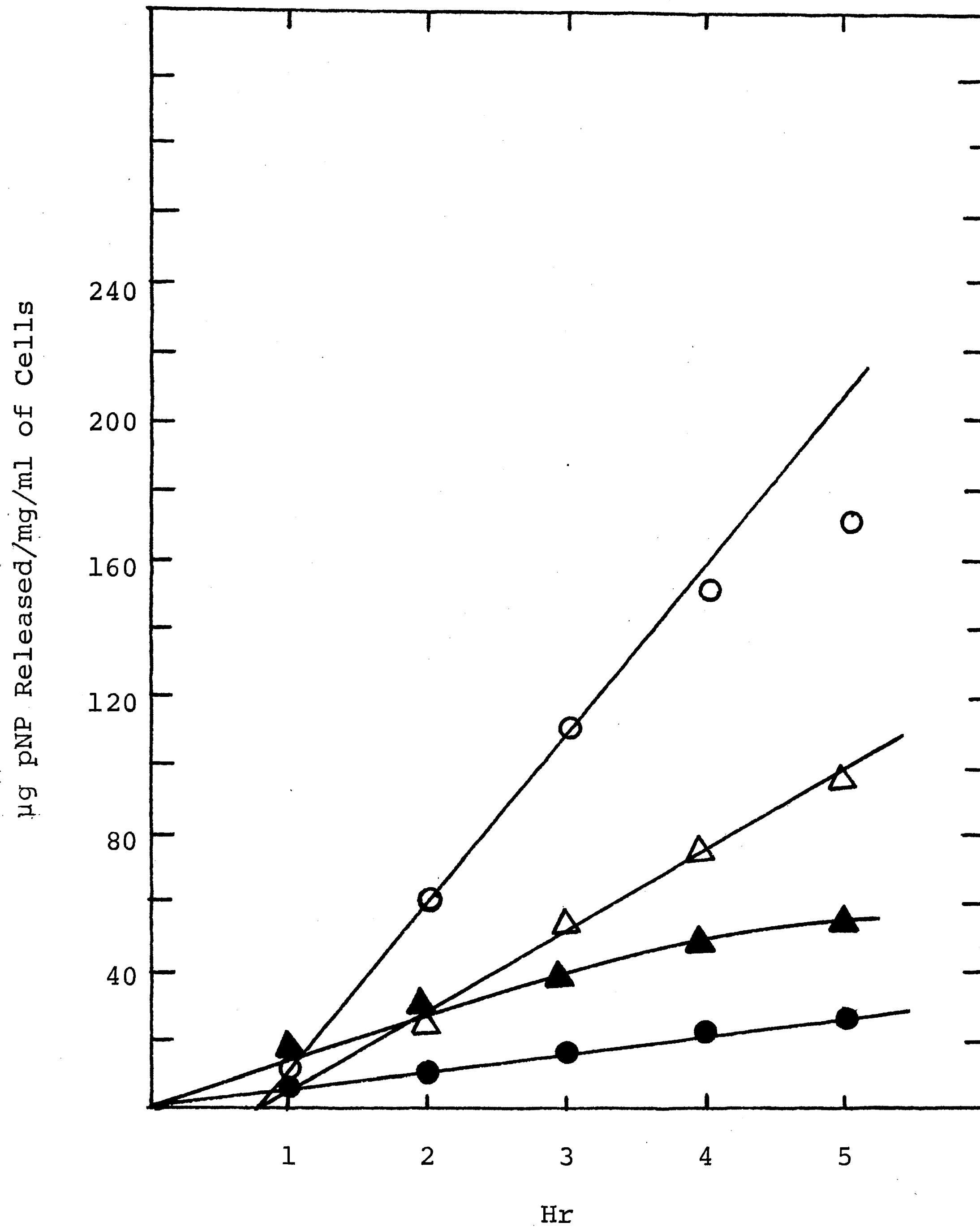
Since the approach used to determine the activity of the phosphatases using the cells as source for both the substrate and the enzyme might be giving an erroneous picture (due to possible cell lysis), it was decided to monitor the activities of the phosphatases using an artificial substrate. As shown in Figure 8, filaments contain significantly more phosphomonoesterase(s) activity than normal cells at pH 6.8. Since alkaline pH prevents filament formation, the activity of the phosphatases was checked at pH 9.0 (maximum activity for the test organism was found to be approximately pH 9.0 [Figure 8]). At this pH value (9.0) normal cells contain extremely high enzymatic activity as compared to their activity at pH 6.8. On the other hand the activity in filaments does not appear to be too great at the two pH values. The

Figure 7. Activity of phosphatases in normal cells and filaments at pH 6.8 determined using the cells themselves as sources of phosphate ester substrates. Normal cells: ●, closed circles; filaments: ▲, closed triangles.



Inorganic Phosphate Released (µg/mg cells)
30
25
20
15
10
5
1
2
3
4
5
Hr

Figure 8. Effect of pH on phosphomonoesterase activities of normal and filamentous cells. Control cells: ○, open circle, pH 9.0; ●, closed circles, pH 6.8. Filaments: △, open triangles, pH 9.0; ▲, closed triangles, pH 6.8.


240
200
160
120
80
40
μg pNP Released/mg/ml of Cells
1
2
3
4
5
Hr

high activity of normal cells is most probably due to their content of periplasmic enzymes. Many of these enzymes are released into the medium during growth of filamentous cells and are therefore not present (Grula and Hopfer, 1972).

To aid in determining if more than one phosphomonoesterase was present in normal and filamentous cells NaF was added at various concentrations (1×10^{-5} - 1×10^{-2} M) to the assay mixture at pH 9.0. At a concentration of only 5×10^{-4} M (at least 4×10^{-4} M is required to inhibit cell division in these cells), NaF decreases phosphatase activity by about 80% in normal cells; however, at the same concentration phosphatase activity is not decreased in filamentous cells. This experiment shows that the enzyme(s) from filaments is highly resistant to NaF at pH values of 6.8 and 9.0. Thus it appears that there are at least two types of enzyme(s) in the periplasmic space of these cells. One is sensitive to NaF and is the major enzyme present in normal cells. Even though it exhibits its optimum activity at pH 9.0, the enzyme is completely different from alkaline phosphatase (alkaline phosphatase is an inducible enzyme which is repressed by inorganic phosphate). The other type of enzyme appears to be most active at neutral pH and is present in near equal amounts in both filaments and normal cells. This enzyme is relatively insensitive to NaF at pH 6.8. Because filaments contain more phosphatase activity at pH 6.8 with the impermeable artificial substrate (p-NPP), it is possible that such expression is a result of the damaged and therefore more

highly permeable envelope known to be present in such cells (Grula and Hopfer, 1972).

Because the phosphatase activity (pH 9.0) is severely depressed by NaF, it is possible that this enzyme activity is directly associated with cell division. This is most likely not the case since NaF does not inhibit division when cells are incubated at pH 8.7 (Figure 3). Nevertheless, to study further the possible involvement of the phosphatase, stimulators of cell division in the presence of NaF (n-propanol, pantoyl lactone, and manganous chloride) were added to the assay mixtures to determine whether or not they are able to overcome the inhibitory effect of NaF on the enzyme activity. It was observed that these division-stimulating compounds do not prevent the inhibition of the enzyme activity (in fact, n-propanol and pantoyl lactone increased the activity by 50% in normal cells at pH 6.8).

Therefore, even though NaF can inhibit the activity of the phosphatase, such enzyme activity is probably not directly associated with cell division in _E. carotovora_.

Effect of Membrane-Perturbing Agents

Certain membrane-perturbing agents (short-chained water-miscible alcohols, detergents, chelating agents and anesthetics) were studied to determine if some further insights could be obtained relating to involvement of the cell membrane in the cell division process.

Short-chained alcohols have been found to increase membrane fluidity and act as antifreezes (Paterson et al., 1972; Woodson, Traynor, Schlapfer, and Barondes, 1976). Of these short-chained alcohols, isopropanol is also known to activate alkaline phosphatase. In order to study their effect on filament formation 0.2 to 2.0% concentrations of several alcohols were added to the GA medium. Even though methanol did not display any significant effect on filament formation, ethanol at a final concentration of 2% in the medium delayed formation of filaments for over 19 hours (filamentous growth normally ensues 10-12 hours after inoculation). Addition of n-propanol (0.8-2.0% in medium) completely prevented formation of filamentous cells. Like ethanol, lower concentrations of n-propanol (0.4-0.8%) also showed a delaying effect on the formation of filaments. Concentrations of n-propanol higher than 2.0% were observed to be very toxic to growth of cells.

The effectiveness of n-propanol in preventing filament formation, prompted us to include in the study, the branched isomer isopropanol. The minimum effective concentration of the branched isomer (isopropanol) capable of preventing filament formation was approximately twice that of n-propanol (minimum concentration for isporopanol was 1.4%, while that for n-propanol was 0.7%).

To determine if the straight-chain portion of the molecule was important in overcoming the effects of NaF on filament formation, 1,3-propanediol was included in the study.

At a concentration of 3.0%, this compound only slightly delayed the induction of filamentous growth. This result was similar to that obtained using ethanol at 2.0%. Because the presence of hydroxyl groups reduced the effectiveness of the molecule in preventing the emergence of filamentous growth, it indicates that a straight-chained hydrophobic moiety in the alcohol is necessary to correctly perturb the membrane and allow formation of normal-sized cells in the presence of NaF.

Triton X-100 (0.1-4.0% in medium), sodium lauryl sulfate (5-100 μg/ml in medium) and N-ethylmorpholium ethosulfate (0.2-20 μg/ml in medium) were also added in the presence and absence of NaF. Triton X-100, a non-ionic detergent, delayed filament formation for 4-6 hours when used at 1.5-2.0% concentrations; however, it prevented growth when used in concentrations over 2.0%. Sodium lauryl sulfate, an anionic detergent, is unlike Triton X-100 in that it causes cell envelope damage at medium concentrations between 50-100 μg/ml as evidenced by the emergence of bulges in both normal (non-NaF) and NaF-treated cells (more noticeable on the NaF-treated cells). Other than the damage caused by SLS to the cell envelope, filament formation was not stopped. The cationic detergent N-ethylmorpholium ethosulfate prevented growth when present at more than 6.0 μg/ml in the medium; however, at concentrations which allow growth to occur, it does not exhibit any effects on filament formation.

Other compounds such as EDTA, lidocaine, dibucaine and propranolol (1×10^{-2} - 1×10^{-7} M) were also used to study possible inhibition of filament formation in the presence of NaF. Even though damages to the cell envelope were observed (extensive lysis and bulgings at higher concentrations by all compounds) filament formation was never prevented by these compounds regardless of the concentration employed.

Inhibitors of Filament Formation

Many enzymes require the presence of cations (such as magnesium, manganese, zinc) for complete or maximum activity. Fluoride ions are known to interact with the cations and/or enzyme complexes containing cations thereby inactivating such enzyme system(s) (Hodge and Smith, 1965; Slater and Bonner, 1952). It was thought possible that such an inactivated enzyme system(s) or the mere unavailability of an essential cation(s) (especially if insoluble complexes are formed with cations such as magnesium) could be responsible for the filamentous growth in the test organism. Therefore, the role(s) played by cations and anions in the induction and maintenance of the filamentous state was studied using chloride and sulfate salts of mono- and divalent cations. In addition to these studies, we also investigated the effects of phosphorylation inhibitors, antimicrotubule-microfilament drugs, membrane-perturbing agents, sulfhydryl group blocking or disrupting agents and inhibitors of cAMP phosphodiesterases on filament formation.

Chloride salts of divalent cations when used at high concentrations (1×10^{-4} M or more) prevent or retard filament formation. Unlike other chloride salts, manganous chloride completely prevented the induction of filamentous growth at 5×10^{-5} M concentrations. GAF medium supplemented with ammonium chloride (5×10^{-3} M or more), like the glucose-ammonia medium, allowed growth only of normal (short) cells. Even though incorporation of these salts at high concentrations (1×10^{-4} M or more) at the time of inoculation prevents and/or delays filamentous growth, their addition into the medium after filament formation has begun (10-12 hours after inoculation) does not prevent the cells from being filamentous. However, in such cases (with the exception of filaments treated with manganous chloride) the rate of reversion (when incubation is continued for over 17 hours) was found to be faster than in control filaments.

Sulfate salts of divalent cations also prevent filament formation when they are available at concentrations greater than 1×10^{-3} M in the GAF medium. The salts of monovalent cations do not prevent filamentous growth when used at the same molar concentrations.

These studies indicate that a high concentration of either a divalent cation or the chloride anion can overcome the action of NaF.

Cysteine at concentrations greater than 1×10^{-4} M prevents filamentous growth when added into the medium before filamentous growth has begun. Since possible metabolic

block(s) in the synthesis of cysteine in the presence of NaF were suspected, we supplemented the medium with most intermediates (sodium sulfide, sodium bisulfite, O-acetylserine) that are involved in the synthesis of this amino acid. These intermediates utilized did not prevent the development of filamentous cells in the presence of NaF.

Arsenate, which forms highly unstable ester bonds (compared to phosphate ester bonds, delays filament formation for as much as 17 hours at 1×10^{-3} M concentration. Other inhibitors of phosphorylation (azide, cyanide and dinitrophenol) do not prevent filament formation when used at concentrations that permit growth.

Agents known to disrupt sulfhydryl bonds (mercaptoethanol, mersalyl acid, N-ethylmaleimide), antimicrotubular and/or microfilamentous drugs (colchicine, vineblastine, cytochalasin B) and inhibitors of cAMP phosphodiesterases (theophylline, caffeine) also do not prevent filament formation (1×10^{-3} - 1×10^{-8} M).

These studies indicate that impairment of the energy-producing machinery, inactivation of enzymes or proteins whose activities or conformation are dependent on the presence of sulfhydryl bonds or changes in functionality of classical structures (such as microtubules, microfilaments aggregating or disaggregating) do not prevent filamentous growth from occurring in the presence of NaF.

Studies on Reversion

Reversion of filaments was studied, as explained in the Methods and Materials section, by first obtaining filamentous cells and then observing their ability to divide (revert to short form) in different types of media.

Because metal ions activate and/or inactivate most enzymes or proteins *in vitro* and *in vivo* it was essential that the effects of various types of monovalent and divalent cations on reversion of the filaments be evaluated. The chloride salts of calcium, magnesium, manganese, iron and zinc at concentrations of 1×10^{-7} M to 1×10^{-2} M were used separately as the only constituents in the reversion medium. All the metal ions used except manganous chloride improved the rate of reversion when used at 1×10^{-3} M concentrations. Manganous chloride, however, at concentrations greater than 1×10^{-5} M completely prevented reversion. Its inhibitory effect on reversion was of greater magnitude than that of chloramphenicol or inhibitors of oxidative phosphorylation. Magnesium and calcium ions at 1×10^{-3} M concentrations were found to aid reversion far better than any of the other salts tested, and magnesium appeared to be slightly better than calcium.

Sodium and potassium chloride at concentrations of 0.146 to 0.5 M were used to study the effects of ionic strength on reversion. At concentrations used, these salts did not facilitate reversion. At 0.5 M concentration some filaments

formed bulges or were converted to spheroplasts; some of them also underwent lysis. The damage done to the cell envelope was so extensive (especially those treated with KCl) that in less than 2 hours most (>70%) of the filaments lysed.

In addition to the salts used to study reversion, each component of the growth medium was also investigated. Since reversion appeared to occur regardless of the presence or absence of a carbon-nitrogen source it appeared necessary to select the best reversion formulations or conditions to study the phenomenon of reversion.

Effect of Magnesium Chloride

Of all divalent cations used only magnesium was found to enhance the rate of reversion when used at 1×10^{-2} M concentrations. As shown in Table VII, approximately 20% of the filaments had reverted after 5 hours of incubation at 25° C in the presence of magnesium ions only (reversion is defined as division of cells to a size of 10 μm or less).

Effect of Magnesium Chloride and Potassium Phosphate

Even though phosphate alone (2×10^{-2} M, pH 6.8) was not a good medium for reversion, in the presence of magnesium ions (2×10^{-2} M), it dramatically increased the rate of reversion. Cell size determinations of reverted cells after 5 hours of incubation in a medium containing magnesium ions and phosphate at pH 6.8 is shown in Table VII. Reversion in

TABLE VII

REVERSION OF FILAMENTS UNDER VARIOUS CONDITIONS*

Medium Formulations	Cell Length Range (μm) (% in Each Range)†				
	>40	30-40	20-30	10-20	<10
Mg^{++}	23	24	16	16	21
Mg^{++} - $PO_4^{\equiv}$	10	16	20	16	39
Mg^{++} - asp.	2	2	4	4	87
Mg^{++} - asp. - $PO_4^{\equiv}$	-	-	2	3	95

*Concentrations of the various components per 100 ml were as follows: $MgCl_2 \cdot 6H_2O$, 1 x 10^{-2} M; K_2HPO_4, 1 x 10^{-2} M; KH_2PO_4, 1 x 10^{-2} M; aspartic acid 2.25 x 10^{-2} M.

†Size estimations were made after 5 hrs incubation.

the presence of phosphate and magnesium ions was approximately 40%.

As shown in Table VIII, there was no increase in cell mass as observed by OD measurements of filaments incubated in the above media (non-growth-supporting). In fact we usually observed a decreased OD reading due to lysis of some of the filaments.

Effect of Aspartic Acid

Addition of aspartic acid (2.25×10^{-2} M, pH 6.8) to the magnesium chloride or magnesium chloride-potassium phosphate medium allowed reversion to be completed in the shortest time measured. Associated with the reversion was the rapid increase in mass as determined by OD readings (Table VIII). Addition of aspartic acid to the non-growth-supporting media permitted us to obtain over 85% reversion in 5 hours (Table VII). Those filaments which did not revert even after 10 hours of incubation in this medium were considered dead or greatly damaged and thus unable to perform the necessary cellular functions required for cell division.

These studies indicated that a carbon-energy source (aspartic acid), phosphate and magnesium ions are the only requirements for cell division as measured by reversion (division) of pre-formed filaments. Although not absolutely essential, it was also observed that aspartic acid greatly accelerates reversion.

TABLE VIII

MASS INCREASE OF FILAMENTS UNDER VARIOUS CONDITIONS OF REVERSION

Time (Hrs.)	Medium and Optical Density (540 nm)*			
	Non-Growth-Supporting		Growth-Supporting	
	Mg^{++}	Mg^{++}-PO_4	Mg^{++}-asp	Mg^{++}-PO_4-asp
0	0.39	0.39	0.39	0.39
2	0.38	03.8	0.54	0.58
4	0.39	0.39	0.65	0.66
5	0.39	0.395	0.68	0.71

*Concentration of media components is as given under Table I.

Effect of pH

Phosphate in the medium (2 x 10^{-2} M) serves both as a buffer and as a source of potassium and phosphate ions. It appeared essential that we separate the functions and determine which is necessary for reversion of filaments.

As shown in Table IX, reversion of filaments in magnesium-phosphate medium is greatly influenced by pH. At acid pH (6.0-6.6) the filaments formed bulges and tended to lyse. No measurable reversion occurred in the acid pH ranges in non-growth-supporting media even after 10 hours of incubation. As shown in Table IX, reversion increases with a shift in pH towards the alkaline side. The optimum pH for reversion appears to be between 7.2 and 7.6. To maintain the buffering capacity of phosphate we substituted N-2-hydroxyethylpiperazine-2-ethanesulfonic acid (HEPES), as an organic and non-degradable buffer (2 x 10^{-2} M). Even though the rate of reversion increased as the pH was raised towards the alkaline side, the percentage of reversion was poor and not comparable to that which occurred in the presence of phosphate. Addition of a small amount of phosphate (2 x 10^{-4} M) to the HEPES buffered media significantly increased the rate of reversion. These observations indicate that both pH and availability of phosphate play a role in bacterial cell division.

TABLE IX

EFFECT OF MEDIUM pH ON REVERSION*

Medium Formulations¶	Cell Length Range (μm) (% in Each Range)†				
	>40	30-40	20-30	10-20	<10
Mg^{++} - PO_4 (6.8)	19	17	16	13	36
Mg^{++} - PO_4 (7.4)	1	2	8	5	84
Mg^{++} - PO_4 (7.8)	-	1	2	2	95

*Desired pH values were obtained by varying the concentrations of K_2HPO_4 and KH_2PO_4; however, final phosphate concentration was always maintained at 0.02 M in the medium.

†Cell size determinations were made after incubation at 25° C on a rotary shaker for 5 hours.

¶Concentration of magnesium was as given under Table I.

Effect of Phosphorylation Inhibitors

Studies on growth and the rate of reversion of filaments in medium formulations containing various inhibitors of phosphorylation are shown in Table X. In all cases concentrations of 1×10^{-7} to 1×10^{-3} M were studied.

Sodium arsenate ($NaAsO_4$) at all concentrations tested did not prevent the initial reversion which takes place in the first hour and contributes to 20% of the reversion in both the non-growth- and growth-supporting media. However, once the initial reversion had occurred, further reversion was prevented in non-growth-supporting media (1×10^{-3} M, 5 hours). Filaments suspended in media containing aspartic acid (growth-supporting) began reverting after 6 hours. With the overcoming of this inhibition a mass increase of the cells also occurred.

Sodium azide (NaN_3) at all concentrations used did not significantly prevent reversion either in non-growth- or growth-supporting media.

Potassium cyanide (KCN) at 1×10^{-3} M concentration inhibits reversion. Its inhibitory effect is of the same magnitude in both types of media.

2,4-Dinitrophenol (DNP), like all the other inhibitors used, did not prevent the initial reversion that takes place during the first hour of incubation. Like arsenate, however, this compound is able to arrest reversion only for the first 6 hours in both types of media. In media containing aspartic

TABLE X

MASS INCREASE OF FILAMENTOUS CELLS INOCULATED IN THE PRESENCE OF INHIBITORS OF SUBSTRATE OR OXIDATIVE PHOSPHORYLATION*

Reversion Formulations†	Optical Density (540 nm) 0 hr.	2 hrs.	5 hrs.
NON-GROWTH-SUPPORTING:			
$MgCl_2 \cdot 6H_2O$ Only	0.24	0.24	0.23
+ $NaAsO_4$	0.24	0.23	0.22
+ NaN_3	0.23	0.23	0.22
+ KCN	0.22	0.22	0.22
+ DNP¶	0.23	0.22	0.20
Mg^{++} - $PO_4^{\equiv}$	0.22	0.21	0.22
+ $NaAsO_4$	0.22	0.21	0.20
+ NaN_3	0.23	0.23	0.22
+ KCN	0.21	0.20	0.19
+ DNP	0.21	0.20	0.16
GROWTH-SUPPORTING:			
Mg^{++} - Aspartic Acid Medium	0.22	0.27	0.33
+ $NaAsO_4$	0.23	0.27	0.32
+ NaN_3	0.25	0.28	0.32
+ KCN	0.23	0.23	0.25
+ DNP	0.23	0.29	0.28
Mg^{++} - $PO_4^{\equiv}$ - Aspartic Acid Medium	0.22	0.26	0.39
+ $NaAsO_4$	0.25	0.29	0.34
+ NaN_3	0.22	0.26	0.30
+ KCN	0.22	0.20	0.21
+ DNP	0.23	0.23	0.31

*All inhibitors were at 1 x 10^{-4} M concentration.

†Concentration of media components was as given under Table I.

¶2,4-Dinitrophenol

acid, reversion and growth take place after 6 hours of incubation. Phosphate, which appears to aid reversion, enhances the inhibitory effect of DNP substantially.

Since the inhibitors used prevent ATP synthesis, the decrease in the rate of reversion in both types of media indicates that energy is needed for the cell division process.

Effect of Mersalyl Acid (Mercurials)

Mersalyl acid, an organic mercurial, capable of disrupting disulfide bonds, inhibits reversion at 1×10^{-3} M concentrations in all types of media used. Filaments suspended in non-growth-supporting media containing this concentration also exhibited extensive lysis.

Effects of Compounds Which Affect the Cell Membrane

Compounds whose mode of action is directed towards the cell membrane like propranolol (Godin, Wanng, Tuchek, 1976), dibucaine and lidocaine (Papahadjopoulus, Jacobson, Poste and Shepherd, 1975; Poste, Papahadjopoulus, Jacobson and Vail, 1975) at 2% and 1×10^{-4} M concentrations respectively (in the reversion media) neither prevented nor aided reversion and growth.

Effect of Aerobiosis and Anaerobiosis

Reversion occurs under both aerobic and anaerobic

conditions. This is not surprising, since this organism is a facultative anaerobe.

Effect of Chloramphenicol (CAP)

To determine whether synthesis of protein(s) is an essential prerequisite for reversion, chloramphenicol (0.08 mg/ml) was added to both non-growth- and growth-supporting media. Even though reversion to the short form (4-6 μm) was not achieved in both types of media during the entire study period (up to 10 hours) a general decrease in size (over 70% of the filaments are no longer than 40 μm; however, in the first 3 hours of incubation in the presence of CAP the majority of the filaments become 20-30 μm in length) especially in the growth-supporting media was observed. Reversion in non-growth-supporting media was minimal. Even though filamentous cells overcame the initial inhibitory effects of CAP and reverted to shorter forms (30 μm or less), reversion was not observed for the rest of the study period (10 hours). The fact that the filamentous cells divided in the presence of CAP, especially, in non-growth-supporting media in the first 3 hours of incubation suggests that protein(s) necessary for cell division have been synthesized and are present in the filaments. In addition, it seems that upon prolonged incubation in the presence of CAP no more of these protein(s) were snythesized and hence no more division (reversion) occurred.

Effect of Ethylenediaminetetraacetic Acid (EDTA)

EDTA (0.1 M) was utilized in all types of reversion media to determine if divalent cations were involved in the reversion process. At this concentration EDTA completely impaired reversion in all types of media tested. These observations indicate that some divalent metal cation(s) is invoved in the cell division process.

Effect of Agents Affecting Microtubules and Microfilaments

Since it was observed that reversion can occur in the absence of any increase in cell mass, it appeared possible that cell division could be effected through an in-place microfilamentous system. Though classical microtubule and microfilament macromolecules have not been observed in bacteria, it was reasoned that some type of related molecules having a similar type of sensitivity to specific drugs might be present. Various concentrations (ranging from 1×10^{-8} to 1×10^{-3} M) colchicine, vineblastine and cytochalasin B were studied. The most effective concentrations and their effect on growth are shown in Table XI. Regardless of concentration, colchicine, which is known to disrupt microtubules, did not inhibit reversion. Vineblastine, which also disrupts microtubules like colchicine (site of action is, however, different from that of colchicine), prevents

TABLE XI

MASS INCREASE OF FILAMENTS TREATED WITH ANTI-MICROTUBULAR AGENTS* IN VARIOUS REVERSION MEDIA†

Reversion Formulations	Optical Density (540 nm) 0 hr.	2 hrs.	5 hrs.
NON-GROWTH-SUPPORTING:			
$MgCl_2 \cdot 6H_2O$ Only	0.29	0.29	0.28
+ Colchicine	0.27	0.28	0.27
+ Vineblastine	0.27	0.27	0.27
+ Cytochalasin B	0.26	0.25	0.26
Mg^{++} - $PO_4^{\equiv}$ Medium	0.27	0.26	0.26
+ Colchicine	0.23	0.23	0.21
+ Vineblastine	0.23	0.21	0.21
+ Cytochalasin B	0.28	0.27	0.28
GROWTH-SUPPORTING:			
Mg^{++} - Aspartic Acid Medium	0.28	0.35	0.44
+ Colchicine	0.28	0.34	0.44
+ Vineblastine	0.26	0.32	0.54
+ Cytochalasin B	0.24	0.29	0.38
Mg^{++} - $PO_4^{\equiv}$ - Aspartic Acid Medium	0.28	0.33	0.42
+ Colchicine	0.29	0.35	0.44
+ Vineblastine	0.28	0.37	0.64
+ Cytochalasin B	0.25	0.38	0.70

*Colchicine, 1×10^{-3} M; vineblastine 2.5×10^{-4} M; cytochalasin B, 1×10^{-4} M.

†Concentration of media components was as given under Table I.

reversion of filaments caused by magnesium chloride (non-growth-supporting medium) when used at a concentration of 2.5 x 10^{-4} M). However, addition of phosphate into the magnesium chloride medium facilitated the reversion substantially. This same concentration, when present in media containing aspartic acid actually accelerates the rate of reversion far more than filaments suspended in magnesium chloride-aspartic acid medium alone. The effect of phosphate in the magnesium chloride-aspartic acid medium could not be assessed because of the fast reversion that occurs in media containing aspartic acid. Cytochalasin B, which disrupts microfilaments, does not accelerate the rate of reversion when used at a concentration of 1 x 10^{-4} M. Controls and treated cells exhibited the same rates of reversion.

Effect of Cyclic Adenosine Monophosphate (cAMP)

Depending on experimental conditions and the test organism, NaF has been found to either activate or inactivate adenyl cyclase (Perkins and Moore, 1971; Rahmaman, 1974); Neer, 1976). Such actions can result either in inhibiting or causing excessive production of cAMP which might somehow be involved in cell division. To study the effect of cAMP on reversion we used the dibutryl form of cAMP (1 x 10^{-8} to 1 x 10^{-3} M) because the presence of dibutryl substituent aids in moving the compound through the cell membrane. Studies were conducted both in non-growth- and growth-supporting

media. At all concentrations used, there was no effect on cell size, i.e., cAMP neither accelerated nor retarded the process of reversion. Caffeine and theophylline (inhibitors of cAMP-phosphodiesterases), were also studied (1×10^{-8} to 1×10^{-3} M concentrations). Caffeine at a concentration of 1×10^{-3} M significantly inhibited reversion; however, at a concentration of 1×10^{-4} M a slight increase in reversion was observed (at this concentration there was no effect on growth). Theophylline (1×10^{-3} M) prevented reversion in all types of media. Results on growth of cells using effective concentrations of these compounds are given in Table XII.

Effects of Heavy and Light Inocula

Initial experiments on reversion were carried out by using filaments such that an OD of 0.36-0.40 was present in the reversion medium. Stained preparations made during cell reversion showed that significant numbers of the filaments were lysing. Since the contents of these lysed filaments could serve as nutrients to the metabolically active filaments, especially for those in the non-growth-supporting media, we decided to test if any differences existed in the rate of reversion when heavy and light inocula were used. It was observed that both the rate of reversion and increase in mass in the non-growth-supporting media were drastically decreased when light inocula were employed. Although no significant increase in OD occurred during the course of the

TABLE XII

EFFECT OF THEOPHYLLINE, CAFFEINE AND DIBUTRYL cAMP ON GROWTH OF REVERTING FILAMENTS

Medium Formulations*	Optical Density (540 nm) 0 hr.	2 hrs.	5 hrs.
NON-GROWTH-SUPPORTING:			
$MgCl_2 \cdot 6H_2O$ Only	0.33	0.34	0.32
+ Theophylline†	0.36	0.37	0.36
+ Caffeine	0.35	0.33	0.33
+ Dibutryl cAMP¶	0.34	0.31	0.32
Mg^{++} - $PO_4^{\equiv}$ Medium			
+ Theophylline	0.33	0.31	0.29
+ Caffeine	0.31	0.27	0.27
+ Dibutryl cAMP	0.35	0.35	0.33
GROWTH-SUPPORTING:			
Mg^{++} - Aspartic Acid Medium	0.32	0.37	0.41
+ Theophylline	0.36	0.42	0.48
+ Caffeine	0.33	0.33	0.35
+ Dibutryl cAMP	0.35	0.40	0.46
Mg^{++} - $PO_4^{\equiv}$ - Aspartic Acid Medium	0.34	0.41	0.46
+ Theophylline	0.33	0.40	0.45
+ Caffeine	0.31	0.32	0.36
+ Dibutryl cAMP	0.33	0.37	0.41

*Concentration of media components was as given under Table I.

†Inhibitors were tested at 1×10^{-3} M.

¶Dibutryl cyclic adenosine monophosphate was used at 1×10^{-4} M concentration.

experiments, this does not indicate that growth was not occurring. Obviously, the growth increases were balanced by cell lysis, and no change in OD was evident. This type of occurrence (lysis and release of nutrients for limited growth) does not negate the data presented as they relate to cell division. Since some utilizable nutrients are required for reversion, it is additional evidence that the simple contraction of an in-place and previously synthesized microtubular or microfilamentous system is not the operative triggering mechanism for cell division in bacterial cells.

To more adequately study the problem of lysis and nutrient reutilization, labelled glutamic acid was incorporated into the growth medium during formation of filamentous cells. Non-labelled aspartic acid was also added to this medium to ensure that diaminopimelic acid (DAP) present in mucopeptide of Erwinia would not be labelled or labelled only to a slight extent. After harvesting the filaments they were placed in a non-growth-supporting medium (magnesium chloride-phosphate only) and incubated. Mucopeptide was isolated from control cells, filamentous cells and filamentous cells incubated in the non-growth-supporting medium for 5 hours. It was reasoned that if nutrients are made available by lysis of some filaments for the reversion process the radioactive carbon from the previously utilized glutamic acid would be randomized and some of it used for synthesis of any new DAP formed. It was observed that radioactivity in the labelled DAP in reverted cells undergoes a dramatic increase. The ratio of

radioactivity in DAP to glutamic acid in filamentous cells was about 0.3 to 1, whereas in reverted cells it was 6 to 1. This experiment conclusively demonstrates that reutilization of nutrients from lysed cells is occurring in this cell system.

Effect of Repeated Washing of Filaments

Fluoride has a capability of complexing with metal ions and phosphate. Since it appeared that a surface complex consisting of a protein, magnesium ion, phosphate and fluoride ion might somehow be responsible for inhibition of division, it was thought necessary to extensively wash the filaments before testing them for reversion to see if such a complex could be removed. The rate of reversion decreased as the number of washings increased (1 to 4) regardless of the reversion medium employed. Examination of the washed cells revealed that extensive lysis of them occurred during the washing procedure even though mineral salts (exactly the same as employed in the growth medium) were used. Obviously, the filamentous cells are too fragile to permit conventional washing and resuspension procedures, and this line of experimentation was not pursued further.

Effects of Pantoyl Lactone (PL)

Pantoyl lactone (6.8×10^{-2} M), an inhibitor of filament and spheroplast formation, prevented lysis and accelerated reversion of filamentous cells in short periods of time

(usually less than 4 hours) in non-growth-supporting media; however, reversion in $MgCl_2$ (non-growth-supporting medium) was only minimal. Since excessively washed filaments (more than 2 or 3 times) showed decreased rates of reversion in the various medium formulations, we also checked what effects (if any) PL might have on filaments that had undergone repeated washing. As the number of washings of the filaments increased (0 to 4), the capability of PL to protect the cells from lysis and to aid in reversion decreased dramatically. These decreased activities of PL (protection from lysis and acceleration of reversion) were observed throughout the incubation period, especially in the non-growth-supporting medium ($Mg\ Cl_2$). These types of reversion studies which utilize PL seem to indicate that the group(s) of compounds it reacts with are either removed during the washing process or somehow altered such that it loses its capability to aid in reversion.

CHAPTER IV

DISCUSSION

Dependency for filament formation on a range of concentration of the carbon/nitrogen source (aspartic acid) in the growth medium has been demonstrated (Figure 4). Enriching this growth medium (GAF), by adding casamino acids or a combination of individual amino acids, prevents filament development regardless of the concentration of NaF used. Filament formation is usually associated with rapid growth of cells (Slater and Schaetcher, 1974); however, under our conditions, addition of extra medium components such as casamino acids (to increase the growth rate) inhibits the formation of filamentous cells. Despite trials to induce filament formation using different media formulations (Table II), it was possible only to reduce growth (as measured by OD readings) but not to induce filament formation.

Since aspartic acid catabolism is initiated by its entrance into the TCA cycle at the 4-carbon dicarboxylic state (fumarate or oxaloacetate), TCA cycle intermediates were studied. Compounds such as succinate, malate, fumarate and citrate do not help in inducing filamentous growth when used singly (as carbon source) nor do they prevent induction of filamentous growth when used in combination with aspartic

acid in the GAF medium. β-Alanine, a product of α-decarboxylation of aspartic acid, and pyruvate, a product of both deamination and β-decarboxylation of aspartic acid also does not induce filamentous growth in GAF medium when used in place of aspartic acid. Carbamylaspartic acid, an essential biosynthetic intermediate for the synthesis of pyrimidines was added to the GAF medium when aspartic acid was present only in low concentrations (1×10^{-3} M). It was also used to completely replace aspartic acid (0.02 M). In no instance did this compound aid in filament formation.

These findings indicate that metabolic blocks preceding aspartate transcarbamylase are not involved in the division inhibition process(es). Taken together, they further indicate that aspartic acid, once it undergoes deamination and/or decarboxylation, is ineffective in inducing filamentous growth.

Analogs of aspartic acid (L-glycyl-L-aspartic acid, L-aspartyl-diethyl ester) whose amino or dicarboxylic acid moieties are masked, when used in excess concentrations in the GAF medium where aspartic acid is available only in low concentrations (1×10^{-3} M) (low enough to allow growth only of short cells), also do not enhance filament formation. On the other hand, the aspartic acid analog, α-methylaspartic acid does allow development of filamentous cells. The only difference between α-methylaspartic acid and the other analogs is the existence of the free amino and carboxylic groups in α-methylaspartic acid.

L-Asparagine, the closest amino acid to aspartic acid in structure, supports growth as well as aspartic acid; however,

the mere change of the β-carboxylic group to an amide moiety completely prevents filamentous growth (Table II). L-Glutamic acid, which has an extra methylene group but all of the same charged moieties aspartic acid contains, also does not allow growth of filamentous cells.

These findings all indicate that aspartic acid is directly involved in inducing filamentous growth and must have its reactive groups in the free form to facilitate the division inhibition phenomenon. This conclusion seems unusual because the cell size of E. carotovora growing in a medium where aspartic acid is the sole source of carbon, nitrogen and energy is very small (<3 μm). Therefore, whatever happens to bring about inhibition of division in the aspartic acid or glucose-aspartic acid medium must be looked at from the point of view of what happens to the growing cells in the presence of NaF. As documented in this thesis, inorganic phosphate, divalent cation(s) (especially magnesium ions) and pH are also important parameters to consider (Figures 2, 3, 4 and 5).

If the initial pH of the GAF medium is adjusted to values greater than 7.4, development of filamentous cells does not occur. As shown in Figure 3, further increases in alkalinity cause development of small cells. This pH dependence of the cell division process is not confined to growing cells only. Reversion (Table IX) of filamentous cells, especially in non-growth-supporting media, also show a pH dependency. Even though no apparent increase in cell mass is observed (determined by OD readings) in the non-growth-supporting

media during reversion (Table VIII), data are presented which show that nutrients from some lysing cells have been incorporated into cellular components during the reversion process. Since the quantity of available nutrients is probably limited, it is unlikely to be the sole cause for bringing about 80% reversion (size of cells became less than 5 μm) in only 5 hours (Table IX). The possibility must be considered that pre-formed "cell division molecule(s)" made non-functional because of complex interaction involving NaF, metal ion(s), inorganic phosphate, and aspartic acid in the medium is dissociated at alkaline pH values.

Some evidence for the presence of a cell division (molecule(s) sensitive to inhibitors of protein synthesis have been gathered using chloramphenicol (CAP). Addition of CAP to reversion media (non-growth- and growth-supporting) does not prevent the initial general decrease in cell size of filaments (first 3 hours); however, continued incubation (10 hours) in the presence of this protein inhibitor prevents further reversion. Thus, the dramatic pH-dependent reversion and the initial reversion in CAP-treated filaments support the argument that the necessary molecule(s) needed for division are present in filamentous cells. The sensitivity of the reversion process to CAP demonstrates that some portion of the system is proteinacious in nature.

Grula and Smith (1965) have demonstrated that cell division in _E. carotovora_ is a constrictive process. One possible mechanism for this type of division could involve a

microtubule-microfilament type of structure undergoing contraction and relaxation. Tubular structures composed of proteins are known to play a role in chromosome replication and cell division in eukaryotic cells (Olmstead and Borisy, 1973). Drugs that affect such proteins (Table XI) do not affect cell division in E. carotovora. The demonstrated insensitivity to the drugs does not negate the contention that a portion of the division system possesses characteristics of a microtubular-microfilamentous system, since such a system in bacteria could possess a different drug sensitivity pattern.

Even though inhibitors of oxidative phosphorylation (azide, cyanide and dinitrophenol) and a substrate phosphorylation inhibitor, arsenate, all prevent reversion (Table X), only arsenate delays filament formation (GAF medium). Since arsenate competes with inorganic phosphate for ester bond formations, arsenate may be preventing the utilization of inorganic phosphate in a substrate-type phosphorylation.

As shown in Figures 1 and 2, NaF decreases growth and prevents division only within a range of concentration. Fluoride ion interacts with enzyme complexes containing conjugated metal cations (especially magnesium ions) thereby effectively inactivating the complexes. Usually, the presence of inorganic phosphate exacerbates the inactivation (Warburg and Christian, 1942; Taitzer and Himoe, 1974; Spearman, Khandelwal and Hamilton, 1973; Lin, Mosteller and Hardesty, 1966; Hodge and Smith, 1965). Possible mechanisms involving phosphate are as follows: 1) Fluoride ions

can form tightly bound complexes with metal ions (especially magnesium ions) in the presence of inorganic phosphate; hence, certain enzymes will be deprived of metal cations for activity (Hodge and Smith, 1965; Haguenauer and Kepes, 1972; Taitzer and Himoe, 1974. 2) Fluoride ions may inhibit dephosphorylation by direct interaction and thus inactivation of phosphatases (Gazitt, Ohad and Loyter, 1976; Hamilton, 1969; Gracia, Suarez, Aragon and Mayor, 1972).

Some support for the involvement of a phosphorylation/ dephosphorylation type of reaction in the process of filament formation was obtained in the nutritional study of inorganic phosphate (Figure 5) made using both the GA and GAF media. Interestingly, even though lowering the inorganic phosphate concentration by a hundredfold prevents filamentous growth completely, rate of growth is not significantly reduced in the absence of NaF. The total phosphate content of cells is about the same regardless of the phosphate concentration in the growth medium (Table VI). Although the total phosphate content of filamentous cells is higher than in normal cells, inhibitors of filamentous growth (pantoyl lactone and n-propanol are exceptions) prevent this increase. It appears possible that these two compounds function after phosphorylations have occurred. Such phosphorylations may be responsible for division inhibition since the other inhibitors tested do inhibit the increased phosphate content of filamentous cells.

To further define the phosphate involvement, different fractions of normal and filamentous cells were assayed for their phosphate content. Nucleic acid phosphate (about 80% of the total content of the cell) was approximately the same in both types of cells. Significant quantitative differences in phosphorylated compounds were observed in the cold TCA extractable (soluble pool) fraction. Identification of these compounds has to be carried out before a possible role can be given to them in the cell division process(es). At this time, it appears that they are 3-, 5-, and 6-carbon compounds.

The increased concentration of phosphorylated compounds in filaments also made it necessary to study the possible role(s) of phosphatases (especially phosphomonoesterases) in the cell division process. The most active phosphatase(s) (optimum activity at pH 9.0) was found in control cells (Figure 8). At pH 9.0, NaF inhibits this phosphatase activity about 80%; however, our investigations have shown that high pH (Figure 3) always prevents filamentous growth when _E. carotovora_ is grown in the GAF medium. It appears, therefore, that the absence from filamentous cells of this enzyme activity is not responsible for the inhibition of division. Moreover, the concentrations of NaF required to significantly inhibit the enzyme activity are much lower than those required to induce filamentous growth. Grula and Hopfer (1972) demonstrated that ATPase activity in filaments induced by D-serine is not involved in the cell division process. Thus two types of phosphatase enzyme activity, although largely

absent from filamentous cells, are not involved in the cell division process in *E. carotovora.* Since both enzyme activities are present in the periplasmic space area of the cells, it is most likely that they escape the cell periphery through the damaged cell envelope. Thus their absence is the result and not a cause of cell division inhibition.

Cellular phosphatases capable of bringing about the release of phosphate from intracellular substrates were also studied. These studies were done at pH 6.8, since filament formation occurs best at this pH. It was observed (Figure 7) that the enzyme activity in filaments is depressed during the first few hours of incubation; however, the total amount of phosphate released by the third to fourth hour is equal to and becomes greater than that released in normal cells.

These results are different from those obtained using the artificial phosphomonoesterase substrate, *para*-nitrophenyl phosphate (p-NPP) at the same pH (Figure 7). One is led to believe, therefore, that different enzyme activities are being measured. This is not unexpected since p-NPP most likely cannot pass through the outer membrane of the cell because of charge repulsion effects (negative cell surface and the negative phosphate group in p-NPP). This membrane is damaged in filamentous cells (Grula and Hopfer, 1972).

Because the decreased phosphatase activity occurs within the pH range wherein excellent inhibition of cell division is effected by NaF, such decreased activity could be related to

inhibition of cell division. It also helps to explain the increased level of phosphate-containing compounds found in filamentous cells.

Whether the decreased pH 6.8 phosphatase enzyme activity occurs because of feedback inhibition by the increased levels of phosphate esters found in filamentous cells or whether the decreased enzyme activity occurs because of complex formation with NaF and is therefore responsible for such increased levels of phosphorylated intermediates cannot be decided at this time. Detailed examination of this enzyme activity could be important since the bulk of the activity (60-70%) is present in the insoluble portion of these cells after three freeze-thaw treatments.

CHAPTER V

SUMMARY AND CONCLUSIONS

This research has focused on attempts to find metabolic or structural differences between normal and NaF-induced non-dividing (filaments) cells of *Erwinia carotovora*.

Amino acids either alone or in various combinations were added in attempts to induce filamentous growth in the presence of NaF; however, none were found to be as suitable as D- or L-aspartic acid. In fact, specific concentrations of aspartic acid were found to be necessary for induction of filamentous growth by NaF.

Intermediates of aspartic acid catabolism neither prevent nor enhance division inhibition by NaF. Use of aspartic acid analogs suggests that the free amino and dicarboxylic groups of aspartic acid are both required for development of filamentous forms in the presence of NaF. In addition, inorganic phosphate, a metal cation(s), especially magnesium ion and pH are also important parameters in the division process of this organism.

Inhibition in the reversion of filaments by chloramphenicol (non-growth-supporting or growth-supporting media) or stimulation of the process at alkaline pH ranges indicate the possible existence of a pre-fromed division molecule(s) in

division inhibited cells. Because reversion was not inhibited by antimicrotubular or antimicrofilamentous drugs, direct evidence for such molecules was not obtained.

Use of phosphorylation inhibitors reveals that only arsenate delays (but does not entirely stop) filamentous growth from occurring. This has been interpreted as an inhibition by arsenate in the utilization of inorganic phosphate for a substrate type phosphorylation. Some support for the involvement of a phosphorylation/dephosphorylation type reaction is further suggested since filaments contain more phosphate than normal cells. Inhibitors of filamentous growth (pantoyl lactone and n-propanol are exceptions) prevent this increase in intracellular phosphate. Some progress has been made in identifying two organic phosphate compounds which are present in increased amounts in filamentous cells. Continued work in this area is planned.

Filamentous cells have a decreased level of a phosphatase active at alkaline pH (it is not, however, alkaline phosphatase). Also, the phosphatase activity at pH 9.0 is strongly inhibited by NaF at much lower concentrations than those required to induce filamentous growth. Because division occurs normally at this pH in the presence of NaF, inhibition in the phosphatase activity appears not to be responsible for inhibition of cell division.

Decreased phosphatase activity has also been demonstrated in filamentous cells at neutral pH ranges using cellular organic phosphate compounds as substrates. Data

obtained relative to such activity may indicate the existence of a relationship between this type of activity and the inhibition of cell division. A decreased intracellular phosphatase activity may help explain why more organic phosphate compounds are present in filamentous cells.

BIBLIOGRAPHY

Adelman, M. R., G. G. Borisy, M. I, Shelanski, R. C. Weisenberg and E. W. Taylor. 1968. Cytoplasmic filaments and tubules. Fed. Proc. 27:1186-1193.

Bandurski, R. S. and B. Axelrod. 1951. The chromatographic identification of some biologically important esters. J. Biol. Chem. 193:405-410.

Bazil, G. W. 1967. Lethal unbalanced growth in bacteria. Nature 216:346-349.

Belfanti, S., A. Contardi and A. Ercoli. 1935. Researches on the phosphatases. II. Inactivation and reactivation of the phosphatases of animal organs. Biochem J. 29: 842-846.

Bennet, E. O., and R. P. Williams. 1957. A comparison of methods for determining bacterial mass with particular emphasis upon the use of total phosphorus. Applied Microbiol. 5:14-16.

Berlin, R. D. and T. E. Ukena. 1972. On the agglutination of polymorphonuclear leucocytes by concanavalin A. Nature New Biology 238:120.

Bleiberg, I., M. Zauderer and C. Baglioni. 1972. Reversible disaggregation by NaF of membrane bound polyribosomes of mouse myeloma cells in tissue culture. Biochim. Biophys. Acta. 269:453-464.

Brinkely, B. R., S. S. Barham, S. C. Barranco and G. M. Fuller. 1974. Rotenone inhibition of spindle microtubule assembly in mammalian cells. Exp. Cell Res. 85:41-46.

Brown, D. L. and G. M. Bouk. 1974. Microtubule biogenesis and cell shape in Ochromonas. III. Effects of herbicidal mitotic inhibitor isopropyl-N-phenylcarbamate on shape and flagellum regeneration. J. Cell Biol. 61: 514-536.

Cavallini, D. and N. Frontali 1954. Quantitative determination of keto acids by paper partition chromatography. Biochim. Biophys. Acta. 13:439-445.

Dehm, P. and D J. Prockop. 1972. Time lag in the secretion of collagen by matrix-free tendon cells and inhibition of the secretory process by colchicine and vineblastine. Biochim. Biophys. Acta. 264:375-382.

Diegelman, R. F. and B. Peterkofsky. 1972. Inhibition of collagen secretion from bone and cultured fibroblasts by microtubular disruptive durgs. Proc. Nat. Acad. Sci. USA. 69:892-896.

Dorough, G. D. and D. L. Seaton. 1954. A method for the extraction and assay of nucleic acid fragments in tissues. J. Am. Chem. Soc. 76:2873-2877.

El Hawary, M. F. S. and R. H. S. Thompson. 1953. Separation and estimation of blood keto acids by paper chromatography. Biochem. J. 53:340-347.

Garilhe, M. R. and M. Laskowski. 1955. Studies of the phosphodiesterase from rattle snake venom. Biochim. Et Biophys. Acta. 18:370-378.

Gazitt, Y., I. Ohad and A. Loyter. 1976. Phosphorylation and dephosphorylation of membrane proteins as a possible mechanism for structural rearrangement of membrane components. Biochim. Biophys. Acta. 436:1-14.

Gillespie, E., R. G. Levine and S. E. Malawista. 1968. Histamine release from rat peritoneal mast cells. Inhibition by colchicine and potentiation by deutrium oxide. J. Pharm. and Exp. Ther. 164:158-165.

Gillespie, E. and L. M. Lichtenstein. 1972. Histamine release from human leucocytes: Studies with deutrium oxide, colchicine and cytochalasin B. J. Clinical Investigation 51:2941-2947.

Godin, D. V. T. Wanng and J. M. Tucheck. 1976. Studies on the interaction of propanolol with erythrocyte membranes. Biochim. Biophys. Acta. 436:757-773.

Goldman, R. D. 1971. The role of three cytoplasmic fibers on BHK-21 cell motility. I. Microtubules and the effects of colchicine. J. Cell Biol. 51:752-762.

Goodman, D. B. P., H. Rasmussen, F. Dibella and C. E. Guthrow, Jr. 1970. Cyclic adenosine 3':5'-monophosphate-stimulated phosphorylation of isolated neurotubule sub-units. Proc. Nat. Acad. Sci. USA 67:652-659.

Gracia, P. E., M. D. Suarez, M. C. Aragon and F. Mayor. 1972. Mevalonate phosphorylation in *Agave americana*. Phytochemistry 11:2495-2498.

Grula, E. A. 1960a. Cell division in a species of Erwinia. I. Initial observations relating to nutritional dependency. J. Bactriol. 80:369-374.

Grula, E. A. 1960b. Cell division in a species of Erwinia. II. Inhibition of division by D-amino acids. J. Bacteriol. 80:375-385.

Grula, E. A. and M. M. Grula. 1962a. Cell division in a species of Erwinia. III. Reversal of inhibition of cell division caused by D-amino acids, penicillin and ultraviolet light. J. Bacteriol. 83:981-988.

Grula, E. A. and M. M. Grula. 1962b. Cell division in a species of Erwinia. IV. Metabolic blocks in a pantothenate biosynthesis and their relationship to inhibition of cell division. J. Bacteriol. 83:989-997.

Grula, E. A. and M. M. Grula. 1962c. Cell division in a species of Erwinia. V. Effect of metabolic inhibitors on terminal division and composition of a "division" medium. J. Bacteriol. 84:492-499.

Grula, E. A. and M. M. Grula. 1964. Cell division in a species of Erwinia. VII. Amino sugar content of dividing and non-dividing cells. Biochem Biophys. Res. Commun. 17:341-346.

Grula, E. A., G. L. Smith and M. M. Grula. 1965. Cell division in a species of Erwinia. VIII. Amino acid composition of the mucopeptide in dividing and non-dividing cells. Can. J. Microbiol. 11:605-610.

Grula, E. A. and G. L. Smith. 1965. Cell division in a species of Erwinia. IX. Electron microscopy of normally dividing cells. J. Bacteriol. 90:1054-1058.

Grula, M. M., R. W. Smith, C. F. Parham and E. A. Grula. 1968. Cell division in a species of Erwinia. XII. A study of nutritional influences in D-serine inhibition of growth and division of Erwinia sp., and of certain specific sites of D-serine action. Can. J. Microbiol. 14:1225-1238.

Grula, E. A. and R. L. Hopfer. 1972. Cell division in a species of Erwinia. XIII. Leakage of proteins from the peripheral area of filamentous cells. Biochim. Biophys. Acta. 255:822-832.

Grula, M. M. and E. A. Grula. 1976. Requirement for pantothenate for filament formation by Erwinia carotovora. J. Bacteriol. 125:968-974.

Haguenauer, R. and A. Kepes. 1972. NaF inhibition of phosphorylation and dephosphorylation involved in alpha-methyl glucoside transport in E. coli K 12. A pH phenomenon sensitive to uncoupling agents. Biochimie 54: 505-512.

Haidle, C. W. and S. G. Knight. 1960. A colorimetric assay for 2-heptanone and other ketones. Biochim. Biophys. Acta. 39:536-537.

Hanes, C. S. and F. A. Isherwood. 1949. Separation of the phosphoric esters on the filter paper chromatogram. Nature 164:1107-1112.

Hamilton, I. R. 1969. Studies with fluoride sensitive and fluoride resistant strains of Streptococcus salivarius: II. Fluoride inhibition of glucose metabolism. Can. J. Microbiol. 15:1021-1027.

Hawary, M. F. S. 1955. Blood keto acid levels during intoxication with inhibitors of carbohydrate metabolism. Biochem. J. 61:348-352.

Heathcote, J. G. and K. Jones. 1965. The rapid resolution of complex mixtures of the naturally occurring amino acids by two dimensional thin-layer chromatography on cellulose powder. Biochem. J. 97:15p

Helmstetter, C. E. 1974a. Initiation of chromosome replication in Escherichia coli. I. Requirements for RNA and protein synthesis at different growth rates. J. Mol. Biol. 84:1-19.

Helmstetter, C. E. 1974b. Initiation of chromosome replication in Escherichia coli. II. Analysis of the control mechanism. J. Mol. Biol. 84:21-36.

Hodge, H. C. and F. A. Smith. 1965. Fluorine chemistry. Vol. IV. Acad. Press, New York, p. 176.

Holmes, K. V. and P. W. Choppin. 1968. On the role of microtubules in movement and alignment of nuclei in virus-induced syncytia. J. Cell Biol. 39:526-543.

Iyehara, H. and N. Otsuji. 1975. Location of the Escherichia coli K-12 ruv gene affecting septum formation after inhibition of deoxyribonucleic acid synthesis. J. Bacteriol. 122:791-793.

Inouye, M. and A. B. Pardee. 1970. Changes of membrane proteins and their relation to deoxyribonucleic acid synthesis and cell division of Escherichia coli. J. Mol. Biol. 52:75-89.

Isherwood, F. A. and D. H. Cruickshank. 1954a. Chromatographic separation and analysis of mixtures of pyruvic, oxaloacetic and alpha-ketoglutaric acids. Nature 173: 121-122.

Isherwood, F. A. and D. H. Cruickshank. 1954b. A new method for the colorimetric estimation of amino acids on paper chromatograms. Nature 174:123-126.

Isherwood, F. A. and C. A. Navis. 1956. Estimation of alpha-keto acids in plant tissue: a critical study of various methods of extraction as applied to strawberry leaves, washed potato slices and peas. Biochem. J. 64: 549-558.

Krebs, H. A. 1943. The intermediary stages in the biological oxidation of carbohydrates. Adv. in Enzymology 3: 191-252.

Kun, E. and M. G. Hernandez. 1956. The oxidation of tartaric acid by an enzyme system of mitochondria. J. Biol. Chem. 218:201-211.

Landman, O. E., A. Ryter and C. Frehel. 1968. Gelatin induced reversion of protoplasts of Bacillus subtilis to the bacillary form: Electron-microscopic and physical study. J. Bacteriol. 96:2154-2170.

Lee, Y. and M. H. Wang. 1968. Studies on the nature of the inhibitory action of inorganic phosphate, fluoride and detergents of 5'-adenylic acid deaminase activity and on the activation by adenosine triphosphate. J. Biol. Chem. 243:2260-2265.

Leibecq, C. and R. A. Peters. 1949. The toxicity of fluroacetate and the tricarboxylic acid cycle. Biochim. Biophys. Acta. 3:215-230.

Lin, S., D. V. Santi and J. A. Spudich. 1974. Biochemical studies on the mode of action of cytochalasin B. J. Biol. Chem. 249:2268-2274.

Lin, S., R. D. Mosteller and B. Hardesty. 1966. The mechanism of sodium fluoride and cycloheximide inhibition of hemoglobin biosynthesis in the cell free reticulocyte system. J. Mol. Biol. 26:51-69.

Loten, E. G. and B. Jeanrenaud. 1974. Effects of cytochalasin B, colchicine and vinecristine on the metabolism of isolated fat cells. Biochem. J. 140:185-192.

Malaisse, L. F., M. H. Greider, W. J. Malaisse and P. E. Lacy. 1971. The stimulus secretion coupling of glucose-induced insulin release. IV. The effect of vinecristine and deutrium oxide on the microtubular system of the pancreatic beta cells. J. Cell Biol. 49: 530-535.

Malawista, S. E. 1965. On the action of colchicine. The melanocyte model. J. Exp. Med. 122:361-384.

Manago, M. G., Ortiz, P. and S. Ochoa. 1956. Enzymatic synthesis of polynucleotides. I. Polynucleotide phosphorylase of Azotobacter vinelandii. Biochim. Biophys. Acta. 20:269-285.

Margulis, L. 1973. Colchicine-sensitive microtubules. International review of Cytology 34:333-361.

Marunouchi, T. and W. Messer. 1973. Replication of a specific chromosome segment in Escherichia coli which is required for cell division. J. Mol. Biol. 78:211-228.

McIntosh, R. J. and K. R. Porter. 1967. Microtubules in the spermatids of domestic fowl. J. Cell Biol. 35:153-173.

Minkoff, L. and R. Damadian. 1975. Biological ion exchange resins. VIII. A preliminary report on actin-like protein in E. coli. and the cytotonus concept. Physiol. Chem. and Physics 7:385-389.

Minkoff, L., J. Abramowitz and R. Damadian. 1976. Biological ion exchange resins. IX. Isolation and partial identification of a potassium sensitive contractile-like protein from E. coli. Physiol. Chem. and Physics 8:167-173.

Mizel, S. B. and L. Wilson. 1972. Nucleoside transport in mammalian cells. Inhibition by colchicine. Biochem. 11:2573-2578.

Monahan, T. M. and C. W. Abell. 1973. The role of adenosine 3'5'monophosphate in the regulation of mammalian cell division. J. Cell Biol. 59:549-558.

Najjar, V. A. 1948. The isolation and properties of phosphoglucomutase. J. Biol. Chem. 175:281-290.

Neer, E. J. 1976. Two soluble forms of guanosine5'(alpha-imino)triphosphate and fluoride-activated adenylate cyclase. J. Biol. Chem. 251:5831-5834.

Newcomb, E. H. and H. T. Bonnett. 1965. Cytoplasmic microtubule and wall microfibril orientation in root hairs of radish. J. Cell Biol. 27:575-589.

Norris, J. R. and D. W. Ribbons. 1971. Methods in microbiology. Vol. 5B, Acad. Press, New York, New York. p. 228.

Olmstead, J. B. and G. G. Borisy. 1973. Microtubules. Annual Review of Biochemistry 42:507-540.

Papahadjopoulos, D., K. Jacobson, G. Poste and G. Shepherd. 1975. Effects of local anesthetics on membrane properties. I. Changes in the fluidity of phospholipid bilayers. Biochim. Biophys. Acta. 394:504-519.

Park, J. T. and R. Hancock. 1960. A fractionation procedure for studies of the synthesis of cell-wall mucopeptide and of other polymers in cells of Staphylococcus aureus. J. Gen. Microbiol. 22:249-258.

Paterson, S. J., K. W. Butler, P. Huang, J. Labelle, I. C. P. Smith and H. Schneider. 1972. The effect of alcohols on lipid bilayers: A spin label study. Biochim. Biophys. Acta. 266:597-602.

Perkins, J. P. and M. M. Moore. 1971. Adenyl cyclase of rat cerebral cortex. Activation by sodium fluoride and detergents. J. Biol. Chem. 246:62-68.

Pollard, T. D. and R. G. Burns. 1974. A denein-like protein from brain. FEBS Letters 40:274-280.

Poste, G., D. Papahadjopoulos, K. Jacobson and W. J. Vail. 1975. Effects of local anesthetics on membrane properties. II. Enhancement of the susceptibility of mammalian cells to agglutination by plant lectins. Biochim. Biophys. Acta. 394:520-539.

Rahmaman, M. and D. Jarett. 1974. Activation of rat adipocyte plasma membrane adenylate cyclase by sodium azide. Biochem. Biophys. Res. Commun. 61:1051-1056.

Rothman, L. B. and E. Cabib. 1966. Inhibition of yeast growth by fluoride and its reversal by phosphoric esters: an effect of pH. Biochim. Biophys. Acta. 117:482-485.

Seeman, P., M. Chau-wong and S. Moyyen. 1972. Membrane expansion by vineblastine and strychnine. Nature New Biology 241:22.

Shapiro, B. M., A. G. Siccardi, Y. Hirota and F. Jacob. 1970. On the process of cellular division in Escherichia coli. II. Membrane protein alterations associated with mutations affecting the initiation of DNA synthesis. J. Mol. Biol. 52:75-89.

Shelanski, M. L., F. Gasku, S. B. Karmer, C. R. Canter and R. Adelstein. 1974. A dynein-like protein associated with neurotubules. FEBS Letters 40:281-285.

Sherma, J. and G. Zweig. 1971. Paper chromatography and electrophoresis Vol. II. Paper chromatography. New York Acad. Press. p. 174-176.

Slater, E. C. and W. D. Bonner. 1952. The effect of fluoride on succinic oxidase system. Biochem. J. 52:185-196.

Slater, M. and M. Schaechter. 1974. Control of cell division in bacteria. Bact. Reviews 38:199-221.

Spearman, T. N., R. L. Khandelwal and I. R. Hamilton. 1973. Some regulatory properties of glycogen phosphorylase from *Streptococcus salivarius*. Arch. Biochem. Biophys. 154:306-313.

Strassman, M., J. B. Shatton and S. Weinhouse. 1960. Conversion of acetolactic acid to the valine precursor, α-dihydroxyisovaleric acid. J. Biol. Chem. 235:700-705.

Suzuki, K. 1974. Cell division and DNA synthesis in uvrA recA double mutants of *E. coli* K12. Molec. Gen. Genet. 129:248-258.

Taitzer, W. and A. Himoe. 1974. Kinetics of the rabbit muscle enolase-catalysed dehydration of 2-phosphoglycerate:-fluoride and phosphate inhibition. J. Biol. Chem. 249:3895-3902.

Taylor, A. C. 1966. Microtubules in the microspikes and cortical cytoplasm of isolated cells. J. Cell Biol. 28:155-168.

Terner, C. 1955a. The metabolism of citric acid in the mammary gland. I. The effect of p-nitrophenol on the synthesis and oxidation of citric acid by homogenates of the mammary gland. Biochem. J. 60:88-95.

Terner, C. 1955b. The metabolism of citric acid in the mammary gland. II. The effect of p-nitrophenol and of fluoride on the synthesis of citric acid in fluroacetate-blocked homogenates. Biochem. J. 60:95-101.

Tilney, L. G., Y. Hiramota and D. Marshland. 1966. Studies on the microtubules in Heliozoa. III. A pressure analysis of the role of these structures in the formation and maintenance of the axopodia of *Actinosphaerium nucleofilum*. J. Cell Biol. 29:77-95.

Ukena, T. E., J. Z. Borysenko, M. J. Kanovsky and R. D. Berlin. 1974. Effects of colchicine, cytochalasin B, and 2-deoxyglucose on the topographical organization of surface bound concanavalin A in normal and transformed fibroblasts. J. Cell Biol. 61:70-82.

Veomett, G., D. M. Prescott, J. Shay and K. R. Porter. 1974. Reconstruction of mammalian cells from nuclear and cytoplasmic components separated by treatment with cytochalasin B. Proc. Nat. Acad. Sci. USA 71:1999-2002.

Wade, H. E. and D. M. Morgan. 1953. Detection of phosphate esters on paper chromatograms. Nature 171:529-530.

Walker, J. R., A. Kovarik, J. S. Allen and R. A. Gustafson. 1975. Regulation of bacterial cell division: Temperature sensitive mutants of *Escherichia coli* that are defective in septum formation. J. Bacteriol. 123:693-703.

Walker, J. R. and A. Kovarik. 1975. Strain specific difference that affects the inhibition of division of *Escherichia coli* filaments by chloramphenicol. J. Bacteriol. 123:752-754.

Warburg, O. and W. Christian. 1942. Isolierung und kristallisation des garungsferments enolase. Biochem. Z. 310: 384-421.

Warr, J. R. and D. Gibbins. 1974. Further studies on colchicine resistant mutants of *Chlamydomonas reinhardii*. Exp. Cell Res. 85:117-122.

Wood, T. 1961. A procedure for the analysis of acid-soluble phosphorus compounds and related substances in muscle and other tissues. J. Chrom. 6:142-154.

Woodson, P. B. J., M. E. Trynor, W. T. Schlapfer and S. H. Barondes. 1976. Increased membrane fluidity implicated in acceleration of decay of post-tetanic potentiation by alcohols. Nature 260:797-799.

Yin, H. H., T. E. Ukena and R. D. Berlin. 1972. Effect of colchicine, colcimid and vineblastine on the agglutination by concanavalin A of transformed cells. Science 178:867-868.

Zacheus, C. W., J. Snyder, D. Smith, K. Summers and J. R. McIntosh. 1974. A functional mitotic spindle prepared from mammalian cells in culture. Microtubule assembly/motility/anaphase *in vitro*. Proc. Nat. Acad. Sci. USA 71:1559-1563.

γ

VITA

Berhanu Abegaz

Candidate for the Degree of

Doctor of Philosophy

Thesis: STUDIES ON FLUORIDE-INDUCED FILAMENTS OF *ERWINIA CAROTOVORA*

Major Field: Microbiology

Biographical:

Personal Data: Born in Ticho, Arussi, Ethiopia, May 21, 1945.

Education: Graduated from H.S.I. Day School in July, 1962; received Bachelor of Science degree in Agriculture from National University, Ethiopia, July, 1968; received Master of Science degree in Microbiology from Oklahoma State University, August, 1970; completed requirements for the Doctor of Philosophy degree in Microbiology at Oklahoma State University in December, 1977.

Professional Experience: Lecturer, National University, Ethiopia, 1970-1973; graduate teaching assistant, 1974-76, graduate research assistant 1976-77, Oklahoma State University.