BACTERIAL STRUCTURE AND RELATED
BIOCHEMICAL FUNCTION

DISSERTATION

Presented in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy in the
Graduate School of the Ohio State
University

By

Leonard Barrett Carr, Hons. B. Sc.

******

The Ohio State University
1958

Approved by

[Signature]
Adviser
Department of Agricultural
Biochemistry
ACKNOWLEDGMENTS

The author wishes to express his appreciation to his adviser, Dr. J. E. Varner; to Dr. Webster and other members of the Department of Agricultural Biochemistry; and to Dr. Prebus, Department of Physics; Dr. Frajola, Department of Physiological Chemistry, for their cumulative help and guidance during the author's period of graduate study.

Financial support for portions of this study was very generously provided by the Research Corporation.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF THE LITERATURE</td>
<td>4</td>
</tr>
<tr>
<td>Bacterial Anatomy</td>
<td>4</td>
</tr>
<tr>
<td>Techniques of Bacterial Rupture and Fractionation</td>
<td>7</td>
</tr>
<tr>
<td>Mineral Nutrition of <em>Azotobacter</em></td>
<td>11</td>
</tr>
<tr>
<td>EXPERIMENTAL METHODS</td>
<td>15</td>
</tr>
<tr>
<td>Introduction</td>
<td>15</td>
</tr>
<tr>
<td>Growth of Cells</td>
<td>15</td>
</tr>
<tr>
<td>Optical Microscope Studies</td>
<td>16</td>
</tr>
<tr>
<td>Electron Microscope Studies</td>
<td>16</td>
</tr>
<tr>
<td>Cellular Fractionation Studies</td>
<td>18</td>
</tr>
<tr>
<td>Biochemical and Radiological Experiments</td>
<td>19</td>
</tr>
<tr>
<td>Mineral Nutrition Studies</td>
<td>21</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>24</td>
</tr>
<tr>
<td>The Anatomy of <em>Azotobacter</em></td>
<td>24</td>
</tr>
<tr>
<td>Bacterial Fractionation Studies</td>
<td>28</td>
</tr>
<tr>
<td>Enzymological Studies on Sonically Disrupted Bacteria</td>
<td>33</td>
</tr>
<tr>
<td>Intracellular Localization of Calcium, Iron, Molybdenum and Tungsten</td>
<td>36</td>
</tr>
</tbody>
</table>
Gytological Studies, the Influence of Metal Ions on Living Cells. .......... 40

Mineral Nutrition Studies. ................................ 45

Calcium, Barium and Strontium .................. 45

Iron, Cobalt and Nickel .................. 47

Iron and Chromium .................. 50

Phosphate and Arsenate .................. 52

Summary - Mineral Nutrition Studies ... 55

SUMMARY ................................................ 57

CONCLUSION ............................................. 61

BIBLIOGRAPHY ........................................... 91

AUTOBIOGRAPHY ....................................... 95
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Enzymatic Properties of Differential Centrifugal Fractions Obtained from Sonicated <em>A. agilis var. vinelandii</em> O</td>
<td>88</td>
</tr>
<tr>
<td>2. Distribution of RNA, DNA and Protein in Centrifugal Fractions of <em>A. agilis var. vinelandii</em> O</td>
<td>89</td>
</tr>
<tr>
<td>3. Capacity of Ba and Sr to Substitute for Ca in <em>Azotobacter spp</em></td>
<td>90</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

**FIGURE**

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Optical Micrographs of <em>Azotobacter agilis</em> var. <em>vinelandii</em> strain O</td>
<td>62</td>
</tr>
<tr>
<td>2. Electron Micrograph of Ultra Thin Sections of Log Phase <em>A. agilis</em> var. <em>vinelandii</em> O</td>
<td>63</td>
</tr>
<tr>
<td>3. Electron Micrograph of Ultra Thin Section of Stationary Phase <em>A. agilis</em> var. <em>vinelandii</em> O</td>
<td>64</td>
</tr>
<tr>
<td>4. Electron Micrograph of Ultra Thin Section of Stationary Phase <em>A. agilis</em> var. <em>vinelandii</em> O View of Two Adjoining Cells</td>
<td>65</td>
</tr>
<tr>
<td>5. Electron Micrograph of Low Speed Centrifugal Fraction Obtained from Sonically Disrupted <em>A. agilis</em> var. <em>vinelandii</em> O</td>
<td>66</td>
</tr>
<tr>
<td>7. Electron Micrograph of the 25p30 Cell Envelope Fraction of <em>A. agilis</em> var. <em>vinelandii</em> O</td>
<td>68</td>
</tr>
<tr>
<td>8. Electron Micrograph of Sonically Disrupted Bacteria 144p60 Fraction</td>
<td>69</td>
</tr>
<tr>
<td>9. Electron Micrograph of the Cell Membrane 144p60 Fraction</td>
<td>70</td>
</tr>
<tr>
<td>10. Electron Micrograph of the Bacterial Cell Envelope 144p60 Fraction</td>
<td>71</td>
</tr>
<tr>
<td>11. Optical Micrographs of Protoplasts and Membranes of <em>A. agilis</em> var. <em>vinelandii</em> O</td>
<td>72</td>
</tr>
<tr>
<td>FIGURE</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>12.</td>
<td>Graph of Release of Ca$^{45}$ from <em>A. agilis</em> var. <em>vinelandii</em> 0 as a Function of Ca, Ba, Sr and Versene</td>
</tr>
<tr>
<td>13.</td>
<td>Optical Micrographs of Ca, Sr and Ba Cells of <em>A. agilis</em> var. <em>vinelandii</em> 0</td>
</tr>
<tr>
<td>14.</td>
<td>Shadowed Electron Micrograph of a Disrupted Strontium Cell of <em>A. agilis</em> var. <em>vinelandii</em> 0 Showing Particulates.</td>
</tr>
<tr>
<td>15.</td>
<td>Optical Micrographs of Ca, Sr, and Ba Cells of <em>A. agilis</em> var. <em>vinelandii</em> 3A</td>
</tr>
<tr>
<td>16.</td>
<td>Optical Micrographs of <em>A. agilis</em> ATCC 7496</td>
</tr>
<tr>
<td>17.</td>
<td>Optical Micrographs of <em>A. agilis</em> 144</td>
</tr>
<tr>
<td>18.</td>
<td>Optical Micrograph of <em>A. beijerinckii</em> B5</td>
</tr>
<tr>
<td>19.</td>
<td>Optical Micrograph of <em>A. macrocytogenes</em> strain 0</td>
</tr>
<tr>
<td>20.</td>
<td>Optical Micrographs of Ca and Sr Cells of <em>A. chroococcum</em> 0 44</td>
</tr>
<tr>
<td>21.</td>
<td>Growth Curves of <em>A. agilis</em> var. <em>vinelandii</em> 0 in Nutrient Media Containing 30 ppm Ca, Sr or Ba</td>
</tr>
<tr>
<td>22.</td>
<td>Growth Response of <em>A. agilis</em> var. <em>vinelandii</em> 0 to Varying Levels of Ca, Sr or Ba</td>
</tr>
<tr>
<td>23.</td>
<td>Growth Response of Iron Deficient <em>A. agilis</em> var. <em>vinelandii</em> 0 to Varying Levels of Iron</td>
</tr>
<tr>
<td>24.</td>
<td>Growth Response of <em>A. agilis</em> var. <em>vinelandii</em> 0 to Varying Levels of Nickel</td>
</tr>
<tr>
<td>25.</td>
<td>Growth Response of <em>A. agilis</em> var. <em>vinelandii</em> 0 to Varying Levels of Cobalt</td>
</tr>
<tr>
<td>26.</td>
<td>Iron and Chromium Interaction in <em>A. agilis</em> var. <em>vinelandii</em> 0</td>
</tr>
<tr>
<td>27.</td>
<td>Growth of <em>Azotobacter</em> spp as a Function of Chromium Level</td>
</tr>
<tr>
<td>28.</td>
<td>Growth Response of <em>A. agilis</em> var. <em>vinelandii</em> 0 to Varying Levels of Phosphate and Arsenate</td>
</tr>
</tbody>
</table>
most popular techniques for bacterial disruption. Such a technique is fully acceptable for the preparation and subsequent purification of specific enzymatically active entities. It does not, however, permit the correlation of biological function with biological structure; and it is in this direction that at least a portion of research effort must proceed if a full understanding of the chemical accompaniments of life are to be uncovered. Subsequent fractionation procedures have resulted in the reported isolation of many fractions capable of a variety of biochemical activities. Some such techniques have been further investigated from the standpoint of resultant cell fraction content and on the basis of this study, potentially profitable fractionation techniques were uncovered.

*A. agilis var. vinelandii O* has near minimal nutrient requirement. Burk's medium, Burk and Lineweaver (1930), made up of 3 per cent sucrose as carbon source, and varying amounts of phosphate, sulphate, chloride, calcium, magnesium, sodium, potassium, iron and molybdenum, supports aerobic growth of this organism in a satisfactory manner. The techniques of variations of and omissions from this basal medium are resorted to in a further attempt to gain additional information on the cytology of this organism; for example, by treating the cells in such a way as to
greatly increase cell size. In addition it was considered that mineral requirement studies may provide further information on some of the cellular functions and inter-relations of the minerals under study.
REVIEW OF THE LITERATURE

Bacterial Anatomy

Prior to the 1940's the anatomy of bacteria had been studied with a precision no greater than the 0.2u limit imposed by the optical system of the light microscope. With the aid of special staining and other techniques this instrument has been, and continues to be, of extreme value in the study of microorganisms. A fundamental advantage of this instrument over the electron microscope, which of course is capable of vastly superior resolution, is that the former permits continuous observation of living cells, and allows continuing study of the cytological events accompanying life processes - for example, the study of cell division and concomitant nuclear division in Mucorales by Robinow (1957a,b). In addition it is possible with this instrument to compare the arrangement of cellular components in living cells with those observed in cells stained specifically for the study of a specific cytological component. By this technique Robinow (1957a) has been able to identify areas, visible within the living cell of Mucor hiemalis, with areas stained specifically for nuclear material in treated cells. Such identification leaves little doubt that the sequence of phase contrast photomicrographs
of living cells included in his publication is indeed illustrative of the sequence of events accompanying nuclear division in this organism.

Many workers in the field of bacterial anatomy continue to use the light microscope as a primary tool in their investigations. Mudd et al. (1958) have used the instrument to good effect in continuing studies on metaphosphate accumulation in *Corynebacterium diphtheriae*. Knaysi (1957) using the light microscope is actively investigating the identity of granules appearing in *Mycobacterium thamnopheous*. Delameter (1956) has long been interested in the mechanism of nuclear division; Bisset (1955) has a divergency of interests.

The development of techniques for the electron microscopy of whole and ultra thin sections of bacteria has permitted the examination of these organisms with object resolution much greater than that obtainable with the light microscope. Observation of whole cells has been of considerable value in the study of bacterial flagellation. Using the electron microscope Hofer (1944) was able to show that some members of the genus *Azotobacter* are peritrichous. Tawara (1957) was able to demonstrate the mode of attachment of the flagella of *Vibrio comma* to the parent cell and Ribi and Shepard (1955) have examined the morphology of *Bacterium tularense* during the growth cycle.
A significant development in the study of bacterial anatomy was the introduction of techniques permitting the examination of ultra thin sections of bacteria in the electron microscope. Birch-Anderson et al. (1953) have published electron micrographs of ultra thin sections of *Escherichia coli*. These micrographs demonstrate the type of structures which have been visualized in bacteria up to the present time. Chapman and Hillier (1955), Chapman (1956), and Chapman and Zworykin (1957) have studied the processes of cell division, spore formation and spore germination, and incidental to these studies, published electron micrographs of *Bacillus megaterium* and *Bacillus cereus*. Other bacteria have been examined in ultra thin section. Chapman and Kroll (1957) have studied *Spirillum serpens* in some detail. Murray (1957) has published an electron micrograph of *Spirillum sp.*, showing a definite cytoplasmic membrane. Ito et al. (1957) have studied *Bacillus aneurinolyticus*, and Shinohara et al. (1957) *Mycobacterium avicenum*. The two latter papers are of interest in that they include identification of structures which have not yet been observed in electron micrographs of ultra thin sections of other bacteria. The first paper above includes an identification of a bacterial nucleus and a dark intranuclear granule tentatively identified by the authors as a nucleolus; the second paper
INTRODUCTION

Of the increasingly numerous bacteria found to be capable of nitrogen fixation, perhaps the organism *Azotobacter agilis var. vinelandii strain Original* has received the greatest attention in the hands of investigators.

In view of this concentration of effort it was thought that if biochemical study of this organism were to be fully effective, particularly in terms of the relation between structure and biochemical function, then an attempt should be made to study the cytology of this organism with the aid of the most recently developed techniques and instrumentation available. In addition to other more specific advantages such a study would contribute to the limited pool of knowledge thus far uncovered on bacterial anatomy as revealed by the electron microscope. Of the various classes of living organisms - plant, animal, and microbial - it is the latter group that has received the least study.

Armed with the knowledge of the cytological nature of *A. agilis var. vinelandii O* one may logically proceed to a study of the biochemical and cytological nature of a variety of fractions obtainable on disruption of this organism. Until recent years mechanical grinding has been amongst the
identifies nuclei and mitochondria. In the opinion of this writer, the data presented in support of these concepts does not justify the conclusions which have been drawn. In both cases the electron micrographs presented show an extremely poor preservation of the specimen and the occurrence of numerous artifacts. It is hoped that further work will demonstrate more convincingly the occurrence of the anatomical features alluded to within these organisms. Of considerable interest are the tube-like structures visible in *M. avicen*.

On the basis of optical and electron microscope studies the following cytological features are now widely accepted as being present within the bacterial cell: cell wall, cytoplasmic membrane, metaphosphate granules, vacuoles, cytoplasm and nucleus. In an attempt to correlate bacterial structure with function, these components deserve special attention.

**Techniques of Bacterial Rupture and Fractionation**

In recent years much effort has been expended toward the goal of preparation of enzymatically active "particulate" fractions. In this area preparations from *A. agilis var. vinelandii* include an electron transporting particle, Breumer et al. (1957); and a particulate fraction capable of phosphorylation, Rose and Ochoa (1956). Jose and Pengra (1957) have described a small but significant cell
free nitrogen fixation in a fraction of A. agilis var. vinelandii O. In other organisms, Asnis et al. (1956) have described a cytochrome containing "particulate" fraction, isolated from Escherichia coli, which is capable of a limited number of oxidative activities. Klein and Booher (1956) have reported on a particulate fraction obtainable from Saccharomyces cerevisiae which is capable of lipogenesis. Wilman and Darter (1956) have reported on the localization of enzymatic activity as a function of particle size in the organism Mycobacterium tuberculosis.

In general, the approach utilized for the preparation of "active" fractions has been the technique of disruption of the parent cell, followed by differential centrifugation. Centrifugal separation of structures within the intact cell has been attempted by Holter and Kopac (1937), and while there are many inherent advantages of cellular fractionation in this way, one is faced with the problem of separation of the stratified cellular content. The technique has received scant application in the microbiological field.

A number of techniques exist for the disruption of bacterial cells. Classically, microbiological rupture has been achieved by grinding the cells with an abrasive material, or by the application of hydrostatically produced high pressures. Buchner (1897) developed the hydraulic
press which carries his name, the application of which was responsible for the first demonstration of cell free alcohol fermentation. Other techniques in use at the turn of the century include that of Rinckelben (1911), plasmolysis of the cells by 8 per cent glycerol; Giglioli (1911), disruption with chloroform; and Dixon and Atkins (1913), freezing in liquid air. Of these techniques, bacterial rupture by grinding with abrasive material continues to be widely used, and a recent report in the literature, Robrish and Marr (1957), has revived interest in the glycerol method of cellular rupture, Carr (1958a,b).

In recent years the development of the Ratheon 10 Kc sonic oscillator has permitted thorough disruption of a great number of bacteria, and the technique has developed a wide following among scientific investigators. Marr and Cota-Robles (1957), and Cota-Robles et al. (1958), have studied the effect of 10 Kc sound waves on the disruption of *A. agilis*.

A significant development in techniques available for bacterial rupture stems from the work of Weibull (1953) on the effect of lysozyme on the cell wall of *Bacillus megaterium*. Weibull observed that spherical protoplasmic bodies were formed when the cell wall of *B. megaterium* was dissolved away, and that the protoplasts could be stabilized in sucrose or polyethylene glycol. Protoplast lysates were observed to consist of empty vesicles or
"ghosts", identified by Weibull (1953b) as probably representing cytoplasmic membranes, together with small granules. Repaske (1957) indicated that the gram negative organisms, as well as the gram positive organisms, successfully lysed hitherto, were also susceptible to the action of lysozyme when Versene was included in the incubation medium.

A further technique which is of interest is that used by Robrish and Marr (1957) and by Rinckelben (1911) as early as 1911, the glycerol-induced disruption of cells.

Each of the techniques above results in the preparation of characteristic subcellular fractions. Some techniques, for instance abrasive grinding or sonication, would be expected to result in heterogeneous cellular preparations, thereby supplying little information with respect to the known knowledge of the biochemical function of specific bacterial cell entities. Marr and Cota-Robles (1957), and Cota-Robles et al. (1958) however, on the basis of sonication studies, were able to suggest that the hydrogenase, cytochrome, and phospholipid of the cells of *A. agilis var. vinelandii* all reside in the cell envelope of this organism. Other techniques may hold considerably more promise. In this category one might include the glycerol disruption of cells, protoplast formation, and careful protoplast disruption, for instance, in the manner utilized by Spiegelman et al. (1958) for the isolation of the nucleus of *B. megaterium*. 
Following bacterial disruption, bacterial fractionation has been achieved largely by differential centrifugation. In recent years the centrifugal forces used have been upgraded to a maximum of 110,000 X gravity. Physico-chemical methods, in combination with centrifugal fractionation, have also been used to a limited extent, for example by Alexander and Wilson (1955) and Keeler and Varner (1958b).

In view of the obvious usefulness of techniques of bacterial rupture and subsequent fractionation, it is desirable to determine critically the cytological constituents in preparations obtained by the application of techniques currently in use.

**Mineral Nutrition of Azotobacter**

Phosphorus, magnesium, potassium, sulphur calcium, iron and molybdenum appear to be specific mineral requirements of the genus *Azotobacter*. Quantitative amounts of these nutrients for optimum growth have not been fully determined for the genus; however, the nitrogen-free mineral salt medium of Burk and Lineweaver (1930), with added sucrose, has been used to culture satisfactorily a large number of strains of *Azotobacter* by Schutter and Wilson (1955). This suggests that this medium contains an assembly of required components in approximate optimal quantity and balance.

Of the required minerals, phosphorus is needed in greatest amount. The level incorporated in Burk's medium
approximates 560 ppm as phosphate. In order to further buffer the nutrient medium against pH change, Keeler and Warner (1957) have increased the phosphate level to 2,200 ppm without apparent adverse effect. Experiments to be referred to later have indicated that approximately 100 ppm phosphate will support maximal growth of *A. agilis var. vinelandii* 0, provided that an alternate buffering system is incorporated in the nutrient medium.

Magnesium is reported to be required in equal amounts by *Azotobacter* irrespective of nitrogen source, Horner and Burk (1934). Jensen (1954) has reported that a magnesium level of 8 - 4 ppm is necessary for optimum growth of *A. drouoczccum* in a nutrient medium in which the phosphate component was added as inorganic phosphate. Addition of phosphate as glycerophosphate or as hexose diphosphate was observed to lessen the magnesium requirement of this organism to the range 4 - 8 ppm. These observations are in accord with the theory that magnesium is a cofactor for cellular enzymes involved in phosphorylation reactions.

The calcium requirement of *Azotobacter* on free nitrogen was shown to be in the range 20 - 40 ppm, by Burk and Lineweaver (1931), and in addition these authors reported that the calcium requirement of *Azotobacter* could be met by strontium. This latter observation has also been made by Esposito and Wilson (1956). The essentiality
of calcium for the process of nitrogen fixation remains uncertain. Burk and Horner (1940) reversed their former opinion, Horner and Burk (1934), to state that calcium is required in equal concentration for growth on free nitrogen, nitrate, ammonia, and asparagine. Esposito and Wilson (1956) have demonstrated a specific calcium requirement for nitrogen fixation by *A. agilis var. vinelandii* O.

Lenhoff et al. (1956) noted that *Pseudomonas fluorescens* incorporates more molybdenum under conditions of high oxygen and on a low iron containing medium than when cultured under low oxygen tension. On the basis of these experiments, Lenhoff et al. have suggested that this organism respires partially by means of an alternate molybdenum containing flavo protein terminal oxidase. Keeler (1957) has observed the same phenomenon in *A. agilis var. vinelandii* O. and has determined that a major portion of molybdate taken up into the bacterial cell is probably incorporated into non-functional molybdoprotein. In view of the low optimum requirement of *A. agilis var. vinelandii* O for molybdenum (0.01 ppm) and iron (3 ppm), it may prove difficult to ascribe important physiological functions to excessive uptake of these ions.

The elucidation of physiological functions of minerals in *Azotobacter* by the technique of seeking mineral substituents or antagonists has received scant
application. The work of Burk and Lineweaver (1931), indicating that strontium could replace calcium in *Azotobacter*, has been referred to above. For some species of the genus, vanadium has been shown to replace the molybdate requirement, Morner et al. (1942), Bové et al. (1957). Tungstate has been reported as an antagonist of molybdate in *A. agilis var. vinelandii O*, Takahashi and Nason (1957), Keeler and Varner (1957). Germanium at a level of 100 ppm has been observed to inhibit cellular growth of *A. agilis var. vinelandii O* by 30 - 50 per cent, Keeler and Varner (1958a).

In relating bacteriological structure in function it would be of interest to investigate further the role of calcium, iron and phosphorus in *Azotobacter*.

To this end calcium, barium and strontium, iron cobalt and nickel, phosphorus and arsenic, and iron and chromium nutrition studies have been initiated and observations made on cytological and growth response of *Azotobacter* to these ions.
EXPERIMENTAL METHODS

Introduction

As indicated in the literature survey, research undertaken in this study lies in three general areas. A study of the anatomy of *Azotobacter* involved electron and optical microscope observation of this organism. Techniques of fractionation of *Azotobacter* were studied and correlated with optical and electron microscope observations. A limited number of biochemical properties were identified with bacterial fractions obtained from sonically disrupted *Azotobacter*. Thirdly, studies on the mineral requirements of *Azotobacter* involved cytological observation with the optical microscope and measurement of growth response.

Growth of Cells

The various strains of *Azotobacter* studied, generously supplied by Drs. P. W. Wilson and R. H. Burris, were all cultured on sterile Burk's, Burke and Lineweaver (1930), nitrogen-free mineral salt medium, with 3 per cent sucrose, 1 ppm Mo added. Four times the suggested Burk's medium level phosphate was added because it was found that without this additional buffering capacity, cultures exhibited, with growth, a tendency toward acid pH. In experiments where
phosphate levels were varied, buffering of these cultures was accomplished by 0.25 M Tris pH 7.4. Growth of cells was followed using an Evelyn Colorimeter measuring culture turbidity using light of 660 nm wavelength.

**Optical Microscope Studies**

Cytological examination of bacterial cells was carried out exclusively on living cells. Both bacteria and bacterial fragments were examined with phase contrast optics (dark field objective) at a magnification of 970x. Bacterial preparations for microscopic examination were made by means of the hanging drop technique.

Photomicrographs were obtained at an initial magnification of 500x and enlarged photographically. The photographic film used was Panatomic X.

**Electron Microscope Studies**

Electron micrographs obtained were of two types, shadowed preparations of whole bacteria and bacterial fractions and sectioned bacteria. Both types of preparations involved essentially the application of current standard techniques.

Shadowed preparations were obtained in the following manner: the specimen was air dried on Formvar covered 200 mesh copper grids, and washed if necessary with distilled water. Fixation was achieved by suspending the specimen in a sealed chamber over 1 per cent osmic acid for ten minutes. Following shadowcasting with metallic platinum at an angle of
The preparation of bacterial ultra thin sections is a relatively more complex procedure. Bacterial cells were collected from growing cultures by the application of low-speed centrifugal forces. The cells were fixed for 1 hour in 1 per cent osmic acid buffered at pH 7.4 with 0.028 M sodium acetate, 0.028 M sodium veronal, Palade (1952). Following fixation the fixed cells were collected again by low-speed centrifugation.

In studies on capsulated stationary phase cells of *A. agilis var. vinelandii* it was thought desirable to eliminate the centrifugation step in order to minimize possible capsular displacement. In this instance a volume of culture medium was mixed directly with fixative. On standing for 15 minutes the cells settled in the bottom of the fixation vessel. The supernatant was decanted and replaced with additional fixative. Further fixation was allowed to proceed for 1 hour.

Dehydration was achieved by passage through a graded ethanol series (10 minutes intervals); the cells were then transferred through two changes of 50 per cent alcohol-50 per cent methacrylate mixture, and embedded in 90 per cent n butyl methacrylate-10 per cent methyl methacrylate mixture, to which 1 per cent benzoyl peroxide (Luperco) had been added. Polymerisation was carried out at 60 C. Sections were mounted
on Formvar or carbon covered grids, and examined in an RCA EMU 2b electron microscope. Electron micrographs were obtained at initial magnifications of up to 20,000x and were further enlarged photographically. Photographic plates used in this study were Ilford Special Lantern Plates of normal of contrasty grade.

Cellular Fractionation Studies

These studies were designed to determine the nature of cellular fractions obtained on sonication, enzymatically catalysed cell wall removal, and osmotic rupture of whole cells.

Sonic disruption of log phase *A. agilis var. vinelandii* Q was achieved by exposing a 30 per cent cell suspension in 0.25 M sucrose 0.1 M phosphate pH 7.4, or 0.85 per cent sodium chloride 0.01 M Tris pH 7.4, to maximum intensity 10 kc sound waves in a Ratheon sonic oscillator.

Cell wall digestion of log phase *A. agilis var. vinelandii* was induced by application of the method of Repaske, (1956). Cells were suspended in Tris buffer, 0.3 M sodium chloride and Versene, and treated with lysozyme. Following an incubation period of 10 minutes the resultant approximately spherical bodies were regarded as bacterial protoplasts. These were characterized by an extreme instability in media of low tonicity. A cytoplasmic membrane fraction was obtained by protoplast lysis induced by the addition of excess distilled water to a medium containing stabilized protoplasts or
alternately by treating whole cells with the reaction mixture above but in the absence of the protoplast stabilizing component 0.3 M sodium chloride.

A cell envelope fraction was obtained by the osmotically induced rupture of *A. agilis var. vinelandii* 0. Log phase cells were collected by centrifugation, transferred to 1.0 M glycerol, and permitted to equilibrate against this medium for a period of 2 hours. Rupture was induced by the dilution of the cell-glycerol mixture with 5x volume of distilled water. The point of completion of rupture of the cells may be ascertained by optical microscope examination.

In the case of each bacterial fractionation technique the resultant preparations were centrifuged in a Spinco refrigerated preparative ultra centrifuge at 25,000 x G for 30 minutes (25p30), and the supernatant fractions thus obtained centrifuged at 144,000 x G for 1 hour (144p60). Specimens were prepared for electron microscopy as indicated previously.

**Biochemical and Radiological Experiments**

Considerable data have been amassed on the biochemical properties of cellular fractions obtained from bacteria, including *A. agilis var. vinelandii* 0, Alexander and Wilson (1955), Keeler, Keeler *et al.* (1956), (1957), (1958), and Robrish and Marr (1957), Cota-Robles *et al.* (1958). This backlog of information permits an estimation of the bio-
chemical capabilities of specific portions of the bacterial cell.

Limited enzymological examination of differential centrifugal fractions obtained from sonically disrupted *A. agilis var. vinelandii* 0 was carried out, and results obtained were seen to correspond with the published information. Succinic dehydrogenase was measured by the method of Slater and Bonner (1952), aconitase and fumarase by the method of Racker (1950a, b), and glutamine transferase by the method of Varner and Webster (1955). An estimation of DNA and RNA content of differentially centrifuged sonically disrupted bacteria was made using the method of Schmidt and Thannhauser (1945).

An interesting avenue of approach to determine the function of specific bacterial components is to examine these for mineral content with the use of radioactive isotopes. The localization of calcium is of interest in view of its essentiality for nitrogen fixation and postulated function in cell wall metabolism; molybdenum, and its antagonist tungsten, for its essential role in nitrogen fixation; and iron as a component of cellular cytochromes. These experiments were conducted in cooperation with Dr. R. F. Keeler and Dr. J. E. Varner, Keeler, Carr and Varner (1958).

Calcium was added as $^{45}$Ca $\text{Cl}_2$ to calcium deficient
cells at a level of 7 ppm. This level is known to be at the lower end of the calcium requirement of _A. agilis var. vinelandii_ 0, and it was reasoned that at this level Ca would be more likely incorporated into functional positions within the cell, rather than incorporated for future needs.

Mo$^{99}$ and W$^{185}$ were added as Na$_2$Mo$^{97}$O$_4$ and K$_2$W$^{185}$O$_4$, Fe$^{59}$ as Fe$^{59}$Cl$_3$, at levels of 4, 40 and 0.1 ppm respectively. Bacterial fractions were prepared as indicated above. Radioactive assays were performed with a Geiger - Muller end window tube, or gas flow Nuclear D47 tube and Nuclear model DL81 scaler.

**Mineral Nutrition Studies**

Mineral nutrition studies were confined largely to observations on the calcium, iron, and phosphorus requirement of **Azotobacter**. In general the technique utilized was to develop a mineral deficient culture of **Azotobacter**, and study the growth and cytological response of the deficient cells on the addition of the deficient mineral or possible analogues and substitutes.

In the development of each class of mineral deficient cells studied, the technique followed was identical. Logarithmic phase cells were repeatedly transferred on nutrient media lacking the appropriate component. In the development of iron deficient cells, transfers were made until the resultant cellular growth reached a constant
minimal level. Where absolute deficiencies were obtained, as with calcium and phosphate, fully deficient cells were added to an equal volume of appropriately deficient nutrient medium, and further incubation permitted. This procedure was resorted to since it was found that the transference of a small quantity of deficient cells to deficient medium failed to initiate cellular propagation, resulting in the loss of a suitable culture for inoculation purposes.

Mineral nutrition experiments were conducted in the following manner. Volumes of Burk's nitrogen-free mineral salt medium, minus the appropriate nutrient, were made up in large glass containers. Deficient media were sterilized in all cases by autoclaving for 10 minutes at 15 pounds per square inch steam pressure. Media were allowed to cool. Appropriate levels of test minerals were pipetted into 125ml Erlenmeyer flasks each fitted with a rubber stopper incorporating a four-inch length of approximately 8mm internal diameter Pyrex glass tube. Cotton plugs were placed in the exterior orifice of the glass tubes. Erlenmeyer flasks were sterilized using the procedure outlined above for the sterilization of deficient media. A 5 per cent inoculum of appropriately deficient cells was then added, with shaking, to a volume of deficient nutrient medium. Twenty-five milliliter portions of the resultant mixture were then added to each flask of the
prepared experimental sequence. Flasks were shaken at approximately 31°C on a Burrell wrist action, or other type, shaker. Measurement of cellular response to added metal ions was assessed at a point of approximately 2/3 maximum potential culture growth. At this level, cells are still in the logarithmic phase of growth, and comparison of optical density measurements of culture turbidity is a valid comparison of growth response, provided that the lag phase of growth is known to be of equal duration in each of the experimental flasks. In studies on calcium, and the substitutive capacities of barium and strontium, entire growth curves were determined. These curves show the equal duration of lag phase of growth in each of the cultures examined. In other experiments, frequent occular examination of growing cultures was used to determine that no significant variation in lag phase occurred in the experimental cultures under study. As an additional check, when it was noted that observed growth, in one, or a series of flasks, was slight or negligible, additional incubation was permitted and further observations made.
RESULTS AND DISCUSSION

The Anatomy of Azotobacter

A major reason for interest in the anatomy of *Azotobacter agilis var. vinelandii strain 0* is the fact that this organism has been used to a very considerable extent in nitrogen fixation studies. A classical approach to the study of complex biochemical reactions has been to attempt isolation of a cell free preparation capable of carrying out the over-all reaction in question. Knowledge of the anatomy of *A. agilis var. vinelandii 0* would permit a more logical approach to the preparation of potentially active fractions.

Optical microscope studies of *A. agilis var. vinelandii 0* reveal little internal detail within the bacterial cell. Figure 1 shows optical micrographs of log phase and stationary phase cells. Examination of log phase cells in the optical microscope indicates the intracellular presence of 1-4 granules per cell. It is thought that these granules, not visible in cells of all cultures, are metaphosphate granules. Mudd (1958) regards similarly identified structures appearing in *Gorynebacterium diphtheriae* as representing the centers of oxidative activity of the cell.
If this is true these structures would be of particular interest for attempted isolation for cell free nitrogen fixation studies. Stationary phase cells appear to contain numerous inclusion bodies. A high viscosity of the growth medium suggests the presence of copious quantities of extracellular material.

Despite the many problems of specimen preparation inherent in techniques leading to electron microscopic examination of ultra thin sections of bacteria, this procedure has been especially useful in gaining a well resolved insight into the nature of the bacterial cell. Figure 2 is an electron micrograph of an ultra thin section of *A. agilis var. vinelandii* Q. While not clearly defined, two membranes are visible. Interior to the cell wall-cytoplasmic membrane complex is an electron scattering component, the cytoplasm, which in part is punctuated by a less electron dense component, tentatively identified as the nucleus. Such a picture provides little encouragement as to the possibility of isolation of specific cell components, beyond the separation of cell wall, cytoplasmic membrane and cellular content. Whitfield and Murray (1956) have reported that the chromatin of the bacterial cell may aggregate in media of high electrolyte concentration, an observation extended by Spiegelman et al. (1956) to the isolation of bacterial nuclei from *Bacillus megaterium*. On the
basis of electron microscope studies on *A. agilis var. vinelandii* Q it appears very likely that a similar isolation could be effected with this organism. Additional cell structures may come to light on further electron microscopic investigation of bacteria; certainly current knowledge of bacterial anatomy fails to provide sufficient complexity at the cytological level to account for the complexity evident at the biochemical level.

Figures 3 and 4 are electron micrographs of ultra thin sections of stationary phase cells of *A. agilis var. vinelandii* Q. These sections show considerable structural detail. Prominently visible in electron micrographs of these cells are numerous inclusion bodies. These are thought to correspond with similarly appearing structures, which are in part visible with the optical microscope (Figure 1). Surrounding the cellular inclusion bodies is an electron scattering component of variable electron scattering capacity, tentatively identified as cytoplasm. The nature of the cell wall-cytoplasmic membrane, in the stationary phase cells illustrated, is not readily evident due to the abundance of adherent extracellular material. The extracellular material, which is considered to be the capsule, appears as a high density component consisting of fibrous strands wound around the periphery of the cell. The strips of electron transmitting material visible within
the capsule, may be interpreted as an artifact of preparation. Alternately, in view of the fact that the "gaps" within the capsular material are found in close proximity with the cell wall surface, it may be suggested that capsule component synthesis proceeds outward from the cell wall and that interwinding of individual layers is completed only as they are pushed towards the outer limits of the capsule. Examination of the capsule periphery reveals the fibrous nature of the capsular material and the absence of a definite limiting membrane.

The chemical nature of the capsule of this organism may be allied to that observed of *Azotobacter indicum*. This was shown by Quinnell *et al.* (1957) to consist of glucose, glucuronic acid and a heptose.

Despite the degree of organization evident in the stationary phase cells such specialization does not permit the identification of a nucleus.

These studies on stationary phase *A. agilis var. vinelandii* have served to cast some light on the nature of the capsule of *Azotobacter*, and have demonstrated the presence of numerous inclusion bodies within cells of this growth phase. The nature of the inclusion bodies is unknown. Many questions remain unsolved, perhaps one of the most challenging of which is the mechanism by means of which such copious quantities of capsular material, as is evident
in the electron micrographs presented here, are synthesized and excreted from the parent cells.

**Bacterial Fractionation Studies**

Three methods of bacterial rupture were examined critically: sonication, protoplast formation and subsequent lysis, and osmotic rupture. The nature of the various fractions studied was determined by electron microscopy. Methods used and fractions examined were as indicated in the methods section above; nomenclature used to denote specific differential centrifugal fractions is that of Alexander and Wilson (1955).

Each of the low speed fractions was found to be heterogeneous in nature, particularly the 25p30 fraction obtained from sonicated bacteria. This fraction (Figure 5) consisted of cell wall fragments and non-descript proteinaceous material. Particulates were occasionally noted. Large electron dense structures 200-400 μm in diameter reported in the 25p30 fraction of Alexander and Wilson (1955) were observed in some preparations of this fraction. Bacterial nuclei or cytoplasmic membranes were not identifiable.

The 25p30 cytoplasmic membrane fraction (Figure 6) obtained by osmotic lysis of protoplasts of *A. agilis var. vinelandii* consisted of two components. Easily recognizable are flattened disks scattered throughout the field,
and identified as membranes. These exist in varying stages of completeness and degradation. Scattered throughout the field and occasionally in close association with the membranes are numerous large spherical granules. Figure 6 shows a partially degraded membrane with several adherent spherical particles. The variation in particle size observable is considerable.

The 25p30 cell envelope fraction (Figure 7) is again contaminated by numerous spherical particles. Figure 7 shows a flattened bacterial cell, most of internal contents of which have been lost. Despite this, most bacterial cell envelopes appear relatively complete and it is usually possible to distinguish the outer edge of the cytoplasmic content within the flattened bacterial cell wall.

The 144p60 fraction obtained by further centrifugation of the 25p30 supernatant fraction from sonically disrupted microorganisms is illustrated in Figure 8. This fraction is made up of regularly appearing particulates having a diameter of about 20 μ. Some aggregates are evident.

The high speed cytoplasmic membrane supernatant 144p60 fraction (Figure 9) is again particulate. In the preparation illustrated aggregation has proceeded to a considerable degree, and individual particles are less readily resolvable.

The cell envelope supernatant 144p60 fraction (Figure 10) appears quite different. In this case, whilst
a few ill defined particulates are visible, the main body of the specimen consists of long fibrous strands of material. In assessing these electron micrographs it is well to remember that specimen preparation for electron microscopy may result in considerable change in physical appearance. The initial stage of specimen preparation, that of centrifugation, may also induce artifact. In the latter instance partial proteolysis of the specimen may occur during the centrifugation process, resuspension of the resultant pellet may be only partially complete, leading to a final image of component aggregates on electron microscopic examination. Subsequent handling, particularly specimen drying with attendant severe surface tension change, may also result in artifact.

The nature of the three 25p30 fractions examined has been described. Of the three, the sonicated preparation appears the most heterogeneous, and while this fraction may be quite reproducible from an enzymatic point of view, it may be of limited value in determining the nature of biological function of specific entities of the bacterial cell.

The bacterial membrane fraction is extensively contaminated with large spherical particles, and care should be taken to remove this contaminant before ascribing biochemical activity to the membranes. A further disadvantage
of this fraction for biochemical study is the fact that the method of preparation involves the use of Versene. As a highly effective chelating agent this substance may, at least partially, remove from the membranes functional metallic ions, thereby serving to mask or decrease inherent enzymatic activity in in vitro experiments involving this fraction.

Most promising from a cytological standpoint is the cell envelope fraction. Advantages here are a comparative ease of preparation, the certainty that all bacterial cells present in the sample are exposed to the same disruptive influences, and the isolation of a cellular fraction with considerable cytological organization. As with other fractions under discussion large spherical particles are a contaminant.

The evidence that the large particulates visible in the electron micrographs presented are discernible components of living cells is minimal. Optical microscope observation of living cells indicates the occasional presence of intracellular granules which are within the appropriate size range. The occurrence of this component, tentatively identified as metaphosphate granules, varies from culture to culture. The numerous occurrence of particulates in the appropriate bacterial fractions is a constant feature, supporting the viewpoint that these
particulates are representative of the cellular cytoplasmic areas which appear homogeneous on optical microscopic examination of living cells. One possibility as to the true nature of the contaminant large spherical particles stems from the work of Weibull (1953b) on *Bacillus megatherium*. Weibull noted the presence of large particulates in low speed centrifugal fractions obtained from disrupted *B. megatherium*. He was able to identify these particulates as being lipoidal in nature. In view of the size and shape differences, it seems unlikely that the contaminant large particles observed in this sequence of electron micrographs are identical with the electron dense granules of a wide range of sizes, shapes and distributions of general diameter 30 - 100 μm, which have been observed in whole *Azotobacter* cells by Hartman, Brodie, and Gray (1957).

The 144p60 fractions examined are not directly comparable. The 144p60 sonicated preparation, as has been succinctly pointed out by Marr and Cota-Robles (1957), is made up of cytoplasmic constituents liberated during cell disruption, and a fraction derived from cell wall breakdown. Presumably the 144p60 cytoplasmic membrane fraction represents cytoplasmic material and solubilized cell wall material; while the 144p60 cell envelope fraction represents cytoplasmic content alone. Differences in appearance of these fractions may be partially ascribed to differences in
nature of constituent components and differences in preparative techniques. Of the three, the 144p60 cell envelope supernatant fraction is most likely to result in the preparation of cytoplasmic component materials free of cell wall and cytoplasmic membrane materials.

Ultra thin sections of *A. agilis var. vinelandii O* fail to reveal the presence of large particulates, other than occasional metaphosphate granules, or of small spherical particles of about 20 μm diameter noted in this study, by Alexander and Wilson (1955), and by Hartman, Brodie and Gray (1957). One must question whether these structures exist in living bacterial cells, or whether they result from the cumulative effects of isolation and fixation procedures subsequent to cell breakage.

**Enzymological Studies on Sonically Disrupted Bacteria**

An early phase of the work reported here was an investigation of the biochemical capacities of various differential centrifugal fractions obtained from sonically disrupted *A. agilis var. vinelandii O*. Results obtained, together with those published by other workers, are listed in Table 1.

Succinic dehydrogenase was found in highest specific activity in a fraction spinning down between 144,000 x G for 30 minutes and 144,000 x G for 360 minutes. Gravitational forces reported here are those pertaining at the
outermost tip of the centrifuge tubes. Assuming that the material spun down is spherical and with a density 1.2 gm/cc using Stoke's Law it is possible to calculate an estimate of range of particle size. In the case of succinic dehydrogenase and glutamyl transferase, highest specific activities were found in particles of size range 50-10 μm. Fumarase and aconitase were found to be associated with particles less than 10 μm in diameter. Other workers have reported that amino acid incorporation is a function of large particles, Burma and Burris (1956). Molybdenum has been shown to be present in highest specific activity in particles of estimated size range 50-12 μm, Keeler, Bullen and Varner (1956). Oxidative phosphorylation is said to be a function of large particles, Hose and Ochoa (1956), or small particles less than 30 μm, Tissieres et al. (1957).

Table 2 presents data pertaining to the localization of RNA, DNA and protein in centrifugal fractions obtained from sonically disrupted A. agilis var. vinelandii O. An approximate size range has been calculated for each of the centrifugal fractions studied. In the experiment reported, protein, measured by the method of Warburg and Christian (1941), occurred in greatest amounts in large particles and in the "soluble" supernatant remaining after centrifugation at 144,000 x g for 6 hours. On a theoretical basis the soluble fraction should contain particulates of
less than 10 μm in diameter, which in turn corresponds very approximately to a unit of 1,000,000 M.W. or less. Components of the soluble fraction therefore are approaching molecular size. RNA content of the cellular fractions occurred in greatest amount in the soluble fraction, only a minimal amount occurred in the large particulate fraction containing a major proportion of cellular protein. DNA was found solely in the high speed supernatant, a finding in accord with the work of Alexander and Wilson (1955).

The techniques involved in studies of this type, random cellular disruption followed by gradation of the liberated cellular materials by means of selected centrifugal forces, are techniques which are conveniently applied. There are however disadvantages to the procedure most serious of which is the fact that, while a fraction obtained in this way can be characterized with respect biochemical capacity, one is unable to state what the fraction represents in terms of cellular structure. This disadvantage has been lessened in the work of Cota-Robles et al. (1958). These authors have attempted cellular rupture on a selective basis. Sonic disruption of bacteria has been permitted only for short periods of time, resulting in the isolation of a "hull" fraction, and secondly, liberated cellular content. A further disadvantage of the procedure is the fact that during differential centrifugal procedures
degradative enzymes may partially degrade cellular components to units having non-characteristic sedimentation properties. As an example, it is unlikely that the localization of approximately 50 per cent RNA and 100 per cent DNA in the 144s360 fraction is a true reflection of the in vivo nature of these bacterial cell components. It is more likely that partial hydrolysis of these structures has occurred during the differential centrifugation procedures resulting in the "solubilization" of these two materials.

**Intracellular Localization of Calcium, Iron, Molybdenum and Tungsten**

This series of experiments was conducted in cooperation with Drs. R. F. Keeler and J. E. Varner.

The method of cellular fractionation utilized was that of enzymatic cell wall digestion followed by protoplast lysis. Optical photomicrographs of whole cells, protoplasts and cytoplasmic membranes are illustrated in Figure 11. Protoplast morphology was found to vary from a rod shape indistinguishable from whole cells, to the more familiar spheroids, the shape assumed being in part a function of the tonicity of the suspension medium, and the time elapsed following the onset of enzymatic treatment. The cytoplasmic membrane fraction was seen to consist of large spherical membranes and granular material. This
fraction appeared morphologically identical in the optical microscope whether prepared directly by the lysis of whole cells, or indirectly by way of bacterial protoplasts. The nature of the cytoplasmic membrane fraction as determined by electron microscopy has been described previously.

Using the technique of Repaske for making protoplasts it was found that Mo$^{99}$, Fe$^{59}$, and W$^{185}$ were present in these structures. Lysis of the protoplasts resulted in the isolation of most of Fe$^{59}$ in the cytoplasmic membrane fraction, and the Mo$^{99}$, and W$^{185}$ in the supernatant. Similar results were obtained on the direct lysis of Mo$^{99}$, Fe$^{59}$, and W$^{185}$ labeled cells, Keeler, Carr and Varner (1958). These results support the thesis that iron is a component of the cytoplasmic membrane. Marr and Cota-Robles (1957) have reported the presence of cytochrome in the "hull" fraction of bacteria. This fraction contains the cytoplasmic membrane of the cell and the finding of cytochrome in this fraction is in excellent accord with the determined localization of radioactive iron. Storck and Wachsman (1957) have reported that the cytoplasmic membrane fraction of Bacillus megaterium contains succinoxidase, inferring that in this organism also at least a portion of the intracellularly located iron is present in the cytoplasmic membrane fraction.

Molybdenum and its antagonist tungsten are both found
in the supernatant fraction following protoplast lysis. This result suggests that molybdenum is essentially located within the cytoplasm of the bacterial cell. *A. agilis var. vinelandii O* is known to have a capacity for uptake and storage of molybdenum, Keeler and Varner (1957). It is unfortunate that the molybdate requirement of *A. agilis var. vinelandii O* for nitrogen fixation is insufficiently high to use radioactive molybdenum as a tool for the identification of a nitrogenase containing bacterial fraction. The localization of tungsten in the same bacterial fraction as molybdenum is in agreement with the observation that the former is a competitive inhibitor of the latter in *A. agilis var. vinelandii O*, Keeler and Varner (1957).

Studies on the intracellular localization of calcium are of interest in that they illustrate the non-desirability of utilization of Versene in the inducement of cell wall removal of *A. agilis var. vinelandii O* as a preliminary step prior to the biochemical analysis of protoplasts or protoplast lysates. It was observed that all the Ca$^{45}$ of Ca$^{45}$ labeled cells was released on protoplast formation. A control experiment with 0.006 M Versene as sole reagent resulted in a similar observation (Figure 12), thus negating the theory that the calcium of the bacterial cell is largely bound in the cell wall and is released only on cell
wall digestion. The study indicates one possible role of Versene in rendering the cell wall of *Azotobacter* susceptible to the action of lysozyme, and suggests that biochemical examination of *Azotobacter* protoplasts and protoplast lysates be prefaced by a consideration of the possibility of cofactor deficiencies in these structures.

Calcium growth experiments have indicated that approximately 7 ppm Ca is required to support cellular growth at near optimal levels. In fully grown cultures the calcium uptake, even at these low levels, was measured at approximately 10 per cent of the calcium added. If one assumes a wet cell volume of about 1 per cent in such cultures, it can be seen that the cell of *A. agilis var. vinelandii* 0 has only a moderate capacity for the concentration of this ion.

Calcium exchange between labeled cells and the external medium was measured in a sequence of studies, the results of which are graphically represented in Figure 12. Labeled cells were immersed in distilled water, solutions of 30 ppm Ca, Sr, Ba, 0.006 M Versene. The radioactive calcium released from the cells in each case was determined. Versene was seen to be the most effective agent for inducing Ca$^{45}$ release. Ninety-five per cent of the total label originally present in the cells was removed in a period of 10 minutes by this reagent. Radioactive calcium
exchange against calcium was effective to a final level of about 83 per cent. Strontium and barium promoted Ca\textsuperscript{45} release to a lesser degree. Finally Ca\textsuperscript{45} leached out from labeled cells to the extent of approximately 50 per cent when these cells were suspended in distilled water alone. In a growing culture of *A. agilis var. vinelandii* 0 one must conclude that extracellular and intracellular calcium ions are in equilibrium, and that to a considerable extent, these ions are freely exchangeable.

**Cytological Studies. The Influence of Metal Ions on Living Cells**

Concomitant with mineral nutrition growth studies, observations were made on the cytology of bacterial cells grown, in many cases, in media of abnormal mineral content. It was hoped that inspection of cytological abnormalities resulting from unusual mineral nutrition might provide information on the cytological role of the mineral under study.

Variation of level of iron, calcium and phosphorus, all components of normal nutrient media, provided little data on the function of these ions. In general both cells grown in excess quantities of one of these nutrients and deficient cells, were observed to be relatively small in size. Arsenate, a possible substitute or antagonist for phosphate, elicited no characteristic cellular abnormality
in *A. agilis* var. *vinelandii O*. Cytological observation of such cells is of particular interest in that arsenate has been shown to partially substitute for phosphate in this organism, and phosphate in the form of metaphosphate granules has been implicated in the process of nitrogen fixation by Esposito and Wilson (1956).

The transition elements, nickel and chromium differ in their toxicity to *A. agilis* var. *vinelandii O*, but in neither case was an abnormal cytological manifestation induced. This is the more surprising in the case of cobalt, which is often thought of as a noxious ion. *A. agilis* var. *vinelandii O*, deficient in iron, was seen to be capable of continuous apparently normal growth in a modified Burk's medium containing 50 ppm Co.

Chromium, a member of the molybdenum - tungsten family of elements, was shown to be an inhibition of iron metabolism in *A. agilis* var. *vinelandii O*. Also demonstrated was the fact that chromium, at a level of approximately 5 ppm, completely inhibited growth of *Azotobacter chroococcum* and *Azotobacter macrocytogenes*. From a cytological standpoint only *A. macrocytogenes* showed a positive response to the presence of chromate ion. At low levels *A. macrocytogenes* appeared abnormally large; at bactericidal levels light growth, followed by complete cellular disruption, was noted.
Investigation of calcium deficient cells of *A. agilis* var. *vinelandii* 0 has revealed that this organism is capable of utilizing barium or strontium ion in place of calcium. Log phase cells grown in Burk's medium containing calcium are normally rod like, 3-5 u in length, and approximately 1 u in diameter. Cells grown in the absence of calcium and in the presence of 30 ppm barium appear spherical with diameter of 1-3 u. Some vacuolation is evident in a few cells. Strontium cells of *A. agilis* var. *vinelandii* 0 are considerably larger than those grown in the presence of calcium. Young cells appear as enlarged rods up to 10-15 u in length. As cells mature "ballooning" of the cells occurs, older cultures contain a preponderance of such cells. Strontium cells are susceptible to changes in osmotic pressure. Lysis of such cells may be achieved by dilution of an appropriate culture with distilled water. Photomicrographs of calcium, barium and strontium cells of *A. agilis* var. *vinelandii* 0 are presented in Figure 13. An attractive theory may be advanced to account for the observed cell wall weakness of strontium cells. If calcium is assigned the cellular role of participation in the maintenance of cell wall rigidity then it is possible that the substitution of strontium for calcium results in an inadequate cell wall structure. Electron micrographs of log phase strontium cells of *A. agilis* var. *vinelandii* 0
fail to show any significant variation in cell wall morphology. Many cells were observed to have disrupted during the preparation of the specimen for the electron microscopy. The cytoplasm which exudes from parent cells has been subject to a very minimum of artifact producing physical forces, and it is likely that examination of such extracellular material will provide an insight of the intracellular organization of the intracellular materials. The exuded cytoplasmic material of young strontium cells does not show characteristic orientation, and is comparable with materials liberated by artificial means from log phase calcium grown cells. As strontium cells become older, and more spherical, cellular rupture on air drying becomes increasingly characteristic. In this case liberated cytoplasmic materials appear organized in spherical bodies of approximately equal diameter. Figure 14 shows a strontium cell which has lost nearly all of the cell content. The few remaining particulates within the cell are spherical in nature, and their presence lends credence to the high degree of organization evident in the exuded cellular material. These exuded particulates may be comparable to those observed as a contaminant in the cell wall-cytoplasmic membrane complex prepared by osmotic rupture of whole cells (Figure 7), or the large spherical particulates present in sonicated bacterial preparations,
or in the cytoplasmic membrane fraction (Figure 6). Each of these particulates are of approximately equal size range. Bacterial anatomy, as it is understood at the present time, fails to provide sufficient cytological complexity to account for the biochemical complexity of the organism. While bacterial organization based on orientation of cytoplasmic materials in large spherical particles may prove an erroneous concept, one must think in terms of a more complex degree of bacterial organization than one based on cytoplasmic particulates of 20-30 μ in diameter. A closer examination of strontium grown A. agilis var. vinelandii 0 cells appears a particularly attractive avenue for further research.

The ability of strontium and barium to substitute for calcium in other species and strains of Azotobacter was examined. Figure 15 shows calcium, barium and strontium cells of A. agilis var. vinelandii 3A. A response comparable to that obtained with A. agilis var. vinelandii 0 is evident. A. agilis var. vinelandii ATCC 7496 (Figure 16), A. agilis 144 (Figure 17) appeared identical whether grown on calcium or strontium containing media. Barium was unable to substitute for calcium in these organisms. With A. beijerinckii B5 (Figure 18) and A. macrocytogenes strain 0 (Figure 19) similar results were obtained. A. chroococcum C44 (Figure 20) grew adequately only on calcium and strontium
containing media. Strontium cells of this organism were extremely large and rod shaped. The results of these experiments are summarized in Table 3. In all the organisms studied strontium could replace calcium, while barium could serve in this capacity in only two. A marked cytological response to the presence of an abnormal mineral ion was noted in three of the organisms studied. If calcium is required for nitrogen fixation, this should be so in each of the organisms studied. Further investigation of species unable to grow on barium containing media should reveal instances where barium serves as an inhibitor of calcium metabolism, thus serving in effect as a specific inhibitor for nitrogen fixation.

At the present time it is not known why cytological responses to metal ions studied here varies so widely in the species of *Azotobacter*.

**Mineral Nutrition Studies:**

**Calcium, Barium and Strontium**

The interesting cytological manifestations of growth of *A. agilis var. vinelandii* on media containing calcium, barium and strontium have already been referred to. Experiments were run to determine the capacity of barium and strontium to substitute for calcium in *A. agilis var. vinelandii*. Growth curves of this organism in a calcium deficient state, grown in Burk's medium containing 30 ppm
Ca, Sr, or Ba are presented in Figure 21. Both the lag phase and the log phase of growth of this organism are of approximately equal duration in each of the three test media. Total growth of cells was determined as being greatest in calcium containing media, growth being less marked in barium and strontium containing media. It should be noted however that growth was determined by optical density measurements. These results may not be directly comparable in that the morphology of barium and strontium cells are quite different from calcium grown cells, and such differences may be reflected in differing light absorption characteristics. Figure 22 shows a graph of the substitutive powers of barium and strontium for calcium, determined by optical density of culture growth, against parts per million ion added. From the graph it can be seen that optimum level of calcium, for calcium deficient cells of *A. agilis var. vinelandii* O approximates 40 ppm, coinciding closely with a published requirement of 30 ppm for normal cells, Burk and Lineweaver (1930). Barium has an optimum requirement of about 50 ppm. Cells of *A. agilis var. vinelandii* O show an extremely wide tolerance to concentration of both barium and strontium, some growth being obtained at levels of as high as 300 ppm of these ions. At these higher levels significant quantities of calcium may be added as impurity, however it must be concluded that cellular tolerance to barium and strontium is of a high order.
These studies on the calcium, barium and strontium metabolism of *A. agilis var. vinelandii* have been conducted with calcium deficient cells. Complete absence of calcium, from such cells or from cells grown in barium or strontium containing media, is unlikely. Traces present in nutrient medium additives or as impurity in added barium or strontium may supply calcium required for critical cellular functions. It must be true however that the majority of cellular functions of calcium in the cell of *A. agilis var. vinelandii* may be carried out by barium and strontium.

**Iron, Cobalt and Nickel**

The iron metabolism of *A. agilis var. vinelandii* has not received extensive study. A level of 3 ppm iron is normally added to the culture medium to grow this organism (Burk's medium). The iron level in nutrient media required to sustain growth of *A. agilis var. vinelandii* must be relatively low, in that it was found that numerous transfers of this organism on iron free media failed to result in the development of a fully deficient culture. Measurement of growth response of iron deficient cells, in nutrient media containing various levels of iron, are represented in Figure 23. In this experiment a level of 8 ppm Fe was required to restore the iron deficient cells to a level of maximum growth. This level is considerably in
excess of the 3 ppm Fe added to a nutrient media used for the maintenance of optimum growth of iron sufficient cells. Levels of 100 ppm Fe were not found to be seriously inhibitory in the case of this organism. This elasticity in the iron requirement of *A. agilis var. vinelandii* 0 must be of inestimable value in permitting continuing growth of this organism in soils of varying iron content. In addition a relationship exists between iron and molybdenum, Keeler (1956), and it appears that an excess of one of the ions may exert a sparing action on the other.

Cobalt and nickel, because of their similarity of atomic structure with that of iron, are logical candidates for examination as possible inhibitors or substitutes for the latter ion. Figure 24 shows the growth response of both iron deficient and iron sufficient cells of *A. agilis var. vinelandii* 0 to varying levels of nickel. Nickel proved to be a most effective bactericidal agent for this organism in that a level of 3 ppm nickel served to inhibit both cultures of iron deficient and iron sufficient cells. The nature of the inhibition was not studied further, but the observation is of interest in that it provides a significant means of inhibiting growth of this organism. The low level of nickel required to inhibit cellular growth infers that the inhibition is relatively specific. Studies with metabolic intermediates might well result in the determination of the mechanism of the inhibition. Nickel
has been shown to be toxic for a number of other organisms, Abelson and Aldous (1950).

Cobalt was found to exert a minimal effect on the growth of *A. agilis var. vinelandii O*. The response of iron deficient cells to various levels of cobalt are indicated by the non-trained cell curve in Figure 25. This growth curve shows a slight fall off of growth at very low levels of added cobalt, followed by a recovery to initial levels at higher levels of the ion. In so far as the experimental cells were maintained over numerous transfers in iron deficient media, and the fall of growth response represents a relatively small change in optical density measurement, it is thought that little significance need be ascribed to this phenomenon. In addition it is possible that the growth phenomenon noted is a function of variation in length of the lag phase of growth over varying concentrations of cobalt. In order to further examine the interaction of iron deficient *A. agilis var. vinelandii O* cells with cobalt a culture of this organism was maintained through several transfers on a nutrient medium containing 50 ppm cobalt. These cells, cobalt-trained cells, were then examined for growth response to varying levels of cobalt ion. The results are plotted in Figure 25. Essentially the response of cobalt trained cells to cobalt ion is identical with that determined of non-trained cells.
In neither instance did a concentration of cobalt as high as 50 ppm exert a significant influence on cellular growth. In addition, as reported previously, no untoward cytological response was observed. A slight increase in culture viscosity and lack of culture pigmentation were the only characteristic manifestations of bacterial cultures containing cobalt. Cobalt has been reported to be toxic for many different species of microorganisms, Abelson and Aldous (1950), Schade (1949). Clearly this toxicity is not extended to *A. agilis var. vinelandii* 0. It would be of considerable interest to determine by means of Co$^{60}$ if cobalt has free access to the intracellular components of the bacterial cell.

**Iron and Chromium**

Studies by Keeler (1957) showed that chromate exerted no influence on the molybdate requirement of *A. agilis var. vinelandii* 0, and that higher levels of the ion were inhibitory to the organism.

A relationship between iron and chromium was noted. It was found that cells of *A. agilis var. vinelandii* 0 grown on low levels of iron were more susceptible to inhibition by chromate, added as Cr$_2$O$_7$-, than cells grown on higher levels of iron. Figure 26 shows the response of partially iron deficient cells of *A. agilis var. vinelandii* 0 grown in the presence of varying levels of
iron and chromate. While the ratio of iron to chromate permitting half maximum culture growth did not remain constant, as would be the case if chromate were a competitive inhibitor of iron, it was found that increments in iron nutrition were accompanied by increments in chromium level required to inhibit cellular growth. The level of chromate required to inhibit growth of *A. agilis var. vinelandii O*, appeared in part to be a function of the iron nutrition of the cells used for inoculation purposes. Iron sufficient cells required more chromate to achieve growth inhibition than iron deficient cells. The data plotted in Figure 26 were obtained using portions of a single culture of *A. agilis var. vinelandii O* as inoculum for each of the experimental systems observed.

Extension of the work on *A. agilis var. vinelandii O* to *Azotobacter chroococcum G44* and *Azotobacter macrocytogenes O* showed that these two latter species were also susceptible to inhibition by chromate ion (Figure 27). *A. macrocytogenes* is somewhat unusual in that slight growth, followed by cellular disruption, occurs at high levels of chromate resulting in the measurement of slight density even at bactericidal levels of the ion.

Chromium, molybdenum and tungsten are members of group VIB of the periodic table. On the basis of similarities in atomic structures chromate would be expected to exert
physiological influence on molybdate metabolism. In point of fact such a relationship was not observed experimentally. It is not known why chromium should interfere with iron metabolism. A similar phenomenon has been observed in plants, Walker and Grover (1956).

**Phosphate and Arsenate**

Numerous reports have indicated that arsenate may, in part, substitute for the phosphate requirement of living organisms. Such a relationship has been found in *A. agilis var. vinelandii 0*.

Figure 28 shows the growth response of phosphate deficient cells of *A. agilis var. vinelandii 0* to varying levels of phosphate, arsenate and a combination of 80 per cent phosphate - 20 per cent arsenate. Maximal growth was achieved at a level of approximately 150 ppm PO$_4$ - one third of the amount usually included in Burk's nitrogen-free mineral salts medium, and one twelfth of that added to the medium of high buffering capacity used by some workers at the present time. Arsenate, up to a level of 500 ppm, failed to permit growth of the organism in the absence of phosphate. A mixture of 80 ppm phosphate - 20 ppm arsenate was found to be more effective in inducing growth of the organism than any level of phosphate alone. Higher levels, above 200 ppm, of the phosphate-arsenate mixture were about as effective as phosphate alone in inducing cellular growth.
It is possible that other ratios of phosphate to arsenate would reveal more striking results.

It is evident that arsenate will not act as a complete substitute for phosphate, and that a partial substitution appears particularly advantageous with respect to cell growth. One may advance theories to explain why this phenomenon exists. A reaction involving phosphate uptake followed by phosphorylation of an ADP molecule, such as is the case in the conversion D-glyceraldehyde-3-phosphate to 3-phospho-D-glycerate, may proceed in more rapid fashion in the presence of arsenate and ADP. In this case the postulated intermediate l-arseno-3-phospho-D-glycerate, thought to be highly unstable, would readily decompose to 3-phosphoglycerate. Transfer of the liberated arsenate to ADP could result in the postulated compound ADP-arsenate, again highly transitory. Spontaneous breakdown of this compound would free both arsenate and ADP. These compounds would then be available for further catalytic function. The net result of arsenolysis of D-glyceraldehyde-3-phosphate would be a speed up of conversion of this compound to 3-phospho-D-glycerate, and the loss of generation of 1 ATP molecule. How serious the partial loss of available energy is to A. agilis var. vinelandii 0 is difficult to estimate. The fact that under favorable growth conditions this organism excretes large quantities of glutamine into the nutrient
medium, and synthesises intracellular metaphosphate, suggests that an excess supply of energy is available to the cell. Other reactions in which arsenate could effect a significant enhancement of reaction rate include the conversion succinyl-coenzyme A to succinate, glutamine to glutamate, and in the process of electron transport in oxidative phosphorylation. If the step succinyl-CoA to succinate is rate limiting, enhanced conversion would permit a more rapid oxidation of other Krebs cycle intermediates. This in turn might well result in a more rapid growth rate of the bacterial cell. Enhancement of the reaction glutamine to glutamate, would provide the cell with additional glutamic acid which would be available for synthetic and transamination reactions and increased participation in the Ornithine cycle. Transamination of glutamate in turn would provide oxidisable \( \alpha \) ketoglutarate. The addition of arsenate to a culture of *A. agilis var. vinelandii O* should therefore be expected to result in a lessened glutamine excretion, and a more efficient utilization of the carbon skeleton previously excreted from the cells in this form. Any stimulation of cellular growth as a result of the effect of arsenate on oxidative phosphorylation should result primarily from the partial uncoupling of oxidation and phosphorylation. Such a phenomenon would increase the supply of available ADP, required as phosphate
acceptor in many phosphate transfer reactions, increase the supply of DPN+ required in certain reactions, and finally permit more rapid oxidation of substrates at the appropriate stages of the Krebs cycle.

The enhancement of growth of *A. agilis var. vinelandii* 0 by arsenate may result from the effect of this ion on these or other metabolic reactions. In each case, other than the conversion of glutamine to glutamate, enhancement may result from an increased interconversion of substrate to product, and the increased availability of ADP and DPN+. Unquestionably a balance exists between the ease of interconversion of metabolic intermediates, and energy production. Arsenate at appropriate levels may induce a more favorable balance in *A. agilis var. vinelandii* 0.

**Summary - Mineral Nutrition Studies**

The results of mineral nutrition studies noted here have included both the expected and unexpected.

The finding that both strontium and barium exert influence on the calcium metabolism of *Azotobacter* is in line with the close structural and chemical properties of these elements. Nickel inhibition of iron metabolism in *A. agilis var. vinelandii* 0 is to be expected from a consideration of the extreme similarity of these two ions. The fact that cobalt exerts no influence on iron metabolism is inconsistent with theoretical expectation. The electronic configuration of each of these ions:
underlines the basic reason for similarity of chemical properties of the transition elements.

The group VI B element chromium bears little similarity to iron:

Cr $2, 8, 13, 1$
Fe $2, 8, 14, 2$.

Nevertheless interaction of the two ions was noted. Chromium failed to interfere in molybdenum metabolism of A. agilis var. vinelandii despite the close similarity of electron configuration:

Cr $2, 8, 13, 1$
Mo $2, 8, 18, 13, 1$.

Phosphate and arsenate fulfilled theoretical expectations;

P $2, 8, 5$
As $2, 8, 18, 5$

in that arsenate appears to replace a part of the phosphate requirement.

It is apparent that much further study is required before we can appreciate the biochemical functions of these elements.
SUMMARY

Some concept of the nature of the cell of Azotobacter agilis var. vinelandii 0 has been gained. Electron micrographs of ultra thin sections of the organism have shown the presence of a relatively thin cell wall and cytoplasmic membrane, and a cytoplasmic content of variable electron scattering capacity. A well defined nucleus was not seen in this organism. Optical microscope studies by Whitfield and Murray (1956) have shown that the chromatin structure of bacteria is a function of ionic environment. Further study on the anatomy of A. agilis var. vinelandii 0 should include observation of ultra thin sections of the organism prepared in such a way as to enhance the possibility of visualizing the bacterial nucleus.

Bacterial fractionation studies have provided a further insight into the nature of the bacterial cell. Preparations of centrifugal fractions obtained from sonicated bacteria were observed to be highly heterogeneous in the electron microscope. A cytoplasmic membrane fraction obtained by enzymatic digestion of the cell wall of A. agilis var. vinelandii 0 was seen to consist of membrane like structures, and was contaminated by large particulates.
Radioactive isotope studies showed that this fraction was almost completely denuded of calcium content by the Versene included in the enzymatic preparation used for cell wall digestion. From the point of view of a correlation of bacterial structure and biochemical function the most useful method of rupture studied was that of osmotic shock. A low speed centrifugal fraction examined was seen to be made up of empty bacterial cell envelopes, each consisting of cell wall and cytoplasmic membrane. These structures, being relatively free from cytoplasmic constituents, provide a potentially excellent means of studying the biochemical capacities of these two structures of the bacterial cell. The application of high speed centrifugal forces following the differential centrifugal removal of the bacterial cell envelopes should permit the isolation of the particulate portions of the cytoplasmic materials of the bacterial cell.

A series of experiments to test the cytological response of Azotobacter to a variety of abnormal mineral nutrients demonstrated the extreme variation in cell size which is possible in A. agilis, Azotobacter macrocytogenes and Azotobacter chroococcum. Strontium and chromium were both found to be effective in increasing cell size to a significant degree. Electron micrographs of ruptured strontium cells of A. agilis var. vinelandii 0 were seen to exude cytoplasmic material arranged in spherical par-
ticulates; these were comparable in size to those observed as contaminants in low speed centrifugal fractions of bacteria. The almost ubiquitous appearance of such particles leads one to suggest that the cytoplasmic material in living bacterial cells may be arranged in spherical structures of the order 100-400 μ in diameter. Current knowledge of bacterial anatomy does not indicate sufficient cytological complexity to account for the biochemical complexity of the bacterial cell.

Growth experiments to test the response of *A. agilis var. vinelandii* O to calcium, strontium and barium, iron cobalt and nickel, iron and chromium, and phosphate and arsenate produced data of a fundamental nature. Strontium and barium were shown to be capable of substituting for calcium. Nickel and chromium were shown to be inhibitory for iron metabolism, and arsenate, in the presence of phosphate, was seen to enhance growth of *A. agilis var. vinelandii* O. Other than the interaction of phosphate and arsenate, the biochemistry of each of these observations is not understood, primarily it is thought, because the mineral metabolism of this organism has not received study beyond a very gross level. A combination of radiological and bacterial fractionation techniques showed that iron is present to greatest extent in the bacterial cytoplasmic membrane fraction, molybdenum occurs in the cytoplasm, and calcium leads a transitory existence.
At the current rate of progress a thorough understanding of bacterial cytology, and bacterial metabolism is many years away. Depending as it does on the fruits of research in each of two major disciplines, a correlated understanding of bacterial anatomy and related biochemical function must inevitably be in the vanguard.
CONCLUSIONS

The application of electron microscopic techniques to the problem of bacterial structure has indicated the presence of certain well defined structures within the bacterial cell.

Of the various methods available for bacterial rupture, that of osmotic shock appears the most promising at this time. This technique, coupled with differential centrifugation, may be used to isolate bacterial cell envelopes consisting of cell wall and cytoplasmic membrane, from cytoplasmic constituents.

Concomitant cytological and mineral nutrition studies appears a particularly fruitful avenue for future research. Such studies could well be extended to include the study of the effects of inclusion of appropriate metabolic intermediates to nutrient media, in instances where it was felt that such a measure would further illustrate the metabolism of specific metal ions.
Figure 1. Optical Micrographs of *Azotobacter agilis* var. *vinelandii* strain 0.

Log phase cells

Stationary phase cells

xl,500
Figure 2. Electron Micrograph of Ultra Thin Sections of Log Phase Azotobacter agilis var. vinelandii O.

x56,000
Figure 3. Electron Micrograph of Stationary Phase \textit{A. agilis var. vinelandii} 0. The Densely Packed Extra-cellular Material is Considered to be the Capsule. $x90,000$
Figure 4. Electron Micrograph of Stationary Phase *A. agilis* var. *vinelandii* O, View of Two Adjoining Cells. x90,000
Figure 5. (25p30) of Sonically Disrupted A. agilis var. vinelandii 0. 25,000 X G pellet obtained after 30 minutes centrifugation of sonically disrupted A. agilis var. vinelandii 0. (25p30 fraction). The fraction is heterogeneous, a cell wall fragment is visible in the center of the field. x60,000
Figure 6. 25p50 Cytoplasmic Membrane Fraction of *A. aërobica* var. *vinelandii* O. Visible are fragments of bacterial flagella, a partially denatured membrane and adherent spherical bodies. These latter are a common contaminant of the fraction.

x25,000
Figure 7. 25p30 Cell Envelope Fraction of \textit{A. agilis var. vinelandii} 0. This fraction was secured by osmotic lysis of whole cells. The extracellular spherical particle is representative of the fraction contaminant.

$x_{50,000}$
Figure 8. Sonically Disrupted Bacteria 144p60 fraction.

x55,000
Figure 9. Bacterial Cell Membrane 144p60 fraction.

x55,000
Figure 10. Bacterial Cell Envelope 144p60 fraction.

x55,000
Figure 11. Protoplasts and Cytoplasmic Membranes of *A. agilis* var. *vinelandii* 0.

x1,500
Figure 12. Graph of Release of Ca$^{45}$ from \textit{A. agilis} var. \textit{vinelandii} as a Function of Ca, Ba, Sr and Versene.
Calcium cells  Barium cells

Strontium cells

Figure 15. Optical Micrographs of *A. agilis var. vinelandii* Grown in the Presence of Ca, Ba or Sr.

x1,500
Figure 14. Electron Micrograph of a Disrupted Strontium Cell of *A. agilis var. vinelandii* 0., the micrograph showing the extreme particulation of the exuded cellular content. Shadowed with metallic platinum at an angle of 1:5.

x10,500
Figure 15. Optical Micrographs of *A. agilis var. vinelandii* 3A Grown in the Presence of Ca, Ba or Sr.
Calcium cells

Figure 16. *A. agilis var. vinelandii* ATCC 7496. Calcium and strontium cells appear identical.

xl,500

Calcium cells

Figure 17. *A. agilis* 144. Calcium and strontium cells appear identical.

xl,500
Calcium cells

Figure 18. *A. Heijerinckii B5*. Calcium and strontium cells appear identical.

xl,500

Calcium cells

Figure 19. *A. Macrocytogenes strain 0*. Calcium and strontium cells appear identical.

xl,500
Figure 20. Optical Micrographs of *A. chroococcum* C 44 grown in the presence of calcium or strontium.
Figure 21. Growth Curves of Calcium Deficient *A. agilis* var. *vinelandii* in Nutrient Media Containing 20 ppm Ca, Sr or Ba.
Figure 22. Growth Response of Calcium Deficient *A. agilis* var. *vinelandii* to Varying Levels of Ca, Ba or Sr.
Figure 23. Growth Response of Iron Deficient *A. agilis var. vinelandii* 0 to Varying Levels of Iron.
Figure 24. Growth Response of *A. agilis var. vinelandii* O to Varying Levels of Nickel.
Figure 25. Growth Response of A. aeolicus var. vinelandii C to Varying Levels of Cobalt.
Figure 26. Iron and Chromium Interaction in A. agilis var. vinelandii Q.
Figure 27. Growth of Azotobacter spp. as a function of chromium level.
Figure 38. Growth Response of A. agilis var. vinelandii to Varying Levels of Phosphate and Arsenate.

Growth Response to Phosphate and Arsenate:
- **PO$_4$**
- **PO$_4$ + 20% AsO$_4$**

**Log Growth**

**Parts** $10^6$
<table>
<thead>
<tr>
<th>Particle Bound Activities</th>
<th>Estimated Size Range</th>
<th>Investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorporation of AA.</td>
<td>large particle</td>
<td>Burma and Burris (1956)</td>
</tr>
<tr>
<td>High Mo</td>
<td>50 - 12 μm</td>
<td>Keeler et al. (1956)</td>
</tr>
<tr>
<td>Cytochrome Oxidase</td>
<td>large particle</td>
<td>Tissieres and Burris (1956)</td>
</tr>
<tr>
<td>Oxidative Phosphorylation</td>
<td>large particle</td>
<td>Rose and Ochoa (1956)</td>
</tr>
<tr>
<td>Oxidative Phosphorylation</td>
<td>30 μm</td>
<td>Tissieres et al. (1957)</td>
</tr>
<tr>
<td>Aconitase</td>
<td>small particle</td>
<td>Alexander and Wilson (1955)</td>
</tr>
<tr>
<td>Aconitase</td>
<td>10 μm</td>
<td>Carr</td>
</tr>
<tr>
<td>Succinoxidase</td>
<td>20 μm</td>
<td>Repaske (1954)</td>
</tr>
<tr>
<td>Succinic Dehydrogenase</td>
<td>small particle</td>
<td>Alexander and Wilson (1955)</td>
</tr>
<tr>
<td>Succinic Dehydrogenase</td>
<td>50 - 10 μm</td>
<td>Carr</td>
</tr>
<tr>
<td>Fumarase</td>
<td>small particle</td>
<td>Alexander and Wilson (1955)</td>
</tr>
<tr>
<td>Fumarase</td>
<td>10 μm</td>
<td>Carr</td>
</tr>
<tr>
<td>Glutamyl Transferase</td>
<td>50 - 12 μm</td>
<td>Carr</td>
</tr>
</tbody>
</table>

Table 1. Enzymatic Properties of Differential Centrifugal Fractions Obtained from Sonicated *A. agilis var. vinelandii* 0.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Approx. size range</th>
<th>% Protein</th>
<th>% RNA</th>
<th>% DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>25p30</td>
<td>1000 A</td>
<td>47</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>60p30</td>
<td>1000-750 A</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>144p50</td>
<td>750-350 A</td>
<td>11</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>144p4 hr</td>
<td>350-150 A</td>
<td>7</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>144p10 hr</td>
<td>150-100 A</td>
<td>5</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>14s</td>
<td>100 A</td>
<td>20</td>
<td>57</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Distribution of RNA, DNA and Protein in Centrifugal Fractions Obtained fromSonically Disrupted *Azotobacter agilis* var. *vinelandii* 0.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Ca</th>
<th>Sr</th>
<th>Ba</th>
<th>Cytological Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. agilis var. vinelandii 0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>A. agilis var. vinelandii 3A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>A. agilis 144</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>A. agilis var. vinelandii 7496</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>A. chroococcum</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>A. beijerinckii</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>A. macrocytogenes</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>No</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY


Bisset, K. A. (1955) The Cytology and Life History of Bacteria. (Williams and Wilkins)


Buchmer, E. (1897) Ber. 50, 117.


I, Leonard Barrett Carr, was born on October 6, 1930, in Detroit, Michigan. From 1932 until 1953, I was resident in England. My secondary school education was obtained at Pocklington School, York, England. Enrolling at Birmingham University, England, in October 1949, I received an Honours B. Sc. degree in Industrial Fermentation from that University in June 1952. In the year 1952-1953 I was enrolled in a postgraduate course in malting and brewing at the British School of Malting and Brewing, Birmingham University, England. Returning to the U. S. A. in July 1955, I was promptly inducted into the U. S. Army, and served as a research assistant at the Department of Virus and Rickettsial diseases at Walter Reed Institute of Research, and in the Biochemistry Department of The Tropical Research Medical Laboratory, at San Juan, Puerto Rico. In 1955, I enrolled in the Department of Agricultural Biochemistry, Ohio State University, and while completing the requirements for the degree Doctor of Philosophy held the position of Graduate Assistant for two years, and that of Research Fellow during my final year of residence.