Modulation of Escherichia coli alkaline phosphatase by Bdellovibrio bacteriovorus.

Mary Ellen McNaughton

University of Windsor

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THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED
This thesis is dedicated to my parents,
my sister and Jess.
MODULATION OF ESCHERICHIA COLI ALKALINE PHOSPHATASE

BY Bdellovibrio Bacteriovorus

by

Mary Ellen McNaughton

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of
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of Masters of Science at
The University of Windsor.

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1978
ABSTRACT

*Bdellovibrio bacteriovorus* inhabits the periplasmic space of its host bacterium. The levels of alkaline phosphatase, a periplasmic enzyme of the host, were found to be higher during periplasmic growth of *B. bacteriovorus*, than in a comparable suspension of *Escherichia coli* alone. A compartmental shift of alkaline phosphatase was also noted from the the periplasm to the extracellular fraction during parasitic growth. *B. bacteriovorus* does not produce alkaline phosphatase since none is produced during its growth with E-15, an *E. coli* mutant which is unable to synthesize alkaline phosphatase. The parasite does not sequester phosphate, thereby further derepressing the host alkaline phosphatase genetic machinery. The host-parasite enzyme and the enzyme from *E. coli* alone were compared by physiochemical methods and found to differ slightly. The isoelectric pH of the host-parasite enzyme was 5.2 compared to 5.0 for the *E. coli* enzyme alone. There was a slight difference in the P.A.G.E. profiles using an enzyme capture stain, however both enzymes displayed identical behavior on gel filtration and ion exchange. The enzymes were purified and the specific activity of the host-parasite enzyme was 3-25 times higher than the host enzyme alone. Enzyme kinetics showed that the Kms were very similar but the turnover number of the host-parasite enzyme was 3 times higher than the *E. coli* enzyme alone.
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TABLE OF CONTENTS

DEDICATION ................................................................. iv
ABSTRACT ................................................................. v
ACKNOWLEDGEMENTS ....................................................... vi
LIST OF TABLES ............................................................. viii
LIST OF FIGURES ............................................................ ix
INTRODUCTION
  B. bacteriovorus Life Cycle ............................................. 1
  B. bacteriovorus Physiology ............................................. 6
  Alkaline Phosphatase .................................................... 13
MATERIALS AND METHODS .................................................. 24
RESULTS ........................................................................... 38
DISCUSSION ..................................................................... 85
BIBLIOGRAPHY ................................................................. 95
GLOSSARY OF TERMS ......................................................... 104
CURRICULUM VITA ............................................................ 105
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Comparison of Enzyme Isolation Methods</td>
<td>47</td>
</tr>
<tr>
<td>II. Affect of A Phosphate Rich Final Media</td>
<td>54</td>
</tr>
<tr>
<td>III. Affect of A Phosphate Free Final Media</td>
<td>56</td>
</tr>
<tr>
<td>IV. Purification of Host-Parasite Alkaline Phosphatase</td>
<td>78</td>
</tr>
<tr>
<td>V. Purification of <em>E. coli</em> Alkaline Phosphatase</td>
<td>79</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>1.</td>
<td>Life Cycle of B. bacteriovorus</td>
</tr>
<tr>
<td>2.</td>
<td>Alkaline Phosphatase &quot;operon&quot;</td>
</tr>
<tr>
<td>3.</td>
<td>Mechanism of Action of Alkaline Phosphatase</td>
</tr>
<tr>
<td>4.</td>
<td>Purification of Alkaline Phosphatase</td>
</tr>
<tr>
<td>5.</td>
<td>The Relationship Between Sonication Time and Alkaline Phosphatase Released</td>
</tr>
</tbody>
</table>
| 6.     | Alkaline Phosphatase Activity vs. Time (Sonication) 
   a) E. coli  
   b) E. coli + B. bacteriovorus | 43   |
| 7.     | Total Alkaline Phosphatase Activity vs. Time (Sonication) | 45   |
| 8.     | Alkaline Phosphatase Activity 
   a) E. coli  
   b) E. coli + B. bacteriovorus | 49   |
<p>| 9.     | Total Alkaline Phosphatase Activity vs. Time (Osmotic Shock) | 51   |
| 10.    | Gel Filtration of the Host-Parasite Alkaline Phosphatase | 59   |
| 11.    | Gel Filtration of E. coli Alkaline Phosphatase | 61   |
| 12.    | Ion Exchange of Host-Parasite Alkaline Phosphatase | 63   |
| 13.    | Ion Exchange of E. coli Alkaline Phosphatase | 65   |
| 14.    | Densitometry Tracings of Alkaline Phosphatase Stained with an Enzyme Capture Method | 69   |
| 15.    | Isoelectric Focusing of Host-Parasite Alkaline Phosphatase | 71   |
| 16.    | Isoelectric Focusing of E. coli Alkaline Phosphatase | 73   |
| 17.    | Immunodiffusion Diagram | 75   |</p>
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>18. Densitometry Tracings</td>
<td>79</td>
</tr>
<tr>
<td>19. Lineweaver-Burke Plot</td>
<td>83</td>
</tr>
</tbody>
</table>
INTRODUCTION
i) Morphology and Life Cycle

*Bdellovibrio bacteriovorus* is a very small (0.25 - 0.4 μm wide by 0.7 - 1.2 μm long) Gram-negative bacterium, which is often vibrio shaped (1-4). The bacterium usually has a single polar flagellum. This flagellum is very thick with an inner core and an outer sheath which appears to be continuous with the cell wall (3-7). However, Konovalova *et al.* (8), have established that the flagellar sheath is not a prolongation of the cell wall but is directly connected to a basal body situated in the cell cytoplasm. A structure called a holdfast has been reported at the anterior end of some strains of *B. bacteriovorus* (6,9-11), and it has been suggested that this holdfast might mediate attachment and penetration of the parasite (11). Burger *et al.* (7), observed that *B. bacteriovorus*, strain W, encysted within the host cell of *Rhodospirillum rubrum* but not in *Escherichia coli*. No other strains have been observed to encyst. These encysted cells, called Bdéllocysts, are a resting stage of the parasite and differ from the vegetative form in morphology, resistance to environmental stress and rate of respiration; (12).

The life cycle of *B. bacteriovorus* (Fig 1) has been studied exhaustively and reviewed extensively (13-16). This life cycle can be divided into 7 categories --- 1) recognition of prey and movement toward it, possibly involving chemotaxis, 2) attachment to the host, 3) entrance into the host (penetration), 4) rounding up or "spheroplasting" of
Fig. 1

1. Chemotaxis?

2. Attachment

3. Penetration

PARASITIC LIFE CYCLE

4. Preparation for growth

5a. Growth (Elongation)

5b. Further Elongation

6. Division

7. Release

Host Independent
the host, called Bdelloplast formation (15), and preparation for growth by the parasite, 5) growth of the parasite, 6) division of the parasite and 7) disruption of the host cell and release of the B. bacteriovorus progeny (14,18).

Chemotaxis has been suggested as playing a role in attachment, since B. bacteriovorus behaves as though it "recognizes" the prospective host cell in a mixed culture of nonsusceptible and susceptible bacteria (19). However, the work of Straley and Conti (20), suggests that B. bacteriovorus does not use chemotaxis to locate prey cells.

Motility of the parasite is essential for attachment (4). The nonflagellated end of the parasite attaches to the host cell (19). The interaction begins with a violent collision of the motile parasite with the host. Although the host cell is usually ten times bigger than the parasite, the impact often moves the host cell several cell lengths (2). Starr and Baigent (19), suggested that the actual collision is necessary for attachment. Snellen and Starr (21), using freese etch, showed the boundary of attachment between B. bacteriovorus and Spirillum serpens, and they suggested that the bond between the two appeared to be extremely firm. However, in the earliest stages, attachment is reversible(19).

After attachment, B. bacteriovorus breaches the host cell wall, and within a few minutes penetrates the host (22). Varon and Shilo (23); suggested that inducible enzymes have to be formed by the parasite before it is able to penetrate the host. Packrell et al. (24,25), isolated two enzymes
released by B. bacteriovorus, strain 6-5-5 into the culture fluid: a peptidase and a protease. They believe that these enzymes are involved with penetration.

Constriction of the parasite is usually seen as B. bacteriovorus advances into the host periplasm, which is the region between the cell wall and the cell membrane of the host (14). The host cell wall then becomes distorted and separated from the cell membrane, forming widened areas in the periplasm, where B. bacteriovorus subsequently develops (11). Snellen and Starr (21), noted wrinkling of the outer wall of Spirillum upon attack by B. bacteriovorus. They suggested that the parasitic attack destroyed the organizational integrity of the lipoprotein of the Spirillum cell wall, which is thought to anchor the outer wall membrane to the underlying peptidoglycan. Whatever the mechanism, the host cell rounds up to form a "spheroplast". This "spheroplast" is not the same as a lysozyme spheroplast in that it does not lyse in a hypotonic medium (13), therefore it is called a Bdelloplast (15).

B. bacteriovorus develops within the periplasm, by thickening and elongating to form a helical filament (14). Rittenberg and Shilo (18) suggested that the intraperiplasmic development of B. bacteriovorus should be divided into two stages: preparation for growth and growth itself. During the preparation for growth stage, the parasite breaks down host material into monomeric units which are subsequently used by the parasite in the growth stage.
After elongation, this helical filament segments by synchronous constriction into individual vibroid units (9). The manner in which the host cell wall is disrupted to release B. *bacteriovorus* progeny is not yet understood (14).

Infection of a single host cell by several B. *bacteriovorus* has been noted; this usually leads to rapid host lysis (9).

ii) Physiology

B. *bacteriovorus* was first believed to be obligately host dependent for its growth (2). Since then several categories of axenically growing B. *bacteriovorus* mutants have been reported (26). Some are incapable of growing in the host while others are capable of growing both parasitically or axenically (26).

B. *bacteriovorus* is an obligate aerobe (2, 27). It has the enzymes for oxidative as well as substrate level phosphorylation (27). Rittenberg and Shilo (18), showed that one of the earliest consequences of parasitic attack is a general disruption of the respiratory activity of the host. Since oxidative and substrate level phosphorylation occur long after this disruption of host respiratory ability, it can be concluded that B. *bacteriovorus* has a complete energy generating system and this system is the exclusive source of energy for parasitic growth (18). Gadkari and Stolp (28), discovered that in B. *bacteriovorus*, strain 109Sa, 88% of its metabolism is oxidative phosphorylation and the rest is
substrate level phosphorylation. B. bacteriovorus cannot oxidize or ferment carbohydrate since it lacks some of the key enzymes involved in the breakdown of carbohydrate (27). Amino acids derived from protein breakdown serve as the major source of energy during intraperiplasmic growth of B. bacteriovorus (29).

Considering the special quality of the intraperiplasmic environment, Pritchard et al. (30), postulated that B. bacteriovorus might have undergone an evolutionary adaptation causing its physiology to take advantage of this particular niche. This niche could provide all the monomers required for the synthesis of macromolecules, so that B. bacteriovorus would not have to synthesize monomers of its own. If B. bacteriovorus were taking advantage of this situation, it would be expected to be insensitive to all metabolic inhibitors that prevent synthesis of monomers. Pritchard et al. (30), then showed that methotrexate, a folate analogue that inhibits the synthesis of dTMP, inhibited the axenic growth of the parasite, but not its intraperiplasmic growth. This would suggest that B. bacteriovorus does not need to synthesize monomers to survive in the periplasmic space, but uses host monomers in its anabolic processes, thus making efficient use of its evolutionary niche.

The results of other experiments also suggest the direct use of monomers by the parasite (29, 31-33). Hespel et al. (29), showed that exogenous substrates are used minimally by B. bacteriovorus during intraperiplasmic
growth. He showed that glutamate or a mixture of amino acids increased the *B. bacteriovorus* population by only about 10% over the control in which *E. coli* served as the only substrate. Matin and Rittenberg (31), illustrated using $^3$H-thymidine, that 73% of the thymidine residues of host DNA were incorporated into *B. bacteriovorus* DNA when *E. coli* was the only source of nutrient. In the presence of dilute nutrient broth, about 23% of the precursors arose from exogenous nutrients. Rittenberg and Langley (33), showed a marked preferential use of *E. coli* phosphorus over exogenous orthophosphate. Their data indicated that nucleoside monophosphates derived from the substrate organism are utilized directly for nucleic acid biosynthesis by *B. bacteriovorus* growing parasitically. Kuenen and Rittenberg (32), demonstrated that most of the fatty acids of *B. bacteriovorus* not homologous with those of the host were derived by metabolic alteration of the preexisting fatty acid of the host. They showed that *B. bacteriovorus* is capable of $\beta$-oxidation and hydrogenation of fatty acids. *De novo* synthesis of lipid from acetate occurs only to a small extent.

Rittenberg and Hespel (34), demonstrated that intraperiplasmic growth of *B. bacteriovorus* is highly energy efficient. $Y_{\text{ATP}}$, the energy efficiency of an organism, is the dry weight of cells formed per mole of ATP produced. This value was determined theoretically and experimentally. For most bacteria the experimental value was about 35% of
the theoretical value. In *B. bacteriovorus*, the theoretical and experimental values were very similar. A "best" \( Y_{\text{ATP}} \) value of 13.5 was obtained for single cycle growth experiments and an average value of 25.9 from multicycle experiments, was obtained for *B. bacteriovorus*. Both values are much higher than the usual value of 10.5 for most bacteria grown in rich medium. Rittenberg and Hespel (34), postulated that part of the reason *B. bacteriovorus* is energy efficient is that it uses host monomers directly. However they stated that this utilization of host monomers directly reduced the ATP requirement for growth by only 30%. The \( Y_{\text{ATP}} \) value for *B. bacteriovorus* is 1.5 times that for other bacteria. Rittenberg proposed therefore that additional factors must be involved in this higher energy efficiency. An underlying assumption in the calculation of the theoretical \( Y_{\text{ATP}} \) value is that catabolism and anabolism are balanced. If the ATP produced by catabolism is not used efficiently, there will be energetic uncoupling (35). The observed \( Y_{\text{ATP}} \) ratio may actually reflect the overall degree of energetic coupling for a particular organism under given conditions. If this is the case, then the degree of coupling for *B. bacteriovorus* appears to be quite high. Rittenberg and Hespel (34) postulated that this efficient energy coupling may account for the difference in energy efficiency in *B. bacteriovorus*.

Considerable work has been done with endogenous
respiration of B. bacteriovor us. Rittenberg and Shilo (18), reported that B. bacteriovor us has an unusually high rate of endogenous respiration — seven times that of E. coli. Hes pel et al. (29), reported that glutamate and mixtures of amino acids are oxidized by B. bacteriovor us, strain 109J. The oxidation of these substrates while suppressing the endogenous respiration of the parasite, caused only a small increase in the rate of O₂ consumption, (20 - 50%). He postulated that one explanation for oxidation of an exogenous substrate without change in the respiratory quotient is that "endogenous respiration of B. bacteriovor us saturates the functional capacity of either its Tricarboxylic Acid Cycle or its electron transport chain".

Gadkari and Stolp (36), discovered that in B. bacteriovor us, the ratio of O₂ uptake during substrate respiration over endogenous respiration was 2.5/1 as compared to 20/1 for E. coli. They also found that during endogenous respiration, the ATP pool turnover rate of B. bacteriovor us was 2.5 times that of E. coli. Considering the difference in ATP pool of B. bacteriovor us (9 nmoles/100μg) and E. coli (4.48 nmoles/100μg), the ATP pool turnover rate of B. bacteriovor us is actually 5 times that of E. coli. Furthermore, Backi and Ettlanger (37), when working with Acetobacter aceti found that there was an abrupt fall in extracellular ATP after substrate exhaustion and a drop in energy charge from 0.87 to 0.14. B. bacteriovor us, on the other hand,
has a constant energy charge between 0.62 and 0.64, during both endogenous and substrate respiration (28). This supports Hespel's idea (29) that endogenous respiration of B. bacteriovorus saturates the energy-producing "machinery" of the organism.

Another peculiarity of endogenous respiration of the parasite was reported by Gadkari and Stolp (28). They found that endogenous respiration of B. bacteriovorus is accomplished by oscillation of the ATP pool. Periods of ATP overproduction occurred at regular intervals of about 60 minutes. The ATP overproduction was started as soon as the ATP pool fell to a minimum value of about 6 nmoles per 100 μg nitrogen. This is consistent with the observation of Hespel (38), that the viability of B. bacteriovorus in the absence of substrate in unusually short, but that addition of respirable substrate prolonged the viability of the culture. In other words, the periodic bursts of ATP probably resulted from B. bacteriovorus' digestion of itself. Addition of exogenous energy sources prevented the organism from respiring its own cellular material. Gadkari and Stolp (34), postulated that since B. bacteriovorus requires motility for parasitism, the energy for motility could be derived from this digestion of self and resultant burst of ATP. If the ATP pool during starvation was kept constantly high by continuous breakdown of its own cellular material, this would result in an even more rapid digestion of itself. Therefore B. bacteriovorus seems to be showing
a degree of control over degradation which allows a more economic use of its cellular material (34).

It seems that *B. bacteriovorus* exhibits control over the degradation of host DNA as well. Martin and Rittenberg (31), showed that when *B. bacteriovorus* was grown on *Pseudomonas* sp. or *E. coli*, the host DNA was degraded 20 to 60 minutes after the initiation of the parasitic cycle, and *B. bacteriovorus* DNA synthesis occurred only after the degradation of host DNA. This clear separation in time between host DNA degradation and *B. bacteriovorus* DNA synthesis points to precise control over degradative processes.

With respect to the degradation of host proteins, Hespel (39), showed that host cytoplasmic enzyme activities decreased differentially during the infection cycle of the parasite. During the first 90 minutes when the glycolytic activities had decreased by 25% to 60%, the \( \beta \)-galactosidase activity had decreased by only 10%. All the above activities decreased to trace amounts by 180 minutes. Hespel postulated that the decrease in *E. coli* enzyme activities could result from inhibition of the enzyme, inactivation of the enzyme by minor alteration of the enzyme or degradation of the enzyme by proteases. His data suggested that degradation by proteases was the most probable event.

Since there seems to be a regulated degradation of host cytoplasmic enzymes and since the parasite actually
resides in the periplasmic space, it would seem logical to postulate that there may also be regulated degradation of host periplasmic enzymes. Alkaline phosphatase is a periplasmic enzyme and since it is well characterized biochemically and genetically, it was chosen as a marker of the host periplasm. This enzyme was used by the author to monitor changes in the periplasm of the host during parasitic attack. The following is a discussion of alkaline phosphatase.

iii) Alkaline Phosphatase

The enzymatically active alkaline phosphatase (E.C.3.1.3.1.) of *E. coli* is a dimer composed of two identical polypeptides, which are coded for by a single structural gene, pho A (40). However there are several isomers of the enzyme (41). Two explanations have been proposed to explain the formation of these various forms of the enzyme. One proposes that there is a modification of the enzyme after synthesis (42), and the other that there is ambiguous translation of the genetic message for the enzyme (43,44). However, during purification, if a shallow salt gradient is used with ion exchange chromatography, a broad peak containing all the isoenzymes can be eluted.

Synthesis of alkaline phosphatase by *E. coli* is repressed under certain environmental and genetic conditions. In the wild type strain, repression occurs when the growth
medium contains a high concentration of orthophosphate (45). Mutations at two loci separated from the pho A structural gene affect the synthesis of alkaline phosphatase (46). One locus consists of two cistrons pho S and pho T (47), while the other locus consists of three cistrons in the R region.

The first locus is involved in orthophosphate transport and therefore affects alkaline phosphatase synthesis indirectly. Pho S is the structural gene for a phosphate binding protein (48), that was originally designated the R2a protein by Garen and Otsuji (47). Willsky et al. (49), showed that in E. coli, there are two major orthophosphate transport systems which are synthesized constitutively; PST and PIT. There are also two inducible transport systems; (50, 51), gly t, an inducible glucose-6-phosphate transport system and an inducible glucose-6-phosphate transport system (50). Both systems are capable of transporting orthophosphate as a secondary substrate (52). Mutations of pho T lead to the loss of orthophosphate transport ability of the PST system (49).

Mutations at the second locus, pho R, have been found to have three phenotypes which have been designated Ra, Rb and Rc (53, 54). Mutations in the Ra or Rb cistrons result in an organism that produces alkaline phosphatase constitutively (55). Mutations in the pho Rc region result in an organism which is not derepressible for alkaline phosphatase
under orthophosphate limiting conditions (55). The \( R_c \) cistron seems to be physically separated from the other cistron of the \( \text{pho R} \) region (55). Kreuzer et al. (46), postulate that the product of the \( \text{pho R} \) cistron is necessary for alkaline phosphatase synthesis. A \( \text{pho B} \) gene has also been reported in the \( \text{pho R} \) area (56). The properties of the \( \text{pho B} \) mutant also suggest a positive control model for alkaline phosphatase regulation. Brickman and Beckwith (57), have demonstrated that a product of the \( \text{pho R-pho B} \) region is required for alkaline phosphatase synthesis. There is a question as to whether the \( \text{pho B} \) and \( \text{pho R} \) regions are the same gene (58).

Recent studies have indicated that lowered internal orthophosphate may not be directly related to alkaline phosphatase derepression. Wilkins (59), showed that pyridine starvation in high orthophosphate media also caused derepression. Just as in the \( \text{Hut} \) operon (60) — the signal that carbon is limiting is transmitted through a rise in cyclic-AMP levels — Wilkins suggested that a nucleotide might be a co-inducer for alkaline phosphatase synthesis. Repression of alkaline phosphatase synthesis may be accomplished by the product of \( \text{pho Rc} \) and/or \( \text{Rb} \) and a co-repressor (Fig 2). However this is speculation and there is no evidence to support it at this time.
Fig. 2

Alkaline Phosphatase "operon"
Structural gene  Phosphate Transport  Repressors  Corepressors?  Inducers  Coinducers?
Morris et al. (61), demonstrated three other periplasmic proteins that are co-ordinately controlled with alkaline phosphatase. Possibly these proteins are involved with the genetic control of alkaline phosphatase.

Inoïye and Beckwith (62), proposed that after synthesis, alkaline phosphatase is transported across the plasma membrane the same way that proteins cross membranes in eukaryotic cells. They proposed that the excretion of alkaline phosphatase occurs according to the signal hypothesis of Blobel and Dobberstein (63). According to this hypothesis, the structural gene for the protein contains a unique sequence (signal sequence), that codes for the amino terminal sequence which is hydrophobic. Translation of the signal sequence into the amino terminus of the growing chain and its emergence from the ribosome triggers the attachment of the ribosome to the membrane. The elongation of the peptide chain on the membrane-bound ribosome proceeds vectorially, discharging the nascent chain across the membrane. The signal sequence is then removed from the polypeptide chain by proteolytic cleavage during or after excretion (63). Inoïye (62) showed that the in vitro translation product of the pho A gene is larger than the subunit of alkaline phosphatase found in the periplasm of E. coli. The extra segment allows alkaline phosphatase to bind tightly to decyl agarose, which is consistent with the notion that the extra segment
is hydrophobic. The large translation product can be processed by a membrane fraction in vitro to about the same size as the subunit of periplasmic alkaline phosphatase.

The biochemistry of alkaline phosphatase is fairly well established. It is a dimeric metal enzyme with a molecular weight of 89,000 daltons (64). Two of its 4 Zn$^{++}$ atoms serve in catalysis and two in maintaining structure (65). Enzyme bound Mg$^{++}$ modulates both the catalytic and structural properties of the enzyme (66). The enzyme has two phosphate binding sites, one on each subunit (67). Since all the substrates of alkaline phosphatase bear a negatively charged phosphate group, the existence of a corresponding positively charged recognition site of the enzyme seems likely (68). The participation of lysyl or hystidyl residues has been ruled unlikely (69) and therefore Zn$^{++}$ and arginyl residues are the remaining possibilities for recognition sites. Phosphate binding is metal ion dependent (70), and Gottesman (71) states that there are indications that Zn$^{++}$ is involved in phosphate binding. Daeman (68) demonstrated that two arginyl residues are essential to both the hydrolase and transferase activities of the enzyme. The manner in which the arginyl residues and Zn$^{++}$ ions participate in the catalytic mechanism of alkaline phosphatase is still unknown (68). Chletowski et al (72), using $^{113}$Cd NMR showed the existence of negative homotropic interactions in phosphate binding. They showed that covalent or non-covalent binding at one site induces structural
changes which prevent tight specific binding of phosphate at
the other site.

Alkaline phosphatase catalyzes the hydrolysis of a
wide variety of phosphate monoesters and the transfer of
phosphoryl groups to suitable alcohol receptors (73).
These monoesters are hydrolyzed at nearly identical rates
and therefore it was postulated that catalysis involved the
rapid formation of a covalent phosphoryl enzyme intermediate.
Rate limiting hydrolysis or breakdown of this common
intermediate would then result in a constant enzyme turnover
rate, regardless of the leaving group. This proposed
intermediate was found to be stable at acid pH and to
contain a phosphoserine group (74). However at alkaline
pH, a noncovalent complex between the enzyme and ortho-
phosphate was found to be more stable than the covalent
complex. Hull et al (73) states that the lifetime of this
complex is so long that it must at least contribute to the
rate limiting step at alkaline pH. Also the phosphate-
purged enzyme underwent a conformational change when
inorganic phosphate was bound at alkaline pH. The NMR
studies of Hull (73) indicated that binding of phosphate
to purged enzyme at low pH (ie to form the covalent
phosphoryl intermediate) did not produce the conformational
change observed at high pH. This led Hull (73) to propose
the following mechanism of action for alkaline phosphatase
(Fig. 3).
Fig. 3

Mechanism of Action of Alkaline Phosphatase
The exact phosphoryl binding or active site stoichiometry of the enzyme is still uncertain. The above mechanism of action refers to a single site. When the enzyme is purged of phosphate it exists in the E' state which is the conformation favored for catalysis. The addition of substrate results in a very rapid bi-molecular association step followed by a rapid dephosphorylation step $k_3$ to give $E' - P$ and an instant burst of ROH. At acid pH, the steady state phase follows with $k_2$ rate limiting. At alkaline pH $k_2$ is fast and the formation of $E'$ from $E'\pi$ complex via $k_1$ becomes the steady state rate limiting step.
MATERIALS AND METHODS
A. BIOLOGICAL

i) Cultures

Both *E. coli* cultures used, were obtained from B. J. Bachman at the *E. coli* Genetic Stock Centre, Yale University, School of Medicine. Each culture has an *E. coli* Genetic Centre Number of CGSC number.

*E. coli*, strain C-90, CGSC #4680, is a pho T mut mutant of Garen and Otsuji (47). This strain produces about 1/3 of the fully derepressed level of alkaline phosphatase when grown on media containing $10^{-3}$ M orthophosphate. Although it carries the PST transport gene, this system is nonfunctional (75). This strain carries the gene pho A, which is the structural gene for alkaline phosphatase and pho S, the gene that codes for the phosphate-binding protein originally designated the R2a protein(47).

*E. coli*, strain E-15, Hfr, CGSC #4829, is also a Garen strain. It has similar characteristics to the C-90 strain but in addition has a deletion in the structural gene pho A (76). This strain is therefore incapable of synthesizing alkaline phosphatase.

*B. bacteriovorus*, strain 6-5-S, isolated from the Thames River in London, Ontario, by Dr. S Maier, is a periplasmic parasite, which will live on *Spirillum serpens* VHL (77). In a mixed culture of host and *B. bacteriovorus*, 6-5-S, all the host cells are killed (27). This facultative parasite can exist host independently on heat
killed cells, or endoperiplasmically in most Gram-negative bacteria. It thrives well on E. coli.

ii) Media

The following media were used for the growth of E. coli or B. bacteriovorus, 6-5-S.

Phosphate-limiting media (PLM), contained 0.12 M THAM-HCl buffer, 0.05 M NaCl, 0.02 M NH₄Cl, 0.02 M KCl, 0.03 M NaSO₄, 0.001 M MgCl₂, 0.002 M CaCl₂, 2 x 10⁻⁶ M ZnCl₂, 0.15% bactopeptone and 0.24% glucose. The final pH of the media was 7.5 (78).

Yeast-Peptone-Sodium Acetate (YPSC) media contained 0.1% bactoyeast extract, 0.1% bactopeptone, 0.05% sodium acetate, 0.005% L-cysteine, 0.25% MgSO₄, and 0.025% CaCl₂ (79).

The following Difco media were prepared in distilled water, Trypticase-soy broth, Trypticase-soy agar, Nutrient broth and Nutrient agar.

THAM buffer, 0.025 M pH 7.5 (Fisher Chemical Co) was supplemented with 0.002 M Ca²⁺ and 0.002 M Mg²⁺ in all cases. The mixed culture of E. coli and B. bacteriovorus was always suspended in this buffer for the six day incubation period. Stock cultures of B. bacteriovorus, 6-5-S, were also suspended in this buffer.

iii) Maintenance of Cultures

Stock cultures of E. coli were incubated at 37°C and
then maintained at 4°C on Trypticase-soy slants and Trypticase-soy broths. The slants were subcultured monthly and the broths weekly.

Stock cultures of *B. bacteriovorus* were obtained by incubating for 6 days a 2-membered culture of *B. bacteriovorus* and *E. coli*, strain C-90. The lysate was centrifuged twice. The first pellet, obtained after centrifugation at 6,000 x g for 20 minutes contained host debris and was discarded. The second pellet, obtained after centrifugation at 16,000 x g for 1 hour contained *B. bacteriovorus*, 6-5-S. This pellet was resuspended in 50 ml of sterile THAM buffer.

The pellet from a 250-ml suspension of *E. coli*, grown in nutrient broth for 16-20 hours was resuspended in 250 ml of sterile THAM. Actively growing cultures of *B. bacteriovorus* were obtained by inoculating 10 ml of *B. bacteriovorus*, 6-5-S stock culture into this 250-ml suspension of *E. coli*. The two-membered culture was incubated for 5-6 days at 30°C on a rotary shaker.

**B. CHEMICAL**

i) **Protein**

The presence and relative concentration of protein in fractions eluted from column chromatography was demonstrated by its characteristic absorbance at 280 nm. Quartz cuvettes (1-cm light path) were used in a Coleman 124 Perkin-Elmer Double-Beam Spectrophotometer. Protein
concentration was also determined by Bailey's modification (80) of the technique of Lowry et al. (81). Crystallized bovine serum albumin (Sigma Chemical Co) was used as a standard.

ii) Alkaline Phosphatase (E.C.3.1.3.1.)

Alkaline phosphatase was assayed by the method of Garen and Leventhal (41). The enzyme was incubated with $1 \times 10^{-3}$ M paranitrophenylphosphate (PNPP) in 1 M Trisma-base, pH 8.0. These were reagent grade chemicals from the Sigma Chemical Co., St. Louis. The increase in absorbance (1-cm path length), which occurred at 410 nm resulted from the dephosphorylation of PNPP to yield nitrophenol. The absorbance change was recorded on a Sargent-Range Precision Recorder. Under the conditions of the assay, the enzymatic activity is linearly dependent on the enzyme concentration and independent of substrate concentration. The enzymatic activity was determined using the equation of Garen and Leventhal (82). One unit of alkaline phosphatase is defined as the activity required to liberate 1 µmole of PNPP per minute under the conditions defined above.

The alkaline phosphatase kinetic studies involved incubation of a constant amount of the enzyme with various concentrations (between $1 \times 10^{-5}$ to $1 \times 10^{-3}$ M) of PNPP. Under these conditions the enzymatic activity was dependent on the substrate concentration. The $K_m$ and $V_{max}$ of the enzyme was obtained using a Linewaever-Burke plot.
iii) **Enzyme Production and Isolation**

A 10-ml Trypticase-soy broth of *E. coli*, strain C-90 (24-hour culture) was centrifuged, and the pellet, containing the bacteria, was resuspended in 500 ml of PLM. This suspension was put on a rotary shaker at 150 rpm at 37 °C for 16-20 hours. The derepressed culture was then centrifuged at 6,000 x g for 20 minutes and the pellet which contained the cells was resuspended in 500 ml of sterile THAM. When the pellet was completely dispersed, the suspension was divided equally into two 500-ml flasks. One suspension was inoculated with 10 ml of *B. bacteriovorus*, 6-5-S stock culture. The other suspension (containing no parasite) was used as a source of *E. coli* alkaline phosphatase. Both flasks were put on a rotary shaker at 150 rpm at 30 °C for 5 days. The periplasmic enzyme, from the parasitic system, was obtained from the supernatant, following centrifugation of the 5-day old suspension at 16,000 x g for 1 hour.

Several methods for obtaining the periplasmic enzyme from the host were compared. These methods included, ultrasonic treatment, osmotic shock, lysozyme treatment, and treatment with toluene.

Ultrasonic treatment involved the disintegration of bacterial cells using ultrasonic vibrations. The pellet from a 5-ml suspension was resuspended in 1 ml of phosphate buffered saline (PBS), pH 7.0 and sonicated with a Bronwill-Blackstone Biosonik Ultrasonic Probe, in 30
second bursts of full power for 3 minutes. All suspensions were sonicated in 10-ml containers packed in ice (78). The sonicated suspension was then centrifuged at 6,000 × g for 20 minutes to remove cell debris and the supernatant, containing the enzyme was assayed for alkaline phosphatase activity.

The method of Malamy and Horecker (83), involved lysozyme spheroplast formation. The cells grown on PLM for 16-20 hours, were harvested by centrifugation and resuspended in 20% sucrose containing 0.033 M THAM buffer pH 8.0. The suspension was kept at 4°C and was treated with 0.1 M EDTA to produce a final concentration of 1 × 10^{-3} M and lysozyme to give a 3.6 × 10^{-8} M concentration. The cells were stirred gently in the cold for 15 minutes. The suspension was then centrifuged at 6,000 × g for 20 minutes at 4°C and the supernatant, which contained the enzyme, was assayed for alkaline phosphatase activity.

The method of osmotic shock by Neu and Heppel (84), differed from that of Malamy and Horecker, in that lysozyme was omitted. After centrifugation of the cell-EDTA mixture, the sensitized cells of the pellet were rapidly mixed with cold water to one fifth of the volume of the original suspension. The lysed cells were then centrifuged again at 6,000 × g for 20 minutes at 4°C. The supernatant which contained the enzyme, was then assayed for alkaline phosphatase activity.

The toluene method involved addition of 3 drops of
toluene to 0.5 ml of the E. coli suspension. This was incubated for 1 hour and then centrifuged at 6,000 x g for 20 minutes. The supernatant which contained the enzyme was assayed for alkaline phosphatase activity.

iii) Immunology

To obtain antisera to the host-parasite enzyme, the crude preparation of enzyme (about 2 mg/ml) from the 2 membered culture was injected into a New Zealand White Rabbit, obtained from the Triangle Farms. A suspension which consisted of 50% crude preparation of enzyme and 50% Freunds incomplete adjuvant was made. The first week, the initial intradermal injections of this mixture were make into the shaved back of the animal. One ml of the injection material was divided into several smaller injections to reduce trauma to the animal. The following week an intravenous injection of 1 ml was made into the marginal ear vein of the rabbit. This intravenous injection was repeated each subsequent week for 5 weeks. The animal was bled prior to injection in each case. The blood was obtained from the marginal ear vein and collected into centrifuge tubes. About 15 to 20 ml were obtained each week and stored overnight in the cold. The serum was obtained the following morning by centrifugation at 2,000 x g for 15 minutes. It was stored at -20°C until used. The same procedure was used for the production of antisera to the host crude enzyme preparation.
To obtain immunodiffusion slides, noble agar (1.5%) was melted in PBS 0.01 M at pH 7.0 and 5 ml was pipetted onto a microscope slide. Five wells were cut out of the agar and the antisera and antigen preparations were placed in these wells and allowed to diffuse in a humidity chamber in the cold for 3 to 7 days. The slides were then washed with saline and distilled water, consecutively and dried at 37°C. The dried slides were stained in 1% Amidoschwartz, destained in 7% acetic acid and a coverslip was applied.

iv) Electrophoresis

Polyacrylamide disc gel electrophoresis (85) was used to characterize crude preparations of each enzyme and as a criteria of purity. A large-pore spacer gel was layered on top of the small-pore separation gel. The spacer gel was photopolymerized using riboflavin as a catalyst. The small-pore gel was chemically polymerized using ammonium persulfate. The electrophoretic buffer was Tris-glycine, pH 8.3. Bromthymol blue was used as a tracer dye. The gels were stained using either Amidoschwartz (1%), Coomassie blue or the enzyme capture method of Allen and Hyncik (86). The enzyme capture method involved incubation of the gels at 25°C for 15 min in a solution containing 5 mM sodium-β-glycerophosphate, 15 mM CaCl₂ and 33 mM Tris-chloride at pH 9.5. The gels were rinsed with water and placed into 3 mM lead nitrate in 80 mM Tris-maleate
at pH 7.0 for 30 minutes. They were then washed for 1 hour in water, with frequent changes of water, and finally placed into a 5% ammonium sulfide solution for 2 minutes. This was followed by a final wash with water. All gels were stored in 7% acetic acid in stoppered test tubes and read on a Beckman Microzone Densitometer, model R-110.

v) Enzyme Purification

The *E. coli* enzyme and the enzyme from the host-parasite interaction (host-parasite enzyme) were purified according to the scheme in Fig. 4. The crude extract of the *E. coli* enzyme was obtained from the cellular fraction by osmotic shock. The crude extract of the host-parasite enzyme was the extracellular fraction of the two-membered culture, obtained after centrifugation at 16,000 x g for 1 hour. The crude extract in each case was fractionated using ammonium sulfate and further purified using gel filtration and ion exchange.

vi) Ammonium Sulfate Fractionation

The crude preparation of the enzyme was separated into 2 ammonium sulfate fractions. Solid ammonium sulfate was added slowly until 50% saturation was obtained. After standing overnight, the precipitate which formed was collected by centrifugation. The pellet, which contained unwanted material was discarded. The supernatant was then
Flow diagram of the procedure for purifying alkaline phosphatase from \textit{E. coli} and \textit{B. bacteriovorus}.

The crude extract of the \textit{E. coli} enzyme was obtained from the cellular fraction by osmotic shock.

The crude extract of the host-parasite enzyme was the extracellular fraction of the two-membered culture.
PURIFICATION OF ALKALINE PHOSPHATASE

Crude Extract

50% ammonium sulfate
16,000 x g - 20 min

Supernatant → Pellet (discard)

70% ammonium sulfate
16,000 x g - 20 min

Pellet ← Supernatant

Resuspend in PBS
Gel Filtration P-150

Buffer ← Active Fraction

Ion Exchange:

Buffer → Buffer + 0.12 M NaCl

Purified Enzyme
brought to 70% saturation by further addition of solid ammonium sulfate. This was again allowed to stand over-night; the precipitate formed was collected by centrifugation. This pellet, which contained the enzyme was dissolved in 2 ml PBS (0.01 M at pH 7.0) and applied to a gel filtration column.

vii) **Column Chromatography**

Bio-gel P-150 (Bio-Rad 50-100 mesh, exclusion limit 150,000 daltons), was prepared by addition fo 45 grams of dry Bio-gel P-150 to an excess of distilled water, while stirring continuously. After settling, supernatant and "fines" were decanted and a 0.025 M PBS buffer pH 7.0 was added to the swollen gel, in a volume 4 times that of the gel. The Bio-gel was stirred, allowed to settle and the "fines" decanted again. This step was repeated 5 times. The washed Bio-gel was packed into a column. The packed equilibrated column measured 33.7 cm and was eluted at a rate of 1 ml per hour. A 5-ml sample of 0.02% blue dextran was used to determine the void volume (14.0 ml) and to discover any irregularities in the column.

The ammonium sulfate precipitated fraction in 2 ml of PBS was applied to a P-150 column. The column was eluted with PBS and 1-ml fractions were collected. Each fraction was analyzed for 280 nm absorbing material and assayed for alkaline phosphatase activity. A qualitative determination of enzyme activity was done on each fraction.
A quantitative assay was performed on fractions showing enzyme activity. This assay was done to determine the peak of activity. The peak was pooled and further purified using DEAE cellulose.

viii) Ion Exchange

The final step of purification was ion-exchange chromatography. A suspension of diethylaminoethyl cellulose anion exchanger (medium mesh, capacity 194 meq/g from the Sigma Chemical Co, St. Louis) was prepared by stirring 20 g of the dry powder into 1 liter of 0.05 M NaCl. After the slurry had been stirred for several minutes, the material was allowed to settle and the "fines particles" were removed by decantation. This washing was repeated several times before the suspension was acidified by addition of a quantity of 1 M HCl equivalent to the exchange capacity of the gel. For 20 g of DEAE-cellulose, 17.4 ml of 1 M HCl was required. This suspension was then used to pack the column. The packed column was washed with 0.05 M NaCl under pressure. The pooled fraction from gel filtration was then added to the column. The flow rate was slow and when the entire fraction had been adsorbed, the column was washed with 20 ml of 0.05 M NaCl. Fractions (4 ml) were collected on an automatic fraction collector at 10-min intervals. The phosphatase was eluted from the column with 0.125 M NaCl and usually appeared in 4 tubes between 20 and 36 ml of eluent (81). Each eluted fraction
was tested for 280 nm absorbing material and assayed qualitatively for alkaline phosphatase activity. Quantitative assays were done on the fractions containing alkaline phosphatase and a peak of activity was determined. The active fractions were pooled.

ix) **Isoelectric Focusing**

The isoelectric pH of each crude preparation was determined using Ampholine 8100 Electrofocusing Equipment.

A sucrose stabilizing solution was put into a LKB 8100-1, 110-ml water jacketed column. The column was filled using a gradient mixer and peristaltic pump and kept cool by circulating refrigerated water through the water jacket. After electrophoresis, the column was emptied by pumping the contents out the bottom. Two-ml fractions were collected and equilibrated to room temperature (5 - 10 minutes) before the pH was determined using a microprobe.
RESULTS
Sonication is an accepted method of releasing protein from bacterial cells (78). Various sonication times were compared to determine the optimal time for the most efficient release of alkaline phosphatase from the host. In Fig. 5, the level of activity of the enzyme released increased to 1 minute, plateaued to 4 minutes and then decreased. The interval of 3 minutes was chosen to release alkaline phosphatase from the host.

Alkaline phosphatase is naturally found in the periplasmic space of *E. coli* (87). This periplasmic space is invaded by *E. bacteriovorus* during parasitization. The parasite reproduces here and then causes the host to burst, releasing the progeny of the parasite (15). This parasitization by *E. bacteriovorus* could be accompanied by a) a shift in compartmentalization of the enzyme from the periplasmic space to the extracellular fraction and/or b) a change in the level of activity of the enzyme in the parasitic system (higher or lower). To determine if any of these alternatives would occur, *E. bacteriovorus* was inoculated into a suspension of *E. coli*, strain C-90, in THAM buffer. An identical suspension of *E. coli* was left uninoculated. Both suspensions were incubated at 30°C on a rotary shaker at 150 rpm for 6 days. Fractions (5-ml) were removed each day from each suspension and centrifuged at 8,000 x g for 30 minutes. The alkaline phosphatase in the pelleted cells was released using sonication and then assayed; the supernatant alkaline phosphatase was assayed
Fig. 5
The relationship between sonication time and alkaline phosphatase activity released.
directly.

The suspension containing the parasite cleared. This indicated that the parasite had grown. Fig. 6a represents the alkaline phosphatase in the suspension of *E. coli* alone. Most of the enzyme occurred in the cellular fraction (about 80%), although some occurred in the extracellular fraction, (about 20%). Fig 6b demonstrates that when the parasite is present, the alkaline phosphatase activity shifts to the extracellular fraction. The cellular fraction here contains about 25% of the activity compared to 80% in the cellular fraction of *E. coli* alone. This indicates that there has been a compartmentalization shift of the enzyme. Fig. 7 is a plot showing the same data as Fig. 6a and 6b except that the extracellular and cellular fractions have been added to show total activity in each system. The peak of activity in both systems occurred at day 4. This graph shows that there is an apparent increase in the total alkaline phosphatase activity in the parasitic system of about 40%. Another interpretation of these data is that the increased activity reflects the altered sensitivity of the enzyme in the 2-membered culture to sonication. For example, some denaturation of the enzyme may be occurring even with a 3-minute sonication time; the enzyme in the parasitic system may be more resistant to this denaturation than the host enzyme alone. Sonication is a very harsh method of enzyme release; extensive cell damage is evident under the
Fig. 6a
Alkaline phosphatase activity vs. time in days in the *E. coli* system alone. The open circles represent the cellular fraction, which has been released by sonication. The solid circles represent extracellular alkaline phosphatase.

Fig. 6b
Alkaline phosphatase activity vs. time in days in the two membered *E. coli* + *B. bacteriovorus* system. The open circles represent the cellular fraction released by sonication. The solid circles represent extracellular alkaline phosphatase.
Fig. 7

Total alkaline phosphatase activity vs. time in days. The open circles represent the two membered, *E. coli* + *B. bacteriovorus* culture. The solid circles represent *E. coli* alone.
microscope and Fig. 5 indicates destruction of the enzyme after a 4 minute exposure to sonication. Since the results shown in Fig 6b could be an artifact of enzyme isolation method, several other methods of enzyme release were then compared to determine the most effective. Specific activity was used as a rough measure of the value of the technique --- a high specific activity demonstrated more enzyme released with less contaminating protein. The results are shown in Table 1. This table indicates that the least desirable method was toluene, which has the lowest specific activity. The specific activity of sonication was also comparatively low. Lysozyme and osmotic shock both had relatively high specific activities. Osmotic shock appeared to be the method of choice.

The growth experiment represented in Figs 6 and 7, was repeated using the method of osmotic shock to extract the enzyme from the cellular fraction. Fig. 8a is a reflection of the typical compartmentalization of the enzyme in the host. More enzyme is found in the cellular fraction than in the extracellular fraction. Fig. 8b shows that again, there has been a shift in compartmentalization from the cellular to the extracellular fraction. Fig. 9 shows the result of adding the cellular and the extracellular fractions in both systems. It is apparent that there is an increase in the total activity in the two membered, host-parasite suspension. Therefore the result of sonication
<table>
<thead>
<tr>
<th>Method</th>
<th>Lysosome</th>
<th>Osmotic Shock</th>
<th>Toluene</th>
<th>Sonication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme released</td>
<td>0.1</td>
<td>0.49</td>
<td>0.04</td>
<td>0.17</td>
</tr>
<tr>
<td>Units / ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>0.03</td>
<td>0.032</td>
<td>0.159</td>
<td>0.072</td>
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<tr>
<td>mg / ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific Activity</td>
<td>3.3</td>
<td>15.3</td>
<td>0.25</td>
<td>2.36</td>
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<tr>
<td>Units / mg</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Fig. 8a
Alkaline phosphatase activity vs time in days in the *E. coli* system alone. The open circles represent the cellular fraction, which has been released by osmotic shock. The solid circles represent extracellular alkaline phosphatase.

Fig. 8b
Alkaline phosphatase activity vs time in days in the two-membered *E. coli* + *B. bacteriovorus* system. The open circles represent the cellular fraction released by osmotic shock. The solid circles represent extracellular alkaline phosphatase.
Fig. 9
Total alkaline phosphatase activity vs. time in days. The open circles represent the two-membered *E. coli* + *B. bacteriovorus* culture. The solid circles represent the *E. coli* culture alone. The enzyme was released from the cellular fraction using osmotic shock.
Total Enzyme Activity

Enzyme Activity (Units/mL)

Time (Days)
growth experiment is probably not an artifact. Osmotic shock was used for all subsequent experiments, where enzyme release was necessary.

The increase in the total enzyme activity seen in the previous two growth experiments could be a result of a) an additional enzyme contributed by the parasite, b) more enzyme synthesized by the host, or c) a change in the structure or conformation of the host enzyme to make it more active.

To test the first hypothesis, B. bacteriovorus was grown on a mutant of E. coli, strain E-15, that lacked the structural gene for alkaline phosphatase. The growth experiment using osmotic shock was repeated except the host in this case was the mutant E-15. No detectable cellular or extracellular enzyme was found throughout the 6-day growth period. Many B. bacteriovorus could be seen under the microscope in a 5-day culture, however, and the optical density of the suspension decreased over time, which indicated that the parasite grew on this strain of E. coli. This experiment demonstrated that alkaline phosphatase was not produced by B. bacteriovorus, in the parasitic situation.

Further, to determine if the parasite could produce alkaline phosphatase while growing saprophytically, B. bacteriovorus, was grown on autoclaved E. coli. Although the suspension decreased in optical density over time, indicating growth, no alkaline phosphatase was detected.
Therefore the first of the three hypotheses was eliminated—the increase in activity was not due to an additional alkaline phosphatase contributed by the parasite.

Considering the second hypothesis, whether more enzyme is synthesized by the host, it was postulated that *E. bacteriovorus* might sequester phosphate during the parasitic interaction, thereby increasing derepression of the *E. coli* alkaline phosphatase gene. If this sequestering occurred, then more enzyme activity would be expected in the host-parasite system than in the host system alone if both cultures were incubated in phosphate-rich medium.

To test this hypothesis, the growth experiment seen in Fig. 8 and 9 was repeated except the host was transferred to sterile THAM supplemented with phosphate (Table 2). The host was grown in either a phosphate-rich or a phosphate-free medium before transfer to the phosphate-enriched THAM. Table 2 shows that there is less activity in the host-parasite system than in the host system alone. Therefore the parasite is not sequestering phosphate. Rather, the results suggest that the change in permeability of the host cell membrane caused by the parasite (18, 83), simply allowed the phosphate to enter the host sooner and repress the gene faster than it was repressed in the host cell alone. The host cell alone must depend on permeases to transport the phosphate into the cell. This data would confirm the work of Rittenberg and Shilo (18) who suggest that there is early host membrane damage in the parasitic
### TABLE II

Affect of a Phosphate Rich Final Media

<table>
<thead>
<tr>
<th>Host Grown in Phosphate Free Media and Then Transferred to Phosphate Rich THAM-HCl</th>
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</thead>
<tbody>
<tr>
<td>Units of Alkaline Phosphatase</td>
</tr>
<tr>
<td>E. coli</td>
</tr>
<tr>
<td>E. coli + B. bacteriovorus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Host Grown in Phosphate Rich Media and then Transferred to Phosphate Rich THAM-HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units of Alkaline Phosphatase</td>
</tr>
<tr>
<td>E. coli</td>
</tr>
<tr>
<td>E. coli + B. bacteriovorus</td>
</tr>
</tbody>
</table>
interaction. It also confirms the work of Crothers et al. (88), who showed that "holes are formed in the membrane of the host during the host-parasite interaction." Table 2 also shows the prior growth of the host on phosphate-rich media further repressed the amount of enzyme subsequently obtained in the growth experiment.

The above two experiments were repeated but this time was to THAM - HCl without phosphate. Again, in Table 3, less enzyme was obtained in the growth experiment if the host was first grown on phosphate rich media than if it was grown on phosphate-free media. Table 3 shows that as the phosphate supply becomes limiting, the derepression of alkaline phosphatase is increased and that increase is always greater in the host-parasite than in the host alone.

To investigate the third hypothesis, whether a change in the structure or conformation of the host-parasite enzyme had occurred, both enzymes were compared using physiochemical techniques. First gel filtration was done to see if there was a shift in the molecular weight of the enzyme. A crude preparation of the host-parasite enzyme (2 ml) was applied to a P-150 gel filtration column. The column had been equilibrated with 0.01 M PBS at pH 7.0. The flow rate was 1 ml / hour, and the fractions were collected in 1 ml aliquots. These fractions were assayed for 280 nm absorbing material and alkaline phosphatase activity. A crude preparation of the host enzyme was
### TABLE III

Affect of a Phosphate Free Final Media

<table>
<thead>
<tr>
<th>Description</th>
<th>Units of Alkaline Phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host Grown in Phosphate Free Media and then Transferred to Phosphate Free THAM-HCl</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>112</td>
</tr>
<tr>
<td>E. coli + B. bacteriovorus</td>
<td>200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Description</th>
<th>Units of Alkaline Phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host Grown in Phosphate Rich Media and then Transferred to Phosphate Free THAM-HCl</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>21</td>
</tr>
<tr>
<td>E. coli + B. bacteriovorus</td>
<td>30</td>
</tr>
</tbody>
</table>
treated in an identical manner. The results are shown in Fig. 10 and Fig. 11. In both instances a single peak was found and this peak was eluted in the same volume as commercially available alkaline phosphatase (Worthington). Therefore, if there was an alteration in the structure of the enzyme, it did not cause a molecular weight change that could be detected by gel filtration.

Ion exchange was then used to see if there was a difference in charge between the two enzymes. A crude preparation of the host-parasite enzyme was put onto an ion exchange column of diethylaminoethyl anion exchanger which had previously been equilibrated with 0.05 M NaCl. The enzyme preparation was applied with a very slow flow rate, in the cold, according to the method of Malamy and Horecker (83), and fractions were collected in 4-ml volumes. A crude preparation of the host enzyme was treated similarly. (See Fig. 12 and 13). In both cases the enzyme in the crude preparations adsorbed to the column and was eluted with 0.125 M NaCl as was the alkaline phosphatase described by Malamy and Horecker (83). It was concluded, from these results, that if an alteration in the enzyme had occurred, the charge was not altered.

The enzymes were then compared by polyacrylamide disc gel electrophoresis and an enzyme capture stain, to determine if there would be a change in the characteristic enzyme capture profile. Sucrose was added to the crude enzyme preparations to make a 40% sucrose solution. This
Fig. 10

Gel Filtration (P-150) of the host-parasite alkaline phosphatase. The cross-hashed area represents the enzyme, the solid line represents 280 nm absorbing material.
Fig. 11

Gel Filtration of *E. coli* alkaline phosphatase.

The cross-hashed area represents the enzyme. The solid line represents 280 nm absorbing material.
Fig. 12

Ion Exchange of host-parasite alkaline phosphatase.

The cross-hashed area represents the enzyme, the solid line represents 280 nm absorbing material.
Fig. 13
Ion Exchange of E. coli alkaline phosphatase. The cross-hashed area represents the enzyme. The solid line represents 280 nm absorbing material.
was layered onto the top of a polyacrylamide gel. A few drops of bromthymol blue (tracer dye) were placed into the upper buffer chamber and a current of about 2 milliamps per tube was applied. The enzyme preparations on the gels were electrophoresed in Tris-glycine buffer pH 8.3 until the tracer dye migrated to the bottom of the gels, usually about 3-5 hours. The gels were then removed from the tubes and stained using the postincubation capture method of Allen and Hynick (86). This method involved incubating the gels sequentially in sodium \(-\beta\)-glycerophosphate (a natural substrate of the enzyme), lead citrate and then ammonium sulfide. A black color was produced wherever the enzyme was present in the gel. The gels were scanned and recorded with a densitometer. Fig. 14 diagrams the patterns that were obtained. It is apparent that although the crude preparations resemble commercial alkaline phosphatase, there are some differences. However, they more closely resemble each other, with the exception of a slight shoulder found in the host-parasite tracing. This shoulder occurred consistently and at all concentrations tested. This result is suggestive of a very minor change in the enzyme structure.

The isoelectric pH of each crude preparation was determined using the LKB Ampholine 8100 Electrofocusing equipment. The ampholine range was 3.5 to 10. The best results were obtained with the cathode at the top. The
Fig. 14

Densitometry tracings of alkaline phosphatase stained by the enzyme capture method of Allen and Hýncík (84).

Fig. 14a  host-parasite alkaline phosphatase
Fig. 14b  *E. coli* alkaline phosphatase
Fig. 14c  Commercially prepared alkaline phosphatase
power was initially set at 5 watts and the system was
electrophoresed until it reached equilibrium which was
indicated by a drop in the power to 0. This usually took
about 1½ to 2 days. Two-ml fractions were collected and
the pH determined. The fractions were assayed for alkaline
phosphatase activity and the results are presented in Fig.
15 and 16. There is a single peak of activity in both
instances indicating that a second enzyme has not been
produced. The isoelectric pH of the host enzyme is 5.0 and
that of the host-parasite is 5.2. The difference in the
isoelectric pH's of the two enzymes suggests a small
structural or conformational alteration in the host-parasite
enzyme. The isoelectric pH of alkaline phosphatase reported
by Gaëron and Leventhal (41) is 4.5. The increase in the
isoelectric pH of both enzyme preparations could result from
the 5-day incubation of the bacteria in THAM at 30°C
before enzyme release.

The following immunological techniques were performed
to further investigate this enzyme change. The antisera to
the crude enzyme preparation were obtained and immunodiff-
usion slides prepared. The results (Fig. 17) show a line
of partial identity between the host-parasite enzyme and
the host enzyme, which is indicative of a small difference
in structure between the two enzymes.

The physiochemical experiments confirm that there is
only one enzyme and that it is not radically altered
alkaline phosphatase. However these experiments did not
Fig. 15
Isoelectric focusing of the host-parasite alkaline phosphatase. The cross-hashed area represents the enzyme, the solid line represents pH.
ENZYME ACTIVITY (UNITS/ML)

E. coli + Bd.
pl. = 5.2

FRACTION NUMBER (2 ML/TUBE)
Fig. 16

Isoelectric focusing of *E. coli* alkaline phosphatase.

The cross-hashed area represents the enzyme, the solid line represents pH.
Fig. 17a
Immunodiffusion diagram showing a line of partial identity between \textit{E. coli} alkaline phosphatase and Host-parasite alkaline phosphatase. The antiserum is Anti-host-parasite alkaline phosphatase.

Fig. 17b
Immunodiffusion diagram showing a line of partial identity between \textit{E. coli} alkaline phosphatase and Host-parasite alkaline phosphatase. The antiserum is Anti- \textit{E. coli} alkaline phosphatase.
rule out a small alteration which could result in modified activity. Therefore the two preparations were purified and the specific activities compared. The scheme used to purify the enzymes is summarized in Fig. 4. Preliminary experiments were done, to establish the optimal conditions for each step in the purification procedure. Ammonium sulfate was used to precipitate the alkaline phosphatase and partially remove unwanted material. Equal volumes of crude preparations were brought to 10, 20, 30, 40, 50, 60, 70, 80, 90, 100% saturation with reagent grade ammonium sulfate. Since the 70% precipitate contained the most activity, a single precipitate from the 50 - 70% ammonium sulfate treatment (described in materials and methods) was collected thereafter. This precipitate was dissolved in 2 ml of 0.01 M PBS at pH 7.0 and applied to a Bio-gel P-150 column. The partially purified enzyme was eluted in a single peak which occurred at the same eluent volume as commercially prepared alkaline phosphatase. The fractions containing the peak of activity were pooled and applied to a DEAE-cellulose column, according to the method of Malamy and Horecker (83). The partially purified preparation also adsorbed to the ion-exchange column and was eluted with 0.025 M NaCl. The peak that resulted from ion exchange was pooled and mixed with sucrose to produce a 40% solution, which was applied to a polyacrylamide gel as described previously. After electrophoresis was completed, the gels
were stained in 10% Amidoschwartz or Coomassie Blue. The densitometry tracings that resulted (Fig. 18) indicate the homogeneity of each preparation. A summary of the purification data can be seen in Table 4 and Table 5. The specific activity of the host-parasite enzyme is 46.5 units/mg compared to 2.5 units/mg for the host enzyme. The values for total enzyme activity and total protein in table 4 and 5 can be compared directly since the data represents equal numbers of host cells. Table 5 shows that the specific activity of the host-parasite enzyme is about 20 times that of the host enzyme alone and replicates of this experiment resulted in a host-parasite specific activity that ranged from 3 to 25 times that of the host enzyme alone.

According to Dixon and Webb (89), factors which influence the velocity of enzyme reactions may produce their results in two distinct ways. They can affect either the formation or the breakdown of the enzyme-substrate complex or both. The determination of $K_m$ and $V_{max}$ enables these two effects to be studied separately. The $K_m$ is a measure of formation of the enzyme-substrate complex whereas the $V_{max}$ is a measure of the breakdown of the enzyme-substrate complex. Enzyme kinetics were done with the two purified preparations. A Lineweaver-Burke plot (Fig. 19) showed the $K_m$ of the host-parasite enzyme to be $4.1 \times 10^{-5}$ l/ml and the $K_m$ of the host enzyme to be $5.45 \times 10^{-5}$ l/ml. The difference between these two values are minimal. On the other hand, the $V_{max}$ or
Fig. 18a
Densitometry tracing of crude *E. coli* alkaline phosphatase.

Fig. 18b
Densitometry tracing of purified *E. coli* alkaline phosphatase.

Fig. 18c
Densitometry tracing of purified host-parasite alkaline phosphatase.
<table>
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<tr>
<th>Purification procedure</th>
<th>Volume treated (ml)</th>
<th>Protein (mg/ml)</th>
<th>Enzyme (U/ml)</th>
<th>Total enzyme (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
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<td></td>
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<td>0.1</td>
<td>0.25</td>
<td>2</td>
<td>0.8</td>
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Fig. 19

Lineweaver-Burke plot showing *E. coli* and host-parasite alkaline phosphatase.
turnover number for the host-parasite enzyme was 100 and for the host enzyme it was 39. Since the turnover number for the host-parasite enzyme is about 3 times that of the host enzyme and the Kms are essentially the same, the alteration in the host-parasite enzyme has affected the speed of hydrolysis of the enzyme-substrate complex and not the affinity of the enzyme for the substrate.

According to Garen and Leventhal (41) the Km for alkaline phosphatase is $1.2 \times 10^{-5}$ and the turnover number is 2700. The Km of both enzymes are close to this reported value but the turnover number of the enzymes are much lower. Protein denaturation during the 5 day incubation of the bacteria at 30 C could be responsible for the low turnover numbers of the host-parasite enzymes and of the host enzyme.
DISCUSSION
The method used to release enzyme from the microorganism is an important variable to consider in bacterial enzyme studies. The apparent increase in activity seen in the parasitic culture in the first two growth experiments, Fig. 7 and Fig. 9, does not appear to be an artifact of the enzyme isolation method. Fig. 2 shows that prolonged sonication for more than 4 minutes may lead to a decrease in activity due to enzyme denaturation. However, both cultures were treated identically and the sonication time was less than 4 minutes. Sonication is one of the harsher methods of enzyme isolation. It disrupts the entire cell, releasing the cytoplasmic contents as well as the periplasmic proteins and leads to a low initial specific activity. Therefore several other methods of enzyme isolation were compared. The most valuable method would be the one that released the most alkaline phosphatase activity with the least amount of total protein. The method using toluene, which dissolves membrane lipid, was the least desirable, because the protein level released was high and the alkaline phosphatase activity recovered relatively low.

Lysozyme treatment (83) and osmotic shock (84) are methods for selective release of periplasmic proteins. The lysozyme method involved making the cells into spheroplasts by treatment with a combination of lysozyme and EDTA, in a THAM-sucrose solution. Lysozyme breaks the β-1,4-glycosidic
bonds between N-acetylglucosamine and N-acetylMuramic acid in the peptidoglycan of the Gram-negative cell envelope and thereby reduces the cell viability. EDTA chelates cations. Costerton et al. (90), noted that EDTA solubilized components of the cell envelope and thus facilitated penetration by lysozyme. Osmotic shock is similar to lysozyme treatment in that it also involves the use of EDTA and sucrose and causes the release of the same group of periplasmic proteins. However, since lysozyme is omitted the cells remain viable after osmotic shock. This would imply that osmotic shock is a gentler method of enzyme release. The data, Table 1 indicates that it indeed produced the enzyme preparation with the highest specific activity. This is consistent with the reports of Neu and Heppel (82) who found, that although both methods released about the same amount of alkaline phosphatase, spheroplast formation resulted in more ribonucleosidase being released. They also obtained the higher specific activity for alkaline phosphatase using osmotic shock.

The use of EDTA during osmotic shock may affect the results. It is known that alkaline phosphatase is a metalloenzyme, containing Mg$^{++}$ and Zn$^{++}$. Csopak and Snajn (91), reported that EDTA, when utilized to release phosphatase from the periplasmic space, remained firmly bound to the enzyme throughout all subsequent stages of purification, providing additional Zn$^{++}$ binding sites.
However, Vallee (91) showed that isolation procedures which never expose the enzyme to chelating agents resulted in preparations with a Zn$^{++}$ content identical to enzymes released in the presence of EDTA. Maximally, alkaline phosphatase in E. coli contains a total of 6 gm-atoms of metal, 4 Zn$^{++}$ and 2 Mg$^{++}$ (92). Phosphatase containing 4 gm-atoms of Zn$^{++}$ will bind 1.4 gm-atoms of Mg$^{++}$ at pH 7.2 and at a higher pH more Mg$^{++}$ is bound (92). Since the pH of enzyme release in my experiments was higher than 7.2, the isolated enzyme was probably very close to having its maximal complement of metal. Also, sonication and osmotic shock yielded similar results in the growth experiments shown in Fig. 7 and Fig. 9, and therefore the EDTA used for osmotic shock is probably not introducing an artifact.

Fig. 2a and Fig. 3a demonstrate that there is a shift in compartmentalization of alkaline phosphatase from the periplasm to the extracellular fraction when the parasite is present. In other words the parasite itself provides a biological method of enzyme release. The mechanism of release is unknown but it is probably enzymatic. The apparent increase in alkaline phosphatase activity may simply be a reflection of this release mechanism.

There was also a lesser shift in alkaline phosphatase from the periplasm to the extracellular fraction in the host system with age. Although this "naturally" released enzyme seemed to have a high specific activity as well, it was very unstable compared with the biologically released
enzyme in the host-parasite system. The parasite may simply be increasing the rate of release of alkaline phosphatase. There was also enzyme in the host-parasite cellular fraction but the amount was so small that it was disregarded. Instead the predominant form of the enzyme in the host was compared to the predominant form of the enzyme in the host-parasite system. Further work should be done with the host extracellular and host-parasite cellular enzyme. The experiments would have to be scaled up and purification methods modified for the unstable host extracellular enzyme.

Data from Fig. 7 and 9 show that there is an apparent increase in alkaline phosphatase activity in the host-parasite system. Since no alkaline phosphatase activity was detected in the E-15 parasite system, it can be assumed that no alkaline phosphatase was produced by the parasite. It was therefore concluded that the increase in alkaline phosphatase activity in the host-parasite system was due to enzyme synthesized by the host. This increase occurs at a time when the components of the host cell are thought to be destroyed (Rittenberg and Shilo, 1970). By the fifth day of incubation of the parasite with \textit{E. coli}, significant destruction of the host can be seen by phase contrast microscopy; the majority of the host cells are only hulks at this time.
To confirm that this apparent increase was not due to an additional enzyme, but rather to more enzyme of the same kind or simply more active enzyme, several physiochemical techniques were carried out. There was a single peak with gel filtration, ion exchange and isoelectric focusing of the host-parasite enzyme, indicating that no new and radically different species of enzyme was present. There was one precipitin line in immunodiffusion, indicating that the main protein present was alkaline phosphatase and that contaminating proteins were not present in sufficient quantities to elicit an antibody response or that the response elicited was not enough to be demonstrated by immunodiffusion. Finally the densitometry tracing for the host-parasite enzyme Fig. 14 was very similar to that produced by commercial alkaline phosphatase, again indicating that no new enzyme had been produced.

However, this physiochemical data also suggests that the enzyme might be slightly modified. The P.A.G.E. graphs Fig. 14 show a slight shoulder on the host-parasite enzyme which does not appear in the host enzyme alone. The isoelectric pH of the host-parasite enzyme is 5.2 which is slightly higher than the isoelectric pH of the host enzyme alone which is 5.0. There is a line of partial identity between the host-parasite enzyme and the host enzyme alone. All these data indicate that a slight structural or conformational modification of the host-parasite enzyme may
have occurred. Possibly digestion by the parasitic proteases caused a nick in the enzyme which made it more active.

Purification of the two enzymes showed that less enzyme protein was present in the host-parasite system -- but that it had a higher specific activity. The increase in specific activity was more than enough to account for the increase found in Fig. 7 and Fig. 9. Therefore this increased specific activity does not suggest that the host is synthesizing more enzyme at a time when most host activities have ceased. This higher specific activity of the host-parasite enzyme is also consistent with the result that *E. bacteriovorus* is not chelating phosphate from the host and thereby causing the host to produce more alkaline phosphatase (in Tables 3 and 4).

An obvious consequence of a change in specific activity should be a change in kinetics. It was found that the *Km* had changed very little but the *Vmax* or turnover number was much higher in the host-parasite enzyme than in the host enzyme alone. According to Dixon and Webb (88), this is indicative of a change in the speed of hydrolysis of the substrate rather than a change in the affinity of the enzyme for the substrate. Again this kinetic change points to a structural or conformational change.

In summary, physiochemical, purification and kinetic data all indicate that there has been a modification and salvaging of the host enzyme during the parasitic cycle. The survival value of this mechanism will now be postulated.
Pritchard et al., 1975, hypothesized that *B. bacteriovorus* has adapted to its ecological niche by utilizing the preformed monomers of the host for synthesis related to growth. Rittenberg and Langley, 1975, showed that *B. bacteriovorus* uses nucleoside phosphates per se from the host for nucleic acid synthesis. Kuenen and Rittenberg, 1975, showed that fatty acids of the host are conserved and used directly and that some fatty acids are even altered to suit the parasite.

The consequences of the above actions of the parasite is the conservation of energy. However, according to Hespell, 1975, these energy saving mechanisms do not account for the total energy efficiency of *B. bacteriovorus*. The synthesis of proteins requires a considerable amount of energy. It takes 25 millimoles of ATP to produce one gram of protein in the parasitic cell whereas it takes only 2 millimoles of ATP to produce one gram of DNA and 6.9 millimoles of ATP to produce one gram of lipid. Therefore any conservation of protein would result in tremendous energy savings -- a greater savings than the conservation of nucleoside-phosphates or fatty acids. Also, since the parasite can modify host fatty acids, perhaps it can modify host enzymes as well, with the result a more active enzyme. A conservation and modification of the host periplasmic enzymes would help to account for the energy efficiency of *B. bacteriovorus*.

The data suggest that the parasite can utilize the alkaline phosphatase of the host. Since by the fifth day
of the parasitic infection cycle there is widespread host
destruction, the fact that the enzyme is present at all
is surprising and suggests that the parasite might be con-
serving it. Further experiments are necessary to prove
that the parasite is in fact using the host periplasmic
enzyme and that the results are not fortuitous. Since it
was shown that \textit{B. bacteriovorus} strain 6-5-5 does not have
an alkaline phosphatase, electron microscopy using reaction
product deposition may be of value. Active association of
the alkaline phosphatase with the parasite would streng-
then the hypothesis that host periplasmic enzymes are
being used by the parasite.

Rittenberg and Langley (33) showed that when ortho-
phosphate was added to a two membered host-parasite cul-
ture, 25\% of the \textit{B. bacteriovorus} phosphorus was derived
from this exogenous source. This result was obtained even
when the exogenous inorganic phosphorus was present at
concentrations up to ten times the quantity of phosphorus
in the substrate \textit{E. coli}. Therefore \textit{B. bacteriovorus} can
use orthophosphate, but there seems to be a preferential
use of \textit{E. coli} phosphorus over exogenous phosphorus. They
also showed that the parasite used nucleoside phosphate
directly from the host in nucleic acid synthesis. However
\textsuperscript{32}P was added to the host so that, host phosphate would be
labelled. This would repress the alkaline phosphatase
of the host since it was not constitutive for alkaline
phosphatase. It would be interesting to repeat their
experiment with a fully constitutive mutant of *E. coli*, to see if the parasite would use host nucleoside-phosphate per se when host alkaline phosphatase was present. At the same time incorporation of radioactive phosphate without the nucleoside could be monitored by means of a double label.

Further work could also include a closer look at the host-parasite enzyme itself to determine the exact structural or conformational difference between it and the unmodified enzyme. It is possible that alkaline phosphatase just happened to be more resistant to the parasitic proteases than other host enzymes. It would be useful to survey the other periplasmic enzymes of the host to see if a similar phenomenon occurred or if there was a controlled degradation of these enzymes as there seems to be with the host cytoplasmic enzymes. Also since *B. bacteriovorus* physiology seems to be based on an amino acid energy source, the proteolytic enzymes of the host periplasm could be examined specifically to see if they are conserved or modified.

On a per cent dry weight basis, *B. bacteriovorus* has four times the DNA content of *E. coli*. Rittenberg and Hespell, 1975, state:

In comparison with other bacteria growing in a complex media, the *B. bacteriovorus* has a much greater ability to modulate the nutritional quality of its environment. Through a regulated degradation of the
substrate cell, the *B. bacteriovorus* apparently controls to a large extent the types, times of appearance, rate of formation and concentration of biosynthetic units.

Whether conservation of host alkaline phosphatase is another aspect of this control or is simply a fortuitous event that does not fit into the picture at all, it is undeniable that *B. bacteriovorus* represents one of Nature's unique puzzles.
BIBLIOGRAPHY


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<th>Abbreviation</th>
<th>Description</th>
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<td>P.A.G.E.</td>
<td>Polyacrylamide Disc Gel Electrophoresis</td>
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<tr>
<td>PLM</td>
<td>Phosphate Limiting Media</td>
</tr>
<tr>
<td>PNPP</td>
<td>Paranitrophenyl Phosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenedinitrilotetraacetic acid</td>
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<tr>
<td>DEAE-celulose</td>
<td>Diethylaminoethyl cellulose</td>
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<td>THAM</td>
<td>Tris Hydroxymethyl Aminomethane</td>
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CIRRICULUM VITA

Born: March 14, 1953, Windsor, Ontario

Educated

Primary: Our Lady of Mount Carmel School, Windsor, Ontario 1959-1966


University: University of Windsor, Windsor, Ontario 1971-1975

Degree: B. Sc. (Hon) 1975

Scholarships:

Ontario Scholarship 1971

1975-1978:

Teaching Assistant, Department of Biology, University of Windsor

1977:

Instructor: St. Clair College of Applied Arts and Technology, Windsor, Ontario

Major Field: Microbiology