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The Encystment of *Azotobacter Vinelandii* in Liquid Culture.

Luther Harold Stevenson

Louisiana State University and Agricultural & Mechanical College

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IN LIQUID CULTURE.

Louisiana State University and Agricultural and Mechanical
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THE ENCYSTMENT OF AZOTOBACTER VINELANDII IN LIQUID CULTURE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
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in

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ABSTRACT

A liquid medium which supports the encystment of Azotobacter vinelandii (A. agilis) was described. Cultures exhibiting greater than 90% cysts were induced by adding 0.6% CaCO₃ to the Burk's basal salts solution with 1% glucose. The cysts grown in the medium supplemented with CaCO₃ were resistant to desiccation and possessed the typical exine and intine capsular components when observed by electron microscopy of ultra-thin sections and carbon replicas. Cells grown in a liquid medium in which the CaCO₃ was deleted and the pH was adjusted periodically by the addition of 0.1 M KOH were not resistant to desiccation and exhibited a loose aggregation of capsular material which was not organized into a typical coat structure. The culture supernatant became very viscous when the cells were grown without the carbonate. The viscous supernatant material could be precipitated with CaCl₂, yielding a thick, polysaccharide-like gel. Encystment was obtained by replacing the carbonate used to supplement the medium with 2×10^{-3} M CaCl₂. The CaCO₃ in the liquid encystment medium apparently serves the dual function of acid neutralization and calcium ion source. Ca⁺⁺ is a structural ion necessary for coordination of the coat components into the rigid cyst coat.

Poly-β-hydroxybutyric acid (PHB) content of the cells reached a maximum during the early stationary phase of growth and subsequently declined. During polymer degradation there was a concurrent increase in the extent of encystment in the cultures supplemented with CaCO₃

and a similar increase in the viscosity of supernatants of cultures grown utilizing KOH for pH control. Nitrogen fixation ceased during the stationary phase. The extent of encystment and the amount of PHB accumulated were directly proportional to and dependent on substrate concentration. The PHB was selectively labeled by the addition of sodium acetate-2-C¹⁴ to late log-phase cells. During polymer utilization and either cyst or viscosity development, 20% of the activity was evolved as CO₂. In non-encysting cultures, 45% of the activity was distributed between residual PHB and other cellular components, and 35% was in the supernatant polysaccharide-like material. Intact cysts retained 80% of the label. Experiments with ruptured cysts indicated that about 35% of the activity was present in the intine material.

It is proposed that the accumulation of large amounts of PHB by A. vinelandii reflects an unbalanced growth condition, which is essential to or enhances the encystment process. Following polymer deposition, the reserve material is utilized endogenously as both a source of energy and as a source of carbon skeletons for the ensuing encystment process. As the coat material is produced, it is coordinated around the developing central body through the mediation of calcium ions. If adequate ions are not available for the formation of complete coats, abortive encystment takes place and the structural units of the coat are liberated into the surrounding medium with a resultant increase in viscosity.

INTRODUCTION

Bisset (5) postulates that most bacterial species produce specialized resting cells. They are generally smaller than actively growing vegetative cells, lack flagella, and are resistant to various deleterious agents. The bacterial resting forms which are most commonly accepted by microbiologists are the endospores produced by Clostridium and Bacillus (90), the microcysts produced by Myxococcus (21), and the cysts produced by Azotobacter (88).

Although they achieve similar goals, the characteristics of the various types of resting cells differ markedly. In general, the endospore is a highly specialized structure formed within a vegetative cell. Following formation of the mature spore, the remnants of the vegetative cell are discarded and the spore is liberated into the surrounding environment (90).

On the other hand, during cyst or microcyst formation a small spherical form of the vegetative cell, the central body, is encased in a dense, multilayered, external coat (88, 115). The coat of the Azotobacter cyst is responsible for the resistance exhibited by this resting cell, since without this structure, the central body is as vulnerable as the young vegetative cell to deleterious agents (72). The rupture of the coat which occurs when Azotobacter cysts are treated with chelating agents indicates that metal ions are involved in maintaining the integrity of this structure (88). Goldschmidt and Wyss (32) have specifically implicated magnesium ions in this role.

The development of the cyst has been extensively studied utilizing solid media in conjunction with various carbon sources (88, 92, 98). Stevenson and Socolofsky (92) observed that it was not the specific carbon source used to grow the organism but its concentration which determined the amount of poly-B-hydroxybutyric acid accumulated by the organism and the subsequent induction of cyst development. They further proposed that the polymer accumulated by the organism served as a carbon or energy source for cyst formation.

Although much progress has been made in the study of cyst development, the examination of this morphogenic process has been hindered by the necessity of cultivating cysts on a solid surface. An encysting system in a liquid medium would present a more convenient system for studying cyst development. Layne and Johnson (50, 51) described resistant forms of Azotobacter produced in liquid culture by the omission of one or more of the minerals from the medium used to grow the organism. However, their resistant forms lacked the characteristic coat structures and attempts to duplicate their results have been unsuccessful (72). Romanow (77) reported cyst formation by A. chroococcum in a liquid medium, however he offered neither quantitative nor cytological evidence of encystment.

The purpose of the research reported in this dissertation was to develop a liquid medium which would support the encystment of A. vinelandii and to examine various environmental factors associated with cyst development.

REVIEW OF LITERATURE

The genus Azotobacter

Description

Members of the genus Azotobacter were first isolated, described, and named by Beijerinck in 1901 (4). The genus has been found subsequently throughout the world in neutral or alkaline soils (10) and in both fresh and salt water (47, 107). Bergey's Manual of Determinative Bacteriology (10) described Azotobacter cells as relatively large, gram-negative, non-sporeforming, and peritrichously flagellated rods or cocci. The cells are obligate aerobes and are capable of nonsymbiotic nitrogen fixation. The name of the genus, Azotobacter, is a modern Latin noun meaning nitrogen rod.

General morphology

During vegetative multiplication Azotobacter cells are ellipsoidal or peanut-shaped, measuring 2 to 6 u in length and 0.5 to 3 u in width (10). Because of the extreme pleomorphic nature of the organism, this can hardly be considered to be a complete morphological description. Löhnis and Smith (59) were the first to recognize that the azotobacter are capable of producing a variety of forms. They described a complex life cycle for the organism having 13 different cell types, some of which were capable of reproduction. Among the types reported were gonidia, buds, arthrospores, endospores, exospores, microcysts, and fungoid forms. However, many of their observations could not be confirmed by subsequent investigation and were attributed to impure cultures (40).

Yet the morphology of Azotobacter in pure cultures is remarkably variable, and because of this feature, the genus has been the object of recent study by investigators equipped with more sophisticated equipment and techniques than were previously available. This work has tended to confirm some, but not all, of the earlier reports (40).

Utilizing light, phase, and electron microscopy, Eisenstark, McMahon, and Eisenstark (24) observed four distinct morphological types in A. agilis cultures grown on nitrogen-free media. The forms included large and small rods, elongated and branched forms, and small cocci. Eisenstark, Ward, and Kyle (25) observed balloon-shaped cells in A. agilis cultures grown on standard nutrient agar or soil extract agar.

The question of gonidia formation by Azotobacter was revived by Bisset and Hale (7) in 1953 when they reported the production of these small elements by A. chroococcum. The gonidia were observed to be capable of independent reproduction and growth, and under some conditions, they reverted to the normal Azotobacter cell. Lawrence (49) supported the question of gonidia formation by demonstrating that particles found in filtrates from old cultures developed into normal cells upon incubation. Utilizing the shadow casting technique and electron microscopy, Van Schreven (103) demonstrated particles that were released from swollen, penicillin treated Azotobacter cells. He considered the particles to be gonidia and reproductive dwarf cells.

By utilizing phase microscopy to observe several strains of A. chroococcum grown under various environmental conditions, Van Schreven (102) conclusively demonstrated that the morphology of the genus is greatly influenced by the media used for cultivation. Many bizarre forms, such as long filaments and giant oval cells, could be induced by the addition of supplemental material to the agar medium. Peptone, milk, potato flour, and nitrate were particularly effective inducing agents.

Carbon metabolism

Carbon metabolism in Azotobacter has been of interest for some time. This interest has been stimulated by the ability of the genus to assimilate molecular nitrogen and to oxidize extensively carbon substrates with a high rate of oxygen consumption (94). The pathway of glucose utilization has been a particular area of dispute over the last few years and the exclusive operation of any of the currently known pathways has not been decisively demonstrated (94).

The operation of the Embden-Meyerhof-Parnas pathway has been suggested since all of the enzymes of the pathway, except phosphohexokinase, have been demonstrated in cell-free extracts of A. vinelandii (94). However, experiments utilizing isotope labeling of the various carbon atoms of glucose have indicated that this is not the primary catabolic pathway utilized by the organism (87, 94).

The operation of the pentose phosphate (PP) pathway was suggested by the demonstration of 6-phosphogluconic dehydrogenase, transaldolase, and transketolase in cell-free extracts of Azotobacter (69, 70).

However, the PP pathway has also been delegated a minor catabolic role because of the results of experiments utilizing isotopic tracing of selected carbon atoms of glucose (94).

Radiorespirometric experiments, as well as enzymatic experiments with cell-free systems, strongly indicate that the Entner-Doudoroff (ED) system is the primary pathway of glucose catabolism in the azotobacter (94). The $C^{14}O_2$ production patterns from individual carbon atoms of glucose by Azotobacter are basically similar to those observed with Pseudomonas fluorescens which appears to utilize the concurrent operation of the ED and PP pathways. A comparison of the theoretical and observed isotope distribution pattern of cellular alanine derived from specifically labeled glucose indicates that the ED pathway alone and not the PP pathway is operative (94). The enzymatic examination of Azotobacter for enzymes important in the ED pathway has revealed that cell-free extracts can convert glucose to 6-phosphogluconate (69) and that the 6-phosphogluconate in turn can be cleaved to form glyceraldehyde-3-phosphate and pyruvate (68). However, the two key enzymes associated with the ED pathway, i.e., 6-phosphogluconic dehydrase and 2-keto-3-deoxy-6-phosphogluconic aldolase, have not been demonstrated in cell extracts of Azotobacter (94).

The terminal oxidation of carbon compounds by Azotobacter has been shown to proceed through the tricarboxylic acid (TCA) cycle. Pyruvic acid, acetic acid, and the member acids of the TCA cycle were found to be readily oxidized (113).

Extracellular polysaccharides

The nature of the extracellular polysaccharides of A. vinelandii and A. agilis has been examined (15, 16). Cohen and Johnstone (15) found that two fractions of extracellular polysaccharide, a free slime material and the true capsular material, could be isolated from A. vinelandii; however, both fractions had the same basic chemical composition. Chemical analysis indicated that, in general, the polymers contained galacturonic acid, glucose, and rhamnose at a ratio of 43:2:1, as well as a hexuronic acid lactone, probably mannuronolactone. Minor differences did exist among the various strains tested. The capsular material isolated from A. agilis (16) appeared to be markedly different from that of A. vinelandii. Chemical tests indicated that only galactose and rhamnose, at a ratio of 1.0 to 0.7, were present in the A. agilis capsule.

Cyst of Azotobacter

Encystment

The presence of thick-walled cells in old cultures of Azotobacter was recognized early in the study of the genus. In 1920 Jones (43) reported observing spherical, thick-walled cells, which he called resting cells or arthrospores, in 14-day old cultures. Batcheskaya (3) reported the formation of a two-layered capsule around aging cells of Azotobacter. The inner layer was thought to be composed of soft slime, while the hard outer layer served as an envelope.

In 1938 Winogradsky (114) published the first detailed description of the encystment process and the first conclusive cytological characterization of the cyst. He reported that encystment occurred when cells of A. vinelandii were grown for 5 or 6 days in the presence of various carbon sources. The first type of cell was observed to be young motile rods. After 3 days of growth, some of the rods were seen to change into rounded forms which became coccoid and diminished in size after 4 to 5 days of incubation. In the last phase, the coccoid cells became mature cysts.

By the use of a complex violamine staining technique, Winogradsky (114) was able to observe and name 3 distinct cytological features of the mature cysts. A brown to black staining inner portion was considered to be the residual vegetative cell and was named the central body. It was surrounded by a dense, double-layered capsule. The outer layer stained black and was called the exine and the inner yellow staining area was called the intine.

Wyss, Neumann, and Socolofsky (115) employed electron microscopy of ultra-thin sections to study the cytology of cyst formation. Their results indicated that encystment occurred after 3 to 5 days of growth on a solid, nitrogen-free medium. Electron micrographs of 1-day old vegetative cells revealed them to be large rods having a homogeneous cytoplasm and a network of membraneous structures (peripheral bodies) along the periphery of the cell. Pockets of material having a low electron density, characteristic of nuclear material, were seen to be interspersed in the cytoplasm. After 2 to 3 days the vegetative cells assumed an oval or spherical shape and the peripheral bodies disappeared.

The next stage of encystment occurred after 36 hr of growth and involved the deposition of a bark-like coat (exine) adjacent to the cell wall. After 48 hr, almost all the cells were encompassed by a layer of exine material which appeared as a loose, fragile envelope surrounding the cell. During this period the intine began to appear as a homogeneous granular material between the exine and central body. The central body retained the cell wall-cell membrane complex typical of the vegetative cell. After 3 to 5 days, the maturation of the cyst was complete. The exine became a thick, laminated structure; the central body became much smaller; and the intine appeared to separate into two distinct layers.

In general, similar studies by Tchan, Birch-Andersen, and Jensen (98) confirmed the observations of Wyss et al. (115). Though the two groups are not in agreement regarding the terminology assigned by Winogradsky (114), electron micrographs contained in the two papers are essentially alike. However, Tchan et al. (98) observed some randomly oriented, triple-layered, cylindrical units within the inner layer or intine.

Factors affecting encystment

The cultural conditions necessary for cyst formation by the azotobacter have been of interest for many years. Winogradsky (114) reported that encystment occurred when A. vinelandii was grown in the presence of butanol, ethanol, butyrate, acetate, and benzoate with butanol promoting the most complete encystment. No cysts were produced in cultures when grown on mannitol or glucose. Socolofsky

and Wyss (88) confirmed this observation when they reported 100% encystment of A. vinelandii when cultured on a medium supplemented with butanol but almost no encystment when cultivated on a sucrose medium.

Layne and Johnson (50, 51) contended that encystment was dependent upon a low carbon and mineral content of the growth medium. However their conclusions were disputed by Stewart, Olson, and Wyss (93). The latter workers reported that both growth and encystment are dependent upon sufficient copper and magnesium ion concentrations in the medium.

Stevenson and Socolofsky (92) reported that cyst development was dependent upon the accumulation of significant amounts of poly-B-hydroxybutyric acid prior to the onset of encystment. The extent of polymer accumulation and the percentage of cyst development were shown to be directly related and to be dependent on the concentration of carbon source in the growth medium.

Eklund, Pope, and Wyss (26) indicated that the presence of capsular polysaccharide is a prerequisite for the formation of mature cysts. By growing A. vinelandii in the presence of either a phage-induced depolymerase enzyme specific for capsular polysaccharide (27) or ammonium ions, they were able to inhibit capsular polysaccharide formation and cyst development. Nonencapsulated mutants of the organism also failed to form cysts.

The development of the cyst, in the main, has been studied utilizing solid media for cultivation of the organism. However, encystment has been occasionally reported in liquid culture. In his original report on cyst development, Winogradsky (114) reported partial encystment in 14-day old standing cultures. Layne and Johnson (50, 51) described resistant forms of Azotobacter produced in liquid medium depleted of one or more divalent metal ions. However, their resistant forms lacked the characteristic coat structures and attempts to duplicate their results have been unsuccessful (72). Romanow (77) reported cyst formation by A. chroococcum in a liquid medium, however he offered no quantitative data and no resistance, lytic or cytological evidence.

Resistance properties

The resistance exhibited by members of the azotobacter group to various deleterious agents is well authenticated in the literature. Löhns and Smith (59) first noted the resistance properties of Azotobacter during their studies on various cell types produced by the genus. Even though much of his work has been disputed by other authors, the resistance properties of the genus are generally recognized.

Although most members of the genus are very sensitive to acid conditions below a pH of 6.0, some species fix nitrogen and grow well in an acidic medium (41). From Indian rice soil, Uppal, Patel, and Daji (101) isolated a variant of A. chroococcum that fixed nitrogen at pH 5.4. Tchan (97) described a strain of A. beijerinckii

that grew vigorously at a pH of 4.8 to 5.1. In 1955 Jensen (41) described a new species named A. macrocytogenes which grew and fixed nitrogen at a pH of 4.6 to 4.8. The reaction of mature cysts to an acidic environment has not been reported.

Some members of the genus have been reported to exhibit a marked thermal resistance (31). Bisset, Baird-Parker, Hale, Jeynes, and Lawrence (6) reported that 4 independently isolated gram-positive variants of A. chroococcum withstood 80 C for 15 min and 2 of the strains survived 100 C for 2-3 min. Garbosky and Giambiage (31) reported extensive heat resistance among several species of Azotobacter. Almost all of the species tested withstood 90 C for 15 min and some survived after a 7 min exposure at 100 C. They stated the heat resistance was not associated with the development of the encysted state but with the presence of a distinct type of internal structure called a "corpuscle". Socolofsky and Wyss (89) reported the cysts to be only slightly more resistant than vegetative cells to thermal destruction.

Socolofsky and Wyss (89) demonstrated that the encysted cell is somewhat more resistant than the vegetative cell to a variety of types of radiations. In order to effect 90% inactivation by ultraviolet light, the cysts required almost twice the dosage required by the vegetative cells. The cysts have also been shown to be 7 times more resistant to gamma rays than the non-encysted cells. Soil populations of Azotobacter have been shown to be much more resistant than laboratory

cultures of cysts to gamma radiation (104). No attempt was made to determine the physiological state of the organisms in the soil.

The cyst has also been shown to be resistant to sonication (89). Only a 4 min treatment of vegetative cells by ultrasonic sound is necessary to inactivate 90% of the population; whereas, 60 min is necessary to inactivate 90% of an encysted population.

The most pronounced difference in resistance properties between cysts and vegetative cells involves resistance to desiccation (89). Winogradsky (114) reported obtaining viable cysts in cultures which had been removed from the growth medium and stored for 2 years. Omeliansky (cited in 105) found that Azotobacter cells stored for 10 years on dried silica gel slants retained their viability. Quantitative experiments conducted by Socolofsky and Wyss (89) indicated that cysts retained almost 100% viability at the end of 12 days of mild desiccation, whereas vegetative cell viability dropped to less than 1% at the end of the first day of treatment. The desiccation resistance property of the cysts was employed by Stevenson and Socolofsky (92) in setting forth a criterion of encystment.

The mechanism involved in the resistance properties of the cyst apparently lies in the protection of the central body provided by the two coat components (72, 89). Acquisition of radiation resistance coincides with the thickening of the cyst coat during encystment, and the cyst becomes radiation sensitive as the coat disintegrates during germination (89). Parker and Socolofsky (72) found that the cyst coat could be removed from the central body with no apparent

injury to the organism. When this was done and the free central bodies produced were exposed to various deleterious agents, they were as vulnerable as the young vegetative cells.

Germination

Azotobacter cysts placed in an appropriate medium undergo a reversion to the vegetative state. This reversion is referred to as germination. Cyst germination was probably first referred to by Jones (43) in 1920. He noted that 14-day old Azotobacter "arthrospores" germinated when placed on fresh medium and the "cell plasma" emerged from the "spore" as a rod-shaped cell. Winogradsky (114) described germination as involving the enlargement of the central body until it breaks out of the surrounding capsule. It then begins to elongate into an oval shape, and finally, into the typical rod-shaped vegetative cell.

Wyss et al. (115) and Tchan et al. (98) utilized electron microscopy of ultra-thin sections to follow the cytology of germination. Wyss et al. (115) reported that the first noticeable change of the cyst during germination was an enlarging of the central body and a corresponding decrease in intine material. The peripheral bodies, characteristic of the vegetative cell, gradually became discernable. Even though the exine became much more fragile during germination, it maintained its shape after release of the rod-shaped vegetative cell.

Tchan et al. (98) indicated that the central body is displaced to an eccentric position within the cyst coat during the early stages

of germination. The central body then becomes slightly pointed and in some cases appears to have membraneous inclusions in the pointed region. They suggested that the inclusions serve as a site for the production of enzymes used for rupturing the cyst coat.

Kramer (46) and Lin (58) have investigated various physiological factors associated with germination. Kramer (46) reported that cyst germination was not inhibited by chloramphenicol and was therefore not dependent on protein synthesis. Lin (58) observed that not all of the carbon compounds which support vegetative growth of Azotobacter, such as mannitol, promote efficient cyst germination. Of the carbon compounds tested, glucose, fructose, and n-propanol were found to be the most active germination promoting agents. When used in conjunction with glucose, casein hydrolysate increased the rate of germination about 20-fold.

Several chemical changes associated with germination were also examined by Lin (58). When cysts were placed in a germination medium, glucose utilization and oxygen consumption began immediately, and calcium and magnesium ions were released into the medium. However, nitrogen fixation and RNA synthesis exhibited about a 4-hr lag period, while DNA and protein synthesis displayed an 8-hr lag. Cell multiplication began about 10 hr after the beginning of germination.

Poly-B-hydroxybutyric acid

Since the very early studies of bacteria, numerous investigators have been interested in the intracellular granules which were commonly observed by light microscopy. Frequently, the granules would not

stain with the common bacterial dyes but would stain with fat soluble dyes such as Sudan black. Because of this property, many of the reported granules were called "fat bodies".

In recent years the "fat bodies" of many bacteria have been shown to be composed of a common bacterial storage compound, poly-B-hydroxybutyric acid (90). Poly-B-hydroxybutyric acid (PHB) is composed of successive molecules of B-hydroxybutyric acid joined by ester linkages between the carboxy group of one molecule and the hydroxy group of the adjacent molecule.

Poly-B-hydroxybutyric acid in bacteria

In 1927, Lemoigne (53) extracted cells of Bacillus "M" with chloroform and recovered a substance having the empirical formula $(C_4H_6O_2)_n$, and additional work indicated that the material was a polymer of B-hydroxybutyric acid. Since this original extraction, the polymer has been found to occur in many bacteria of varying gram-reaction, morphology, and physiology (Table 1). Carr (11) has recently reported the occurrence of PHB in the blue-green alga, Chloroyloea fritschii.

Properties of poly-B-hydroxybutyric acid

Deposits of PHB in bacteria appear as discrete granules and in electron micrographs of ultra-thin sections, the granules appear as very electron-transparent areas each surrounded by a limiting membrane (8). PHB can be extracted by first digesting the cells with alkaline hypochlorite and dissolving the resulting extract in boiling chloroform (110). Evaporation of the chloroform extract

Table 1

Occurrence of poly-B-hydroxybutyric acid in bacteria

Organism	Reference
<u>Azotobacter agilis</u>	29
<u>Azotobacter chroococcum</u>	55, 60
<u>Azotobacter vinelandii</u>	29, 92
<u>Bacillus anthracis</u>	54
<u>Bacillus cereus</u>	54, 61, 106, 110
<u>Bacillus megaterium</u>	54, 60, 61, 62, 64, 85, 108
<u>Bacillus mycoides</u>	54
<u>Caryophanon latum</u>	75
<u>Chromatium okanii</u>	60, 80
<u>Chromobacterium</u> sp.	29
<u>Chromobacterium violaceum</u>	29
<u>Ferrobacillus ferrooxidans</u>	60
<u>Hydrogenomonas</u> sp.	33, 60, 81
<u>Lampropedia hyalina</u>	60
<u>Micrococcus denitrificans</u>	60
<u>Micrococcus halodenitrificans</u>	60, 82, 83
<u>Pseudomonas</u> sp.	13, 29, 67, 73
<u>Pseudomonas antimycetica</u>	29, 36
<u>Pseudomonas lemoignei</u>	19
<u>Pseudomonas methanica</u>	44
<u>Pseudomonas pseudomallei</u>	57
<u>Pseudomonas saccharophila</u>	20, 60
<u>Pseudomonas solanacearum</u>	36
<u>Rhizobium</u> sp.	29, 60
<u>Rhizobium japonicum</u>	36
<u>Rhizobium trifolii</u>	105
<u>Rhodopseudomonas spheroides</u>	12
<u>Rhodospirillum rubrum</u>	8, 20, 60, 64, 91
<u>Sphaerotilus natans</u>	71, 78
<u>Spirillum anulus</u>	63
<u>Spirillum itersonii</u>	60, 63
<u>Spirillum normaal</u>	60
<u>Spirillum serpens</u>	36, 60, 63
<u>Vibrio</u> sp.	35, 36

yields a thin film of white to grayish material somewhat reminiscent of thin plastic sheeting (110). The extracted polymer is soluble in a variety of solvents including chloroform, glacial acetic acid, pyridine, octyl alcohol, aqueous phenol, 1 M sodium hydroxide, and triolein. It is insoluble in alkaline hypochlorite, water, ether, acetone, ethanol, and carbon tetrachloride. On strong heating, the polymer melts, chars, and gives off odorous white fumes. Upon condensation of the fumes on the cooler parts of the tube, needle-shaped crystals of crotonic acid are formed (110).

The melting point and molecular weight of the extracted polymer vary considerably depending on the method of extraction. Melting points ranging from 114 C to 188 C and molecular weights ranging from 1,000 to 256,000 have been reported (60).

The polymer from various bacteria has been found to be crystalline in vivo (1) and to exhibit a consistent morphological form when viewed in the electron microscope after precipitation from a chloroform solution (1, 60). X-ray diffraction studies made on extracted polymer indicate that it is in a helical conformation in the solid state (1, 60).

Poly-β-hydroxybutyric acid metabolism

The enzymatic synthesis and degradation of PHB has been studied by a number of investigators during the last several years. Merrick and Doudoroff (64) described polymer synthesis in cell-free extracts derived from Bacillus megaterium and Rhodospirillum rubrum. PHB synthetase activity from both organisms was associated with a

particulate fraction consisting mainly of "native" polymer granules. The coenzyme A derivative of B-hydroxybutyric acid was readily incorporated into the polymer, however no uptake of the free acid was demonstrated. Very little acid activation was detected when the synthetase fraction was supplemented with the free acid, coenzyme A, and ATP.

Two different types of PHB depolymerase systems have been studied (13, 19, 65). Merrick and Doudoroff (65) described a complex intracellular depolymerase system, consisting of three separate factors, derived from R. rubrum. One factor was a high molecular, thermostable "activator" protein. The "activator" alone did not carry out hydrolysis, however it was necessary for depolymerase activity. In some cases, trypsin could be substituted for the "activator". The depolymerase enzyme was an extremely unstable, thermolabile, high molecular weight protein. A third component of the depolymerase system was a labile factor associated with the PHB granules used as substrate. These granules were prepared from Bacillus megaterium and great care had to be taken during preparation to preserve the granules in their "native" state. Exposure of the granules to various chemical and enzymatic treatments, freezing, heating, or repeated centrifugation resulted in a loss of the substrate associated factor. Merrick, Lundgren, and Pfister (66) have subsequently demonstrated that any treatment of the "native" granules which disrupts their membrane covering results in the loss of the substrate associated

factor. The principal product of the depolymerase system was observed to be D(-)B-hydroxybutyric acid, however a small amount of esterified products was released. The ester dimers were hydrolyzed by a specific esterase.

During studies of organisms which could utilize PHB as a sole carbon source, Chowdhury (13) and Delafield, Doudoroff, Palleroni, Lusty, and Contopoulos (19) detected and described extracellular PHB depolymerase systems. Chowdhury (13) studied a depolymerase produced by an unidentified Pseudomonas species. In marked contrast to the intracellular system of Merrick and Doudoroff (64), the extracellular depolymerase from Pseudomonas consisted of only one component and the PHB utilized as the carbon source could be severely treated (isolated by hypochlorite digestion) and still serve as a utilizable substrate. The depolymerase was inducible only with PHB; however, it had no pronounced substrate specificity. It hydrolyzed PHB, B-hydroxybutyric acid propyl ester, ethyl acetate, p-nitrophenyl acetate, and other ester compounds.

From a number of aerobic pseudomonads capable of utilizing PHB as a sole carbon source, Delafield et al. (19) selected P. lemoignei for study of extracellular depolymerization. P. lemoignei extracellular depolymerase could also utilize as substrate PHB which had been extensively denatured by chemical purification. The extract purified from the organism by Delafield et al. (19) apparently consisted of a mixture of two very similar enzymes. Both enzymes were shown to be basic proteins which digested the polymer at slightly

different rates. The enzymes were reported to be constitutive and were produced during cell growth. B-hydroxybutyric acid suppressed enzyme excretion.

The principal end product of extracellular PHB depolymerization was the dimeric ester of B-hydroxybutyrate, but some monomer was also produced (19). The dimer was rapidly absorbed by the cells where it was hydrolyzed by a specific dimer hydrolase (18, 19).

Physiological role of poly-B-hydroxybutyric acid

Although PHB was originally discovered in 1927 (53), a physiological role for it was not convincingly demonstrated until about 30 years later. Today, the polymer is generally considered to be one of the primary storage compounds in bacteria (17).

The importance of the polymer was first indicated in 1935 by Gaffron (cited in 20) during his studies of the photosynthetic assimilation of organic compounds by the purple bacteria. He succeeded in isolating an assimilatory product with the empirical formula $(C_4H_6O_2)_n$, however the significance of the finding was not immediately recognized.

In 1950, Lemoigne, Grelet, and Croson (56) drew attention to the different amounts of PHB obtained by growing Bacillus megaterium on various media. Macrae and Wilkinson (61, 62) suggested a storage function for the polymer in 1958. They noted that as the ratio of carbon source to the nitrogen source in the growth medium of B. megaterium increased, and as the medium became nitrogen-deficient

instead of carbon-deficient, there was an increase in the amount of PHB per cell.

In 1959 Doudoroff and Stanier (20) reported that 60 to 90% of the carbon assimilated by Pseudomonas saccharophila and Rhodospirillum rubrum initially was deposited in the PHB fraction of the cells. Stanier, Doudoroff, Kunisawa, and Contopoulos (91) subsequently reported that PHB was the major product formed during photosynthetic assimilation of carbon compounds which were convertible to C₂ units by R. rubrum. PHB has also been shown to be the primary assimilatory product when the autotrophic, hydrogen-oxidizing bacterium, Hydrogenomonas, is grown in an atmosphere of CO₂, H₂, and O₂ (33, 81). These observations indicate that PHB has a general physiological function in bacteria as the primary product of carbon assimilation; its role in bacterial metabolism being analogous to that of starch and glycogen in the metabolism of other organisms (91).

The endogenous utilization of PHB by bacteria and the importance of the polymer in maintaining cell viability during starvation is well recognized (17). Macrae and Wilkinson (61) noted that PHB was degraded during starvation of Bacillus megaterium and that the rate of autolysis was twice as high for those cells low in PHB content as compared to cells rich in the polymer. Sierra and Gibbons (82, 83) made a careful study of the endogenous respiration and survival characteristics of Micrococcus halodenitrificans. They reported that the amount of polymer in resting cells decreased with time, and that PHB was the only major cellular component utilized during

endogenous respiration. Cell viability could be correlated with polymer content. PHB-rich cells maintained their viability for longer periods than did PHB-poor cells. They further noted that as the PHB-rich cells utilized their polymer endogenously, viability was reduced. Sobek, Charba, and Foust (86) obtained almost identical results during studies with Azotobacter.

Several authors have suggested a relationship between PHB accumulation and sporulation by Bacillus. Tinelli (99, 100) found that PHB was oxidized to CO₂ and H₂O during the endotrophic sporulation of B. megaterium. She also showed that non-sporulating strains were unable to oxidize the polymer fully, and she concluded that there was an intimate connection between sporulation and polymer metabolism. Further evidence for the interrelation between these two factors was presented by Slepecky and Law (85). These investigators noted that polymer utilization immediately preceded sporulation in B. megaterium.

Structural role of metal ions in bacteria

The practice of incorporating mineral salts into bacterial growth media is ubiquitous and the importance of divalent metal ions as co-factors in enzymatic reactions is well known. Evidence is accumulating which indicates that these ions also have an important role in the structure of bacterial cell walls. Keeler and Gray (45) found that up to 70% of the radioactive Ca⁺⁺ added to the growth medium was incorporated into the cell wall fraction of Listeria monocytogenes. Primosigh, Pelzer, Maass, and Weidel (74) made the interesting

assertion that the polypeptide side chains of the "R" layer (79) contain diaminopimelic acid and would carry an excess negative charge which must be neutralized before they could be packed into a rigid structure; Humphrey and Vincent (38) suggested that Ca^{++} specifically performs this function in Rhizobium trifolii. Eagon and Carson (22) and Eagon, Simmons, and Carson (23) reported that Ca^{++} , Mg^{++} , and Zn^{++} were components of the cell wall of Pseudomonas aeruginosa. Leive (52) and Asbell and Eagon (2) have reported that these ions were specifically involved in bonding with the lipopolysaccharide component of the cell wall.

The presence of divalent metal ions, particularly Ca^{++} , in bacterial spores is well known (96). Calcium ion deficiency leads to reduced spore formation and to a lowered heat resistance of those spores which are formed (34, 95). A high calcium ion content, however, does not appear to be uniquely associated with spore cytology, refractility, or resistance to desiccation, phenol, or ultraviolet irradiation (84). Spores accumulate metal ions other than Ca^{++} when they are added to the culture medium, however these ions cannot completely substitute for Ca^{++} in respect to the thermal resistance and germination properties of the spores (28).

The importance of metal ions in maintaining the structure of the cyst coat was first referred to by Socolofsky and Wyss (88). They noted that the coat ruptured when it was treated with EDTA and proposed that divalent metal ions were involved in coordination of the exine coat materials. Goldschmidt and Wyss (32) have implicated magnesium ions in this role.

MATERIALS AND METHODS

Organism and culture media

Azotobacter vinelandii (A. agilis), ATCC 12837, was the test organism in these studies. The cells were grown in 50 ml of a modified-Burk's nitrogen-free salts solution (112) in 250 ml Erlenmeyer flasks at 33 C with shaking. The salts solution was prepared according to the formulation in Table 2.

Table 2

Composition of a modified-Burk's nitrogen-free salts solution

Component	Grams per liter of distilled water
KH_2PO_4	0.2
K_2HPO_4	0.8
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.085
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.005
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.0003

Because of its greater solubility, CaCl_2 was substituted for CaSO_4 which is normally employed. Unless otherwise indicated, 1% glucose was utilized as the carbon source. Aqueous solutions of CaCl_2 and glucose were autoclaved separately and the FeSO_4 solution was sterilized by filtration (Millipore, Grade HA). All of these solutions were added to the sterile medium just prior to inoculation.

When CaCO_3 was added to the growth medium, measured amounts were sterilized in a hot air oven and added to the basal salts solution before inoculation. The cultures were supplemented with additional divalent metal ions in certain instances by the addition of 1 ml of the appropriate dilution of the ion to the culture at the beginning of the stationary phase. Cysts grown on a solid surface, needed for comparison purposes, were cultivated on the surface of an agar medium prepared by supplementing the basal salts solution with 0.2% n-butanol and 2% agar.

Optical density measurement

The optical density (OD) of the culture was measured at 600 $m\mu$ with a Bausch and Lomb Spectronic 20 colorimeter-spectrophotometer.

Viable cell counts

Viable cell counts were made by the smear plate technique. Dilution blanks contained 0.85% NaCl. Plates used for enumeration of the organisms were prepared by supplementing the growth medium with 2% agar. Triplicate samples were made of each dilution and incubated at 33 C for 2 days before examination.

Determination of encystment

The extent of encystment was determined by the desiccation technique of Socolofsky and Wyss (89). A cyst was considered to be that form of the organism which could survive exposure to predetermined desiccation conditions for a period of 4 days. To assay desiccation resistance, cell suspensions were impinged on the surface of membrane filters with a pore size of 0.45 microns (Millipore, HA).

The membranes were transferred to dry absorbent pads in petri dishes and placed in an incubator at 33 C. At various intervals the cells were washed from the membranes by vigorous agitation in 100 ml of 0.85% NaCl, and the number of viable cells determined. Experiments indicated that the cells could be removed quantitatively by the washing action.

Measurement of pH and pH control

The pH of the medium was measured directly in the culture flasks with a Corning combination electrode in conjunction with a Beckman Zeromatic pH meter. Control of pH in the growth vessel was effected by adding 0.1 M KOH to the culture at necessary intervals.

Viscosity measurement

For measurement of the culture fluid viscosity, supernatants were clarified by centrifugation at 70,000 x g for 2 hr with a Spinco Model L ultracentrifuge. The viscosity of the resulting supernatant material was measured with a Cannon-Fenske number 200 Viscometer in a 33 C waterbath. These measurements were recorded as efflux times. The efflux time of distilled water under these conditions was 10 sec.

Lytic experiments

The susceptibility of the cells to the lytic activities of ethylene diaminetetraacetic acid (EDTA) and lysozyme was determined according to the method of Socolofsky and Wyss (88). Two ml samples of a cell suspension at an optical density of 0.7 were treated with a 1 ml quantity of the appropriate agents. Changes in OD were

determined at 600 $m\mu$ in a Bausch and Lomb Spectronic 20 colorimeter-spectrophotometer. The total system included cells, 100 μ moles tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0, 100 μ g crystalline lysozyme, and 400 μ g of trisodium EDTA, pH 8.0 in a total volume of 3 ml.

Glucose determination

The changes in the glucose concentration in the medium were determined utilizing the "Glucostat" reagent (Worthington Biochemical Corporation), a coupled enzyme system composed of glucose oxidase and peroxidase. The reagent was prepared by dissolving the contents of the "Chromogen" and "Glucostat" vials in 90 ml distilled water. The test was performed utilizing a series of tubes containing the unknown samples and a glucose standard. To 9.0 ml of reagent, 1 ml of the sample (0.05 to 0.3 mg of glucose) was added and the mixture was allowed to react 5 min at room temperature. At the end of this period, 1 drop of 4 M HCl was used to stop the reaction. The absorbancy was determined with a Klett-Summerson colorimeter equipped with a number 42 filter.

Determination of cell nitrogen

Nitrogen was measured by the Kjeldahl method as recommended by Hiller, Plazin, and Van Slyke (37). Five ml of culture were placed in a 30 ml micro-Kjeldahl digestion-distillation flask along with 0.5 ml of a $HgSO_4$ solution, of 0.5 g K_2SO_4 , 1 ml conc. H_2SO_4 , and several Norton 14X boiling chips. The $HgSO_4$ solution was prepared by dissolving 10 g of red mercuric oxide in 100 ml of 12% H_2SO_4 .

The water was evaporated and the suspension digested for 30 min after clearing. The suspension was cooled, water added, and the flask placed on a steam distillation apparatus. Zinc powder and 10 ml of 10 M NaOH were added to liberate the ammonia. The mixture was distilled into 1% boric acid solution and back-titrated to the original pH using standard HCl.

Poly-B-hydroxybutyric acid extraction and quantitation

PHB was extracted from the cells and measured by the method of Law and Slepecky (48). After the desired period of growth, 5 ml of the culture were removed from the flask and placed in 50 ml polypropylene test tubes. A quantity of 5 ml of commercial sodium hypochlorite (Clorox) was added to the tubes and the mixture digested for 24 hr at 35 C.

Following digestion, the tubes were centrifuged, the supernatant fluid discarded, and the precipitate washed sequentially in 5 ml each of water, 95% alcohol, and acetone. Following the acetone washing, the resulting precipitate was allowed to dry overnight. The PHB was then extracted from the residue with boiling chloroform, and the chloroform filtered through a coarse sintered glass filter. After each filtration the filter was washed with a small portion of hot chloroform to remove any residual polymer. The chloroform was dispensed into assay tubes and allowed to evaporate overnight. After evaporation, 10 ml of reagent grade conc. H_2SO_4 were added to each assay tube. The tubes were capped with aluminum foil and heated at 100 C for 10 min. The heating of the polymer in conc. H_2SO_4

has been shown to convert the polymer to crotonic acid quantitatively (48). After cooling, the absorbancy of the acid solution was determined at 235 $m\mu$ using silica cells of 1 cm light path in a Beckman model DB spectrophotometer.

Electron microscopy

For the preparation of ultra-thin sections, 30 ml of culture were centrifuged and the resulting cell pellet was mixed with 1 ml of 2% warm agar. The suspension was spread on the surface of glass slides and allowed to solidify. After hardening, the agar was cut into small blocks and placed in screw cap tubes and fixed with 2% unbuffered $KMnO_4$ for 1 hr. The diced agar blocks were washed twice with distilled water and dehydrated by passage through a graded alcohol series. Alcohol was removed by washing twice with propylene oxide. The blocks were suspended in a 1:1 propylene oxide and Maraglas epoxy resin mixture (30) for 30 min. The blocks were removed from the 1:1 mixture and placed in a Maraglas plastic mixture for 1 hr at room temperature and then at 4 C overnight. The agar blocks were then removed from the plastic solution and transferred to "BEEM" capsules (Better Equipment for Electron Microscopy, Inc., P. O. Box 132, Jerome Av. Station, Bronx, N. Y. 10468). Fresh Maraglas plastic mixture was added and the capsules were held in a vacuum oven at 60 C for 72 hr to remove any bubbles and to polymerize the embedding material. The blocks were sectioned with a DuPont diamond knife using an LKB Ultratome. The sections were placed on unsupported 400 mesh copper grids and post-stained with either 2% unbuffered $KMnO_4$ or lead hydroxide.

Carbon replicas were prepared according to the method of Bradley and Williams (9). The cells were harvested from the culture by centrifugation and a turbid suspension was prepared by resuspending the cell pellet in distilled water. One drop of the suspension was placed on the surface of a parlodion coated copper grid (300 or 400 mesh) and blotted dry. This process was repeated several times to insure an adequate sample on each of the grids. The carbon replicas were prepared by placing the grids in a Kinney high vacuum evaporator and evaporating the tip of one carbon rod placed directly over the grids at a distance of approximately 14 cm.

After coating the specimen with carbon, the grids were removed from the instrument and the parlodion film on each grid dissolved with a few drops of amyl acetate. The cell material was then removed from the grids by treatment with an acid mixture formulated by dissolving 1.5 g KMnO_4 and 1.5 g $\text{K}_2\text{Cr}_2\text{O}_7$ in 15 ml conc. H_2SO_4 . A few drops of the mixture were placed on the inner surface of a small crucible cover, and the grids were placed on the surface of the acid with the carbon film up. The acid solution dissolved the cell material; however, it did not harm the copper grids or the carbon film. After all the cells had been dissolved, the grids sank beneath the surface of the acid solution and were removed with a pair of forceps. The excess fluid was blotted off and the grids were then dipped into distilled water to wash off any remaining acid. The MnO_2 formed by the decomposition of the acid solution was removed by dipping

the grids in conc. HCl. After the HCl treatment, the grids were rewashed with distilled water and allowed to air dry.

To enhance the contrast of the replicas, the grids were shadowed with 25-30 mg of germanium metal at an angle of 18 degrees. All preparations were viewed with an RCA-EMU-3G electron microscope.

Radioisotope experiments

Radioactivity was measured with a Beckman liquid scintillation system. The scintillation fluid employed consisted of 6 g 2,5-diphenyloxazole (PPO) and 0.25 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per liter of dioxane or toluene. Each sample vial contained 15 ml of the solution. Each growth flask was supplemented with about 0.5 μ c of sodium acetate-2-C¹⁴ (Volk Radiochemical Co., 803 North Lake Street, Burbank, Cal.). Whole cells were sampled by collecting, washing, and drying the cells from 0.1 ml of culture on Millipore (Grade HA) membrane filters. The extracellular polysaccharide-like material was sampled by precipitating it from 0.1 ml of culture supernatant with 0.1 ml of 1 M CaCl₂. The material was washed and dried before the addition of scintillation fluid. The presumptive intine material was prepared by lysing the cysts with EDTA (88), followed by centrifugation and precipitation of the released material with 1 M CaCl₂. PHB was extracted by the method of Law and Slepecky (48) and the activity was measured by incorporating 0.1 ml of the chloroform extract into the scintillation fluid prepared with toluene. Carbon dioxide activity was measured by the method of Jeffrey and Alvarez (39).

RESULTS

This investigation is divided into three major phases. The first phase concerns the development of a liquid medium which supports the encystment of the organism and the characterization of the cells produced in liquid culture. The second deals with an examination of the role of divalent metal ions in cyst coat development, and the third phase involves a study of the relationship between PHB metabolism and encystment.

The encystment medium

When Azotobacter vinelandii was cultured in a liquid medium supplemented with 1% glucose, the pH of the culture decreased to below 5 (14) and the majority of the cells in the culture lost viability (Fig. 1). The death of the cell population was not evident from the observation of the OD of the culture. In order to study encystment in this type of medium, care had to be taken to maintain a neutral or slightly alkaline pH. Three methods were attempted to accomplish this. One involved raising the phosphate concentration to increase the buffering capacity of the Burk's salts solution. At the high phosphate concentrations, however, the extent of growth was reduced and the cells developed abnormal shapes including giant cells, chains, and filaments. A second method used was control of pH by periodic adjustment. The pH of the medium was carefully monitored and sufficient 0.1 M KOH was added at appropriate intervals to maintain it at 6.8 to 7.5 (Fig. 2). The cells cultivated in this manner maintained

Fig. 1. The relationships observed between viable cell count, optical density, and pH of the culture when cells of Azotobacter vinelandii 12837 were grown in a liquid medium supplemented with 1% glucose.

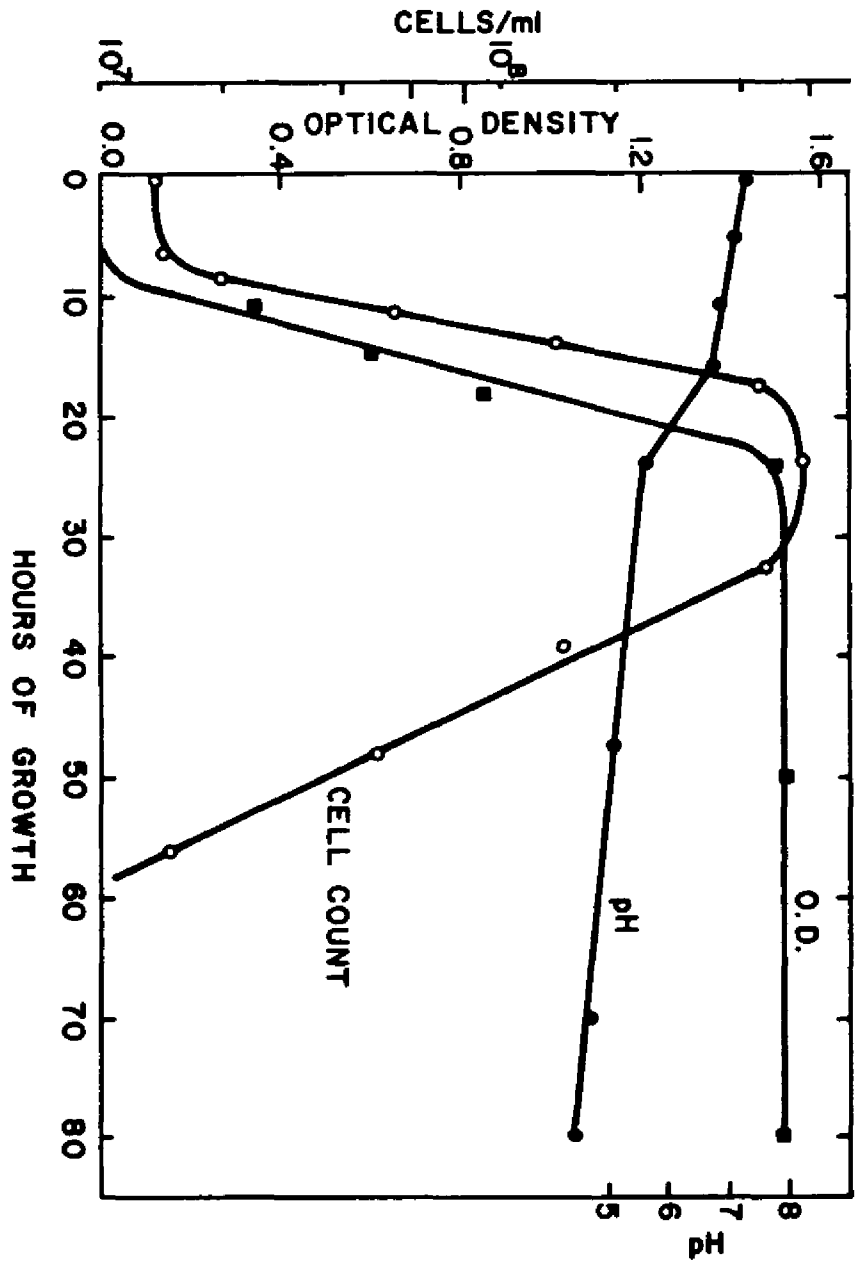
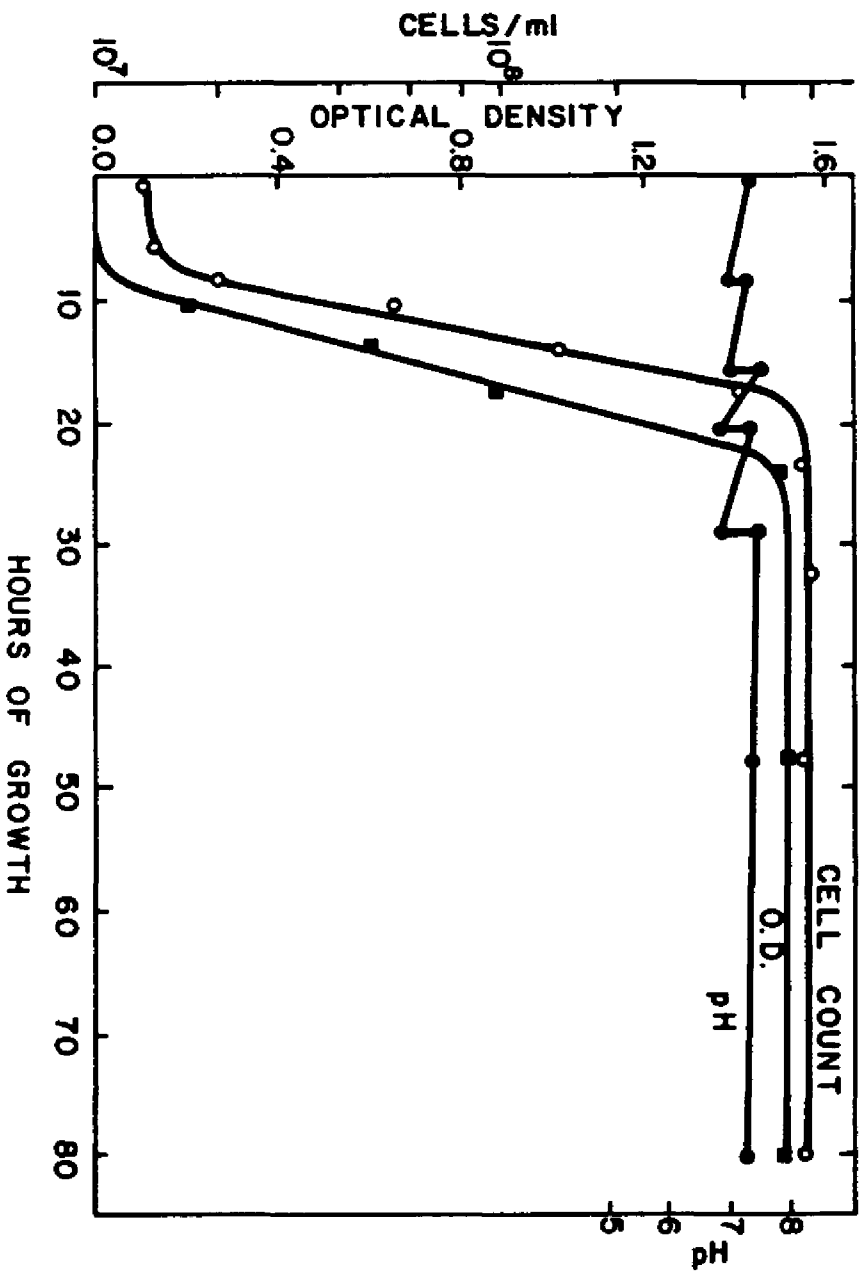


Fig. 2. The relationships observed between viable cell count, optical density, and pH of the culture when cells of Azotobacter vinelandii 12837 were grown in a liquid medium supplemented with 1% glucose. The pH was adjusted periodically by the addition of 0.1 M KOH. The abrupt increases in the pH of the culture fluid noted in the graph correspond to the periods of KOH addition.



their viability; however, they did not appear to encyst and the medium became very viscous during the stationary phase (14). A third method involved the addition of 0.6% solid CaCO_3 to the basal medium (114). When this was done, the cells maintained their viability and the culture fluid remained at a slightly alkaline pH (Fig. 3). Phase contrast microscopy indicated that the cells grown in the medium supplemented with CaCO_3 encysted. Since the cells remained viable throughout the experimental period when the pH was controlled (Fig. 2 and 3), the OD of the culture was considered to be an adequate measure of growth and was used for that purpose during the remainder of the investigation.

Characterization of the cells grown in liquid culture

The cells grown in liquid culture were compared to typical cysts produced on a solid medium in respect to their cytological, lytic, and resistance properties.

The cytological examination was done by electron microscopy of ultra-thin sections and carbon replicas. Figure 4 is a composite photograph showing 4 electron micrographs of ultra-thin sections of 4-day old cells grown under conditions of pH adjustment with KOH. The cells have shortened, rounded up, and resemble the central body of the cyst; however, no organized coat was observed. Some loose material, presumably unorganized exine coat components, is seen in the surrounding medium. Figures 4A and 4B were post-stained with lead hydroxide and Fig. 4C was post-stained with potassium permanganate. Figure 4D, post-stained with lead hydroxide, clearly illustrates that the exine fragments are triple layered. The

Fig. 3. The relationships observed between viable cell count, optical density, and pH of the culture when cells of Azotobacter vinelandii 12837 were grown in a liquid medium supplemented with 1% glucose and 0.6% CaCO₃.

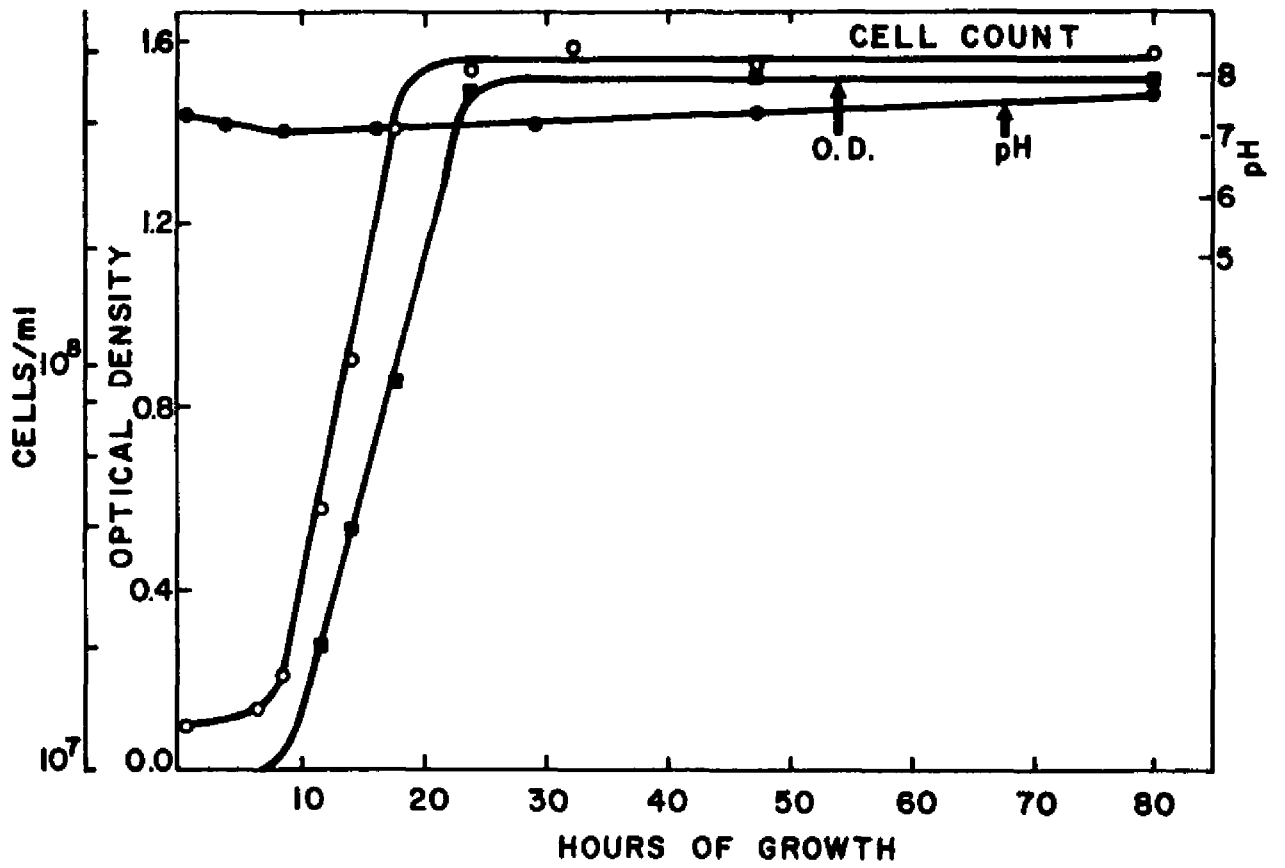


Fig. 4. Electron micrographs of ultra-thin sections of 4-day old Azotobacter vinelandii 12837 cells grown in a liquid culture in which the pH was adjusted periodically by the addition of 0.1 M KOH. A cell resembling a central body (cb) and free exine (ex) fragments are evident. (A) The cells were post-stained with lead hydroxide. (B) The cells were post-stained with lead hydroxide, (ex) exine (cb) central body. (C) The cells were post-stained with KMnO_4 . (D) The cells were post-stained with lead hydroxide. A small exine (ex) fragment is indicated by the arrow. Unless otherwise indicated, the line on each of the micrographs in this and the remaining figures represents 1 micron.



A



B



Cx



0.5μ



D

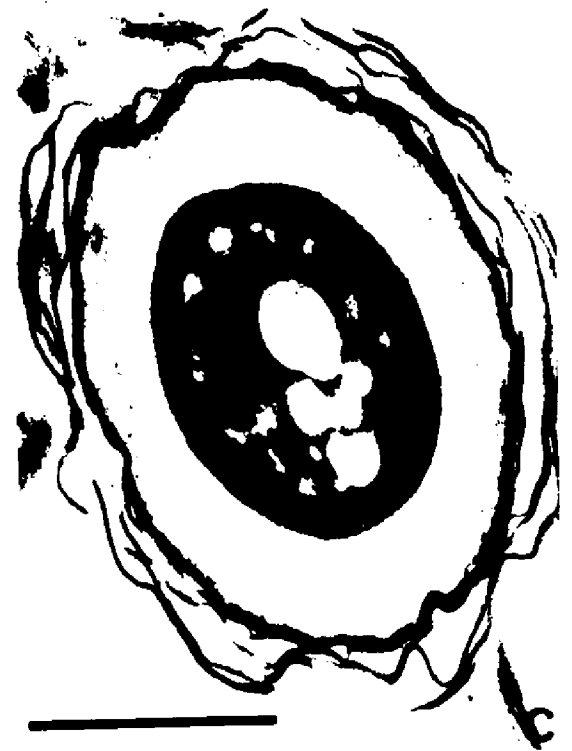
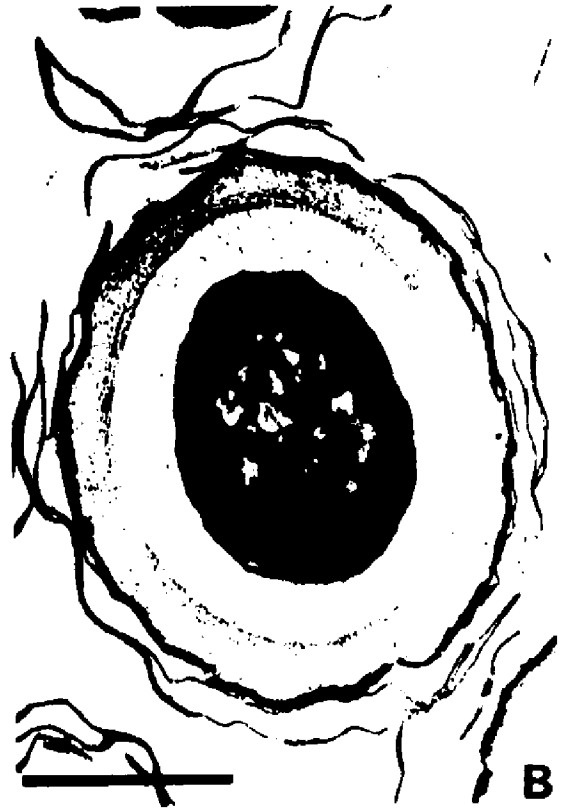
individual units are composed of two electron dense outer layers and a less electron dense inner layer. A very small, triple-layered exine fragment can be seen next to a cell which resembles a central body (arrow).

An electron microscopic examination of ultra-thin sections of 4-day old cells grown in liquid culture with the addition of CaCO_3 revealed that they exhibit the typical cyst structures, exine, intine, and central body (Fig. 5). The exine layer of the cyst does not appear as compact as that observed in solid-grown cysts (88, 98, 115). Figure 5 is a composite of 4 representative micrographs showing the varying amounts of PHB granules commonly observed in mature cysts. A cyst with extensive intracellular deposits of PHB, seen as the light, electron-transparent areas within the central body, is shown in Fig. 5A. Figure 5B shows a cyst with no PHB granules. Figures 5C and 5D are electron micrographs of cysts with intermediate amounts of PHB granules. Each of the micrographs shows the two-layered appearance of the intine observed by Wyss et al. (115).

The cells produced in liquid culture were also examined by the carbon replica technique. Since no information was available concerning the surface appearance of Azotobacter vegetative cells or cysts grown on solid substrates, replicas were prepared from these types of cells in order to form a basis for the evaluation of the replicas of cells grown in liquid culture.

The surface configuration of vegetative cells is seen in Fig. 6 which consists of 2 micrographs at slightly different magnifications.

Fig. 5. Electron micrographs of ultra-thin sections of Azotobacter vinelandii 12837 cells grown in a liquid culture supplemented with 0.6% CaCO₃. (A) Mature cysts which exhibit extensive intracellular deposits of PHB. (B) Mature cyst which exhibits no intracellular deposits of PHB. (C) Mature cyst which exhibits an intermediate amount of PHB deposits. (D) Mature cyst which exhibits an intermediate amount of PHB deposits. The typical cyst structures of exine (ex), intine (in), and central body (cb) are labeled.



D

B

C

Fig. 6. Electron micrographs of carbon replicas of actively growing Azotobacter vinelandii 12837 cells.
(A) 15,500 x. (B) 14,000 x.



The cells are peanut-shaped, which is typical of actively growing Azotobacter, and measure about 3.5 μ by 1.5 μ . Their surface is smooth with no indication of extracellular material.

Figure 7 is an electron micrograph of a carbon replica showing the surface appearance of typical cysts grown on a solid medium. In marked contrast to the vegetative cells, the surface of the cyst is very irregular and appears to be composed of several layers of material. This is in agreement with the exine coat observed with KMnO_4 fixed cysts when seen in thin sections (115).

A carbon replica of a cell taken from a 4-day old liquid culture in which the pH was adjusted by the periodic addition of 0.1 M KOH is seen in Fig. 8A. The cell is small and smooth with no indication of extracellular coat material. The surface is similar to that of young vegetative cells.

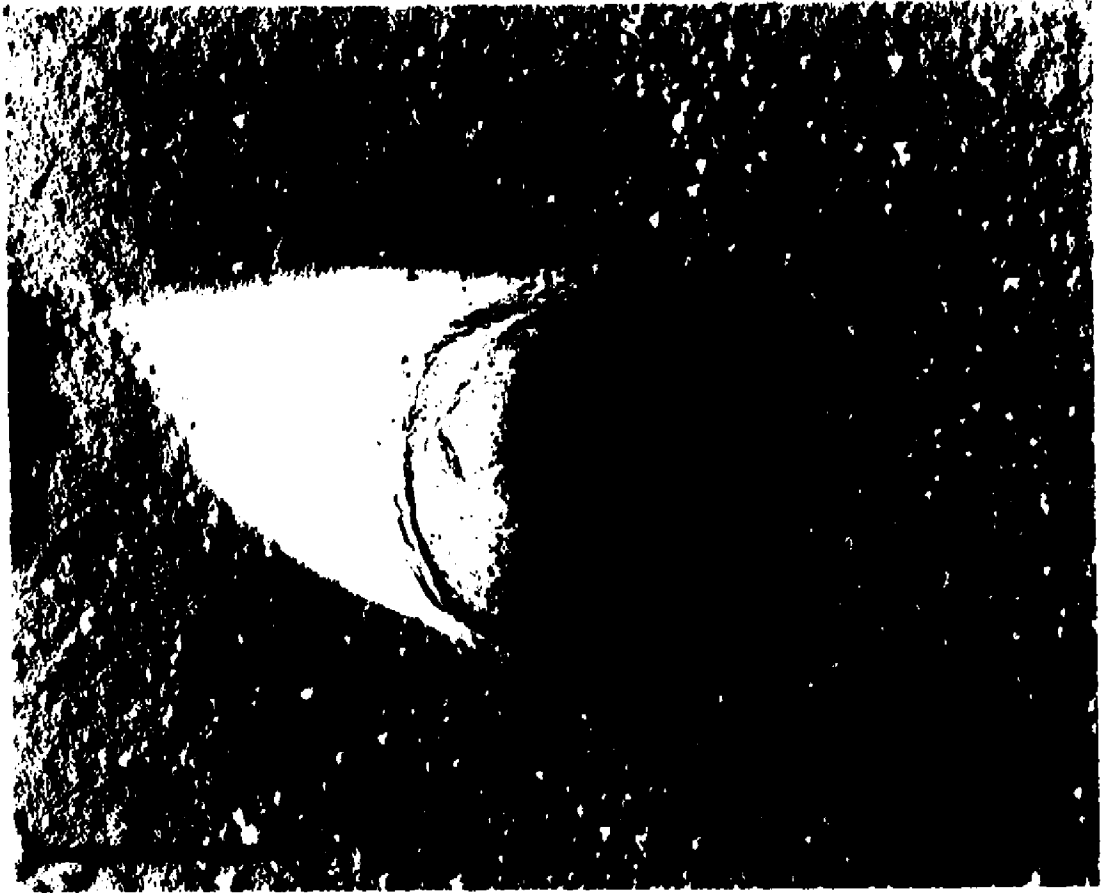
The resemblance of the KOH grown cells and the central body of the mature cyst which was noted in Fig. 4 is also demonstrated in Fig. 8B. This electron micrograph was made from a carbon replica of an EDTA-ruptured Azotobacter cyst. The collapsed exine coat and the ejected central body are apparent. The central body is similar to the cell seen in Fig. 8A.

Figure 9 is composed of 2 electron micrographs showing replicas of 4-day old cells grown in liquid culture supplemented with CaCO_3 . They appear to be identical to the cysts grown on a solid substrate, showing an irregular, layered surface. A thin covering which is

Fig. 7. Electron micrograph of a carbon replica of Azotobacter vinelandii 12837 cysts grown on Burk's nitrogen-free agar utilizing 0.2% n-butanol as carbon source. Flagellar remnants (or debris) are apparent.



Fig. 8. Electron micrographs of carbon replicas of Azotobacter vinelandii 12837 cells grown under various environmental conditions. (A) Non-encysted cell which was taken from a culture in which the pH was controlled by the periodic addition of 0.1 M KOH. (B) An EDTA-ruptured cyst which exhibits a collapsed exine coat (ex) and an ejected central body (cb).



B

Fig. 9. Electron micrographs of carbon replicas of 4-day old cells of Azotobacter vinelandii 12837 grown in a liquid culture supplemented with 0.6% CaCO₃. A thin covering which is sometimes noted around the exterior of the cyst coat in carbon replicas is indicated by the arrow in B.



B

sometimes noted around the exterior of the cyst coat in carbon replicas is clearly seen in Fig. 9B (arrow).

The reaction of cells grown under different cultural conditions to the lytic activities of EDTA and lysozyme is shown in Fig. 10. The first graph illustrates the results obtained with cysts produced on a solid medium. The drop in OD with the addition of EDTA alone corresponds to rupture of the cyst coat. The increased drop in OD with both EDTA and lysozyme results from lysis of the central body by lysozyme after coat rupture by EDTA. The cells grown in a liquid medium supplemented with CaCO_3 exhibited the same pattern. The cells grown with external adjustment of pH exhibited different characteristics. Very little change in OD was noted with EDTA exposure alone, indicating little cyst coat development. The drop in OD with EDTA and lysozyme treatment is apparently due to lysis of the cells.

The pattern of desiccation resistance of 4-day old cells grown under the different cultural conditions is shown in Fig. 11. The cysts cultivated on butanol-agar plates and the cells grown in a liquid medium supplemented with CaCO_3 show almost complete resistance to the treatment. The culture in which the pH was controlled by the periodic addition of KOH produced less than 10% cysts as determined by desiccation resistance. Examination of the sonication resistance of the 3 types of cells showed similar results. The cysts produced on a solid surface and the cells grown in liquid medium supplemented with CaCO_3 were resistant to sonication, whereas, the cells grown with KOH control of pH were no more resistant than young vegetative cells.

Fig. 10. Lysis of cells of Azotobacter vinelandii 12837 grown under different cultural conditions. The degree of lysis was measured as a reduction in the optical density at 600 m μ and plotted against the minutes of exposure to the lytic agents.

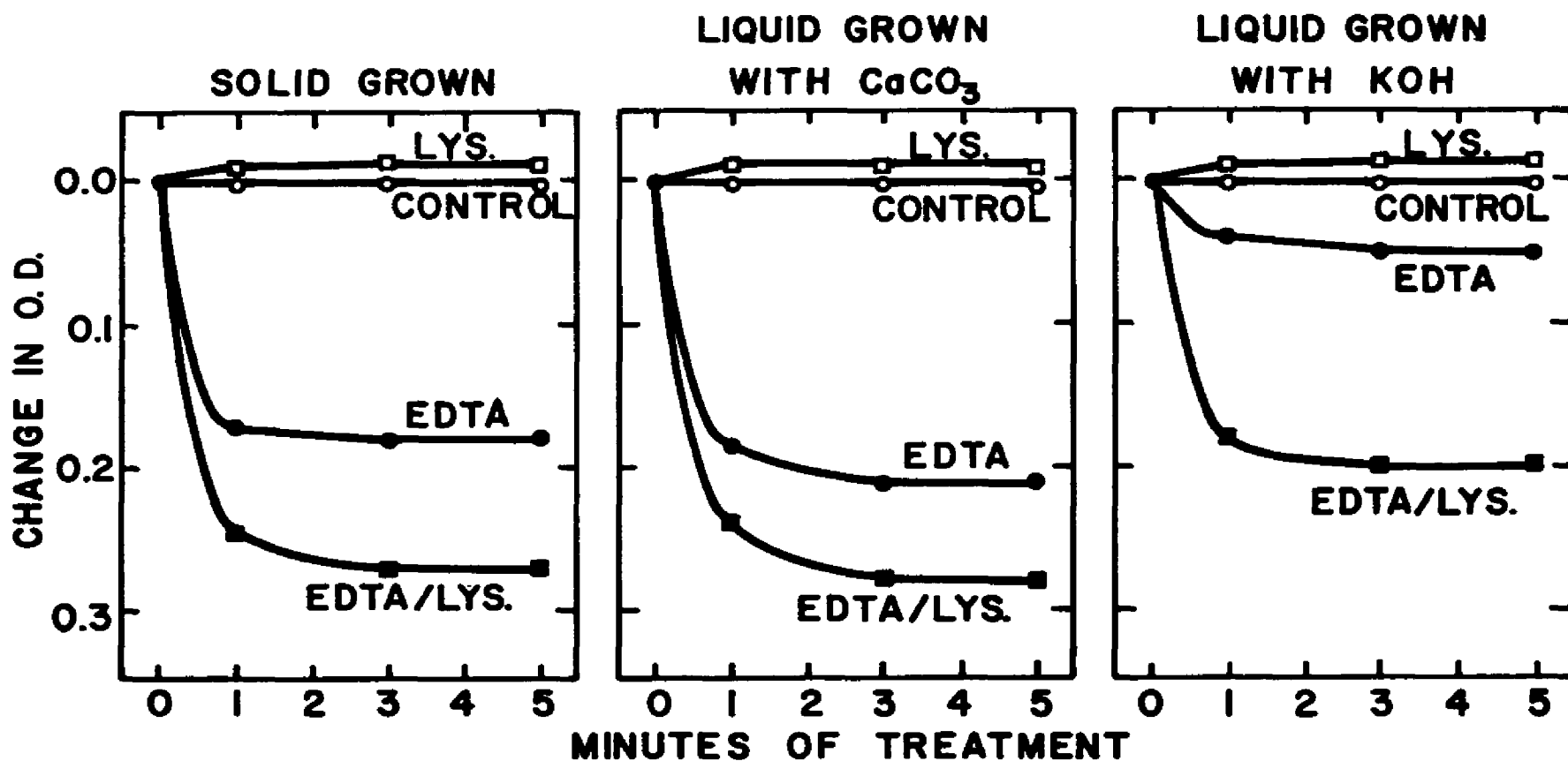
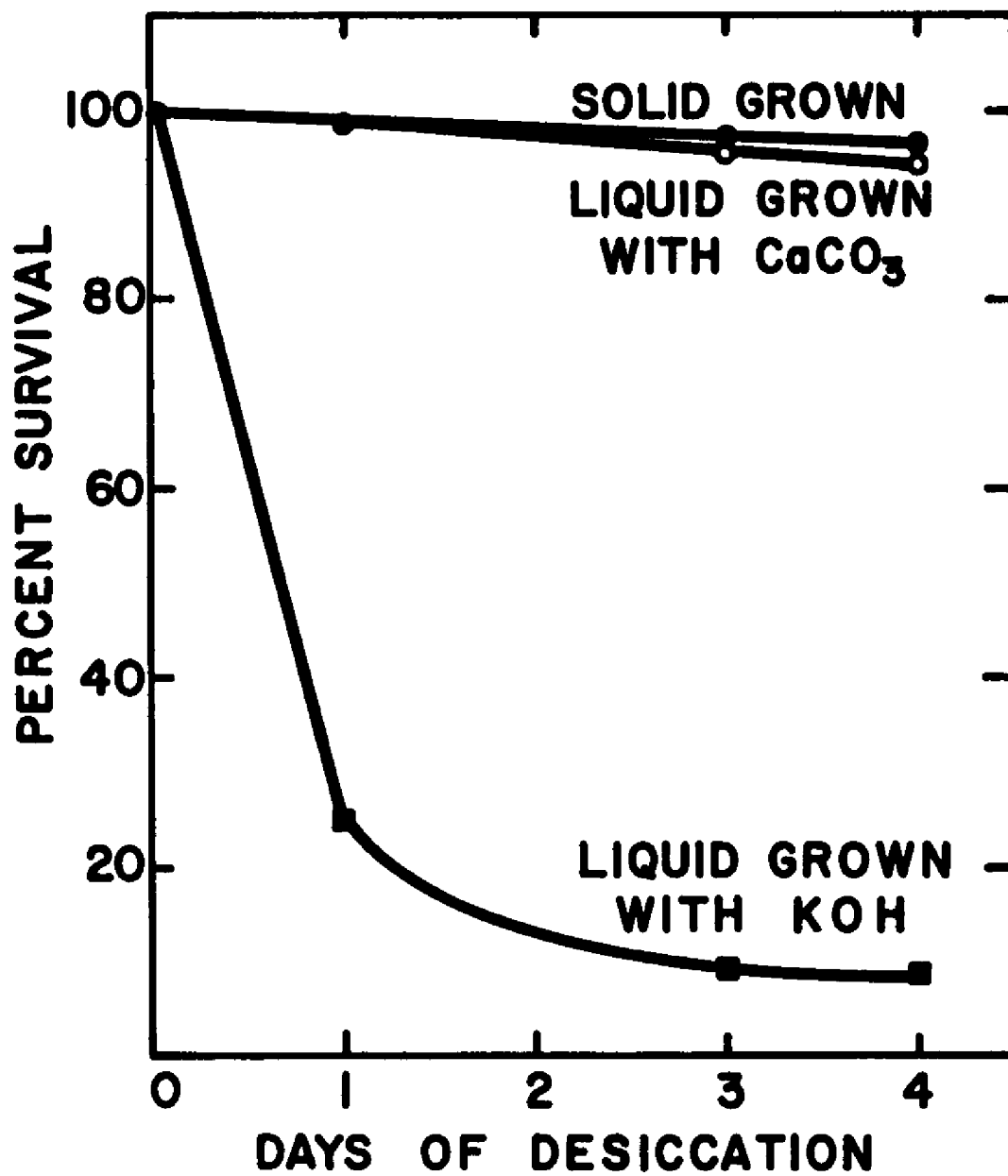


Fig. 11. Effect of desiccation on 4-day old cells of Azotobacter vinelandii 12837 grown under different cultural conditions.



Role of divalent metal ions in encystment

Since the CaCO_3 in the encystment medium apparently functioned in a role greater than that of acid neutralization, several experiments were completed in order to investigate its function. One approach involved cultivating the organisms under varying concentrations of CaCO_3 . The results are illustrated in Fig. 12. There was a sharp increase in the percentage of cysts in cultures with up to 0.2% CaCO_3 . At higher CaCO_3 concentrations, only a gradual increase in encystment was noted. It is apparent that the CaCO_3 functions in a role in addition to pH control from the observation that a concentration of 0.1% CaCO_3 was sufficient to maintain the culture pH at 7.5; however, it supported only 35% encystment.

Several experiments were conducted to specifically investigate the function of the calcium ions. Because of its high solubility, calcium chloride was used as a calcium ion source. The effect of calcium ions on the viscous material remaining after growth in a liquid medium without CaCO_3 was investigated by the addition of 1 ml of a 1 M CaCl_2 solution to 20 ml of clarified supernatant. A thick polysaccharide-like material separated from a watery residue (Fig. 13). After dehydration of the gel with absolute alcohol, a fibrous white material was recovered (Fig. 13). The gel could be dissolved by treating it with 5% EDTA. Preliminary chemical analysis has indicated that the polysaccharide-like material is a high molecular weight substance consisting of both carbohydrate and protein components.

Fig. 12. The relationship observed between the concentration of CaCO_3 used to supplement the growth medium and the extent of encystment by Azotobacter vinelandii 12837. The extent of encystment was measured after 4 days of growth. The vertical lines indicate the range of results obtained from several experiments.

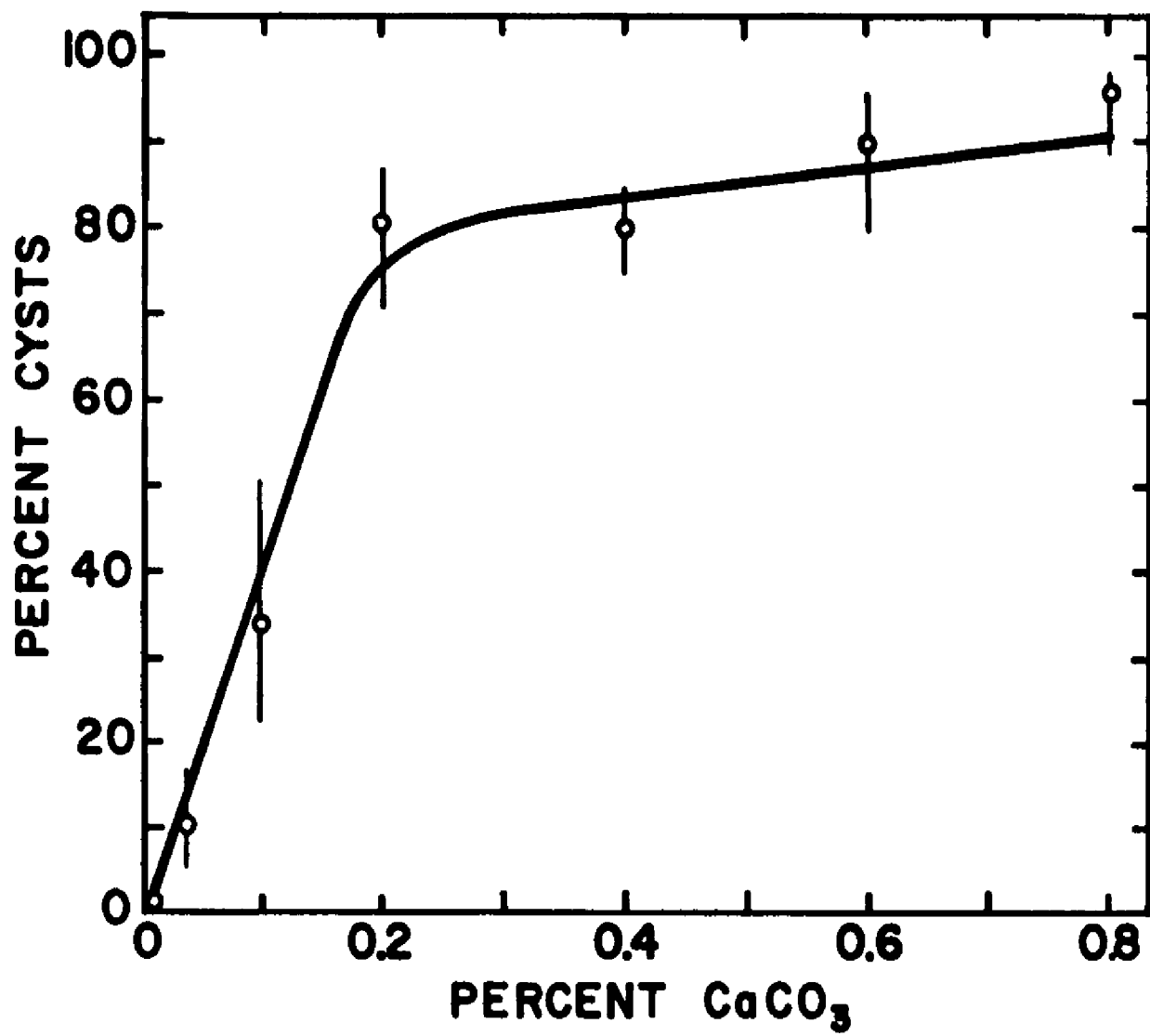
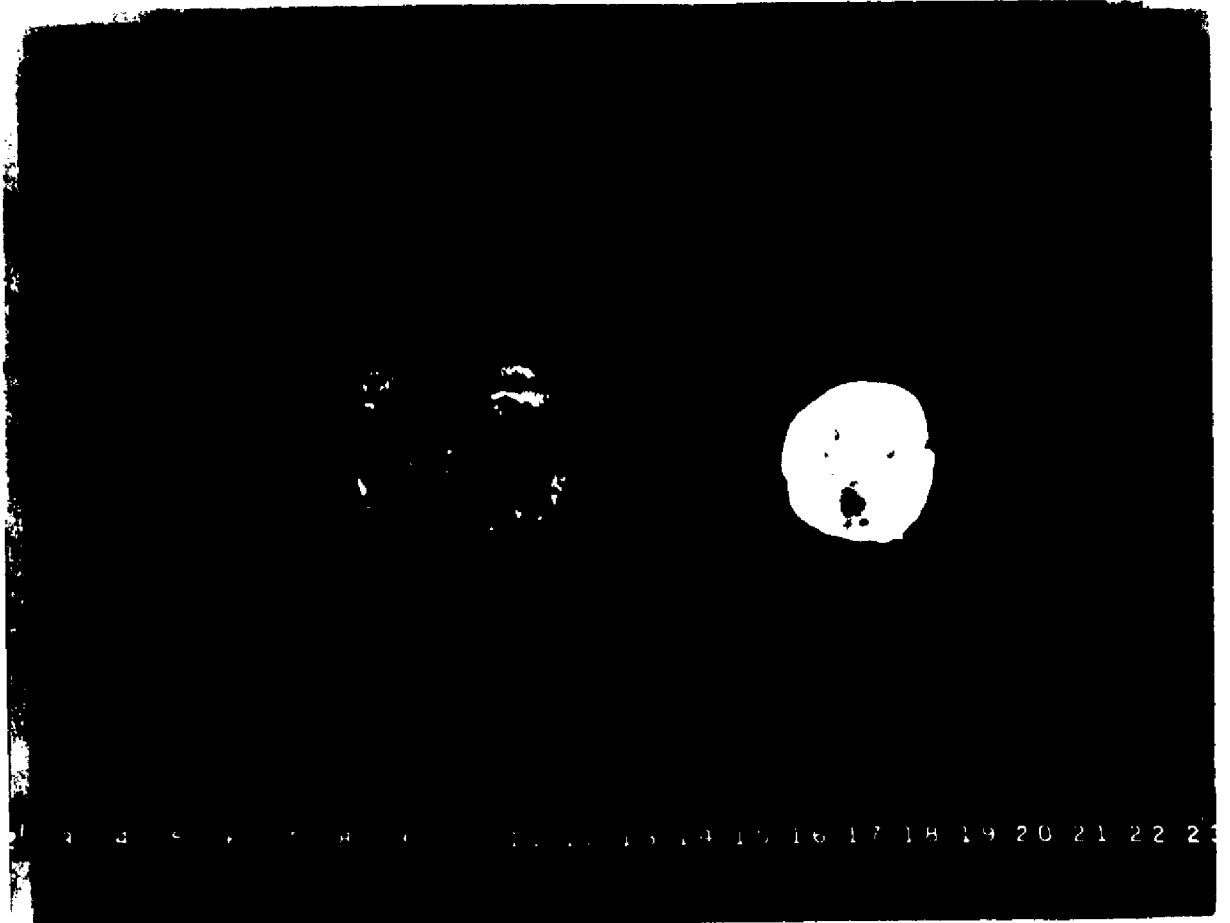


Fig. 13. Polysaccharide-like material produced by cells of Azotobacter vinelandii 12837 grown in a liquid culture in which the pH was adjusted periodically by the addition of 0.1 M KOH. The material was precipitated by adding 1 ml of 1 M CaCl₂ to 20 ml of culture supernatant. Each mark on the scale represents 1 mm. The sample at the right has been dehydrated with ethyl alcohol.



Since calcium ions were involved in the aggregation of the polysaccharide-like material, experiments were performed to determine the effect of calcium ion concentration on cyst coat formation. In these experiments 1 ml of the appropriate dilution of CaCl_2 was added to the growth medium at the beginning of the stationary phase, a time at which there were less than 0.1% cysts. One ml of distilled water was added to the control culture. The pH of the cultures was adjusted periodically by the addition of 0.1 M KOH. The results are illustrated in Fig. 14. As the concentration of supplemental CaCl_2 was increased, there was a decrease in the viscosity of the supernatant fluids of 4-day old cultures and an increase in the percentage of cysts produced. Maximum encystment was observed at a supplementation of 2×10^{-3} M CaCl_2 with a sharp drop in the percent encystment at higher concentrations. The higher concentrations caused severe precipitation and altered the medium to such an extent that further cyst development was hindered. Figure 15 illustrates the surface appearance of cysts produced by supplementing the basal medium with 2×10^{-3} M CaCl_2 . The contrast between the encysted cell and the central body type cell is markedly demonstrated in Fig. 15B.

Several other metal ions were tested for their ability to replace Ca^{++} (Table 3). Of the ions tested, Ni^{++} , Mn^{++} , Cu^{++} , and Zn^{++} precipitated the polysaccharide-type material from viscous supernatants. No precipitation was observed with Na^+ , Li^+ , Mg^{++} . However, Cu^{++} , Ni^{++} , and Zn^{++} proved to be too toxic for use as a substitute for Ca^{++} in the encysting system. At concentrations of 10^{-5} M and above,

Fig. 14. Relationship between the efflux time of culture supernatant fluids and the extent of encystment when cells of Azotobacter vinelandii 12837 were cultivated in a liquid medium supplemented with varying concentrations of CaCl_2 . The percent encystment was determined by desiccation resistance and the efflux times of the culture supernatant fluids were measured with a Cannon-Fenske number 200 Viscometer at 33 C. Both parameters were measured after 4 days of growth.

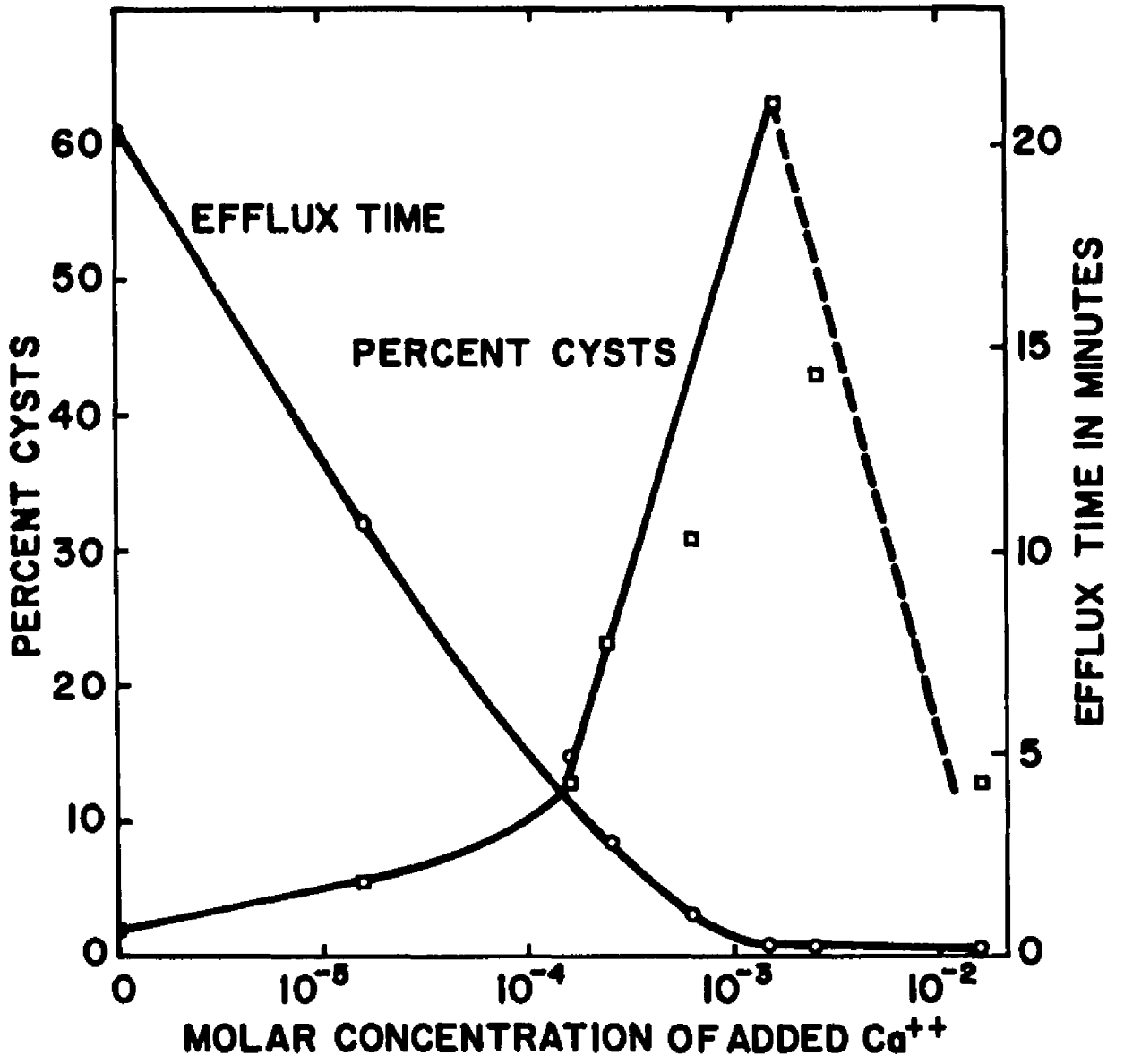


Fig. 15. Electron micrographs of carbon replicas of cells of Azotobacter vinelandii 12837 grown in a liquid medium with 2×10^{-3} M supplemental CaCl_2 . (A) encysted cell. (B) encysted cell (upper right) and non-encysted cell (lower left).



Table 3

Effect of cations on cyst formation and on the supernatant polysaccharide-like material produced by Azotobacter vinelandii 12837

Cation	Salt utilized	Concentration range employed	Effect when added to polysaccharide-like material liquid culture	
calcium	CaCl ₂	10 ⁻¹ to 10 ⁻⁷ M	precipitation	cyst development and loss of culture viscosity
sodium	NaCl	10 ⁻¹ to 10 ⁻³ M	no effect	no effect
lithium	LiCl ₂	10 ⁻¹ to 10 ⁻³ M	no effect	no effect
magnesium	MgSO ₄	10 ⁻¹ to 10 ⁻⁷ M	no effect	loss of culture viscosity, no encystment
manganese	MnCl ₂	10 ⁻¹ to 10 ⁻⁷ M	precipitation	loss of culture viscosity, no encystment
nickel	NiSO ₄	10 ⁻¹ to 10 ⁻⁹ M	precipitation	toxic
	NiCl ₂	10 ⁻¹ to 10 ⁻⁹ M	precipitation	toxic
cupric	CuSO ₄	10 ⁻¹ to 10 ⁻⁹ M	precipitation	toxic
zinc	ZnSO ₄	10 ⁻¹ to 10 ⁻⁹ M	precipitation	toxic

these ions caused death and lysis of the cells. Concentrations of 10^{-6} M and below had no effect on either the viscosity or encystment. Mg^{++} and Mn^{++} concentrations of 10^{-2} caused a drop in the viscosity of the culture medium, however, there was no increase in the extent of cyst formation.

Role of poly-B-hydroxybutyric acid metabolism in encystment

Stevenson and Socolofsky (92) have proposed a direct relationship between the PHB accumulation by cells of A. vinelandii and subsequent cyst development. An attempt was made to utilize the encysting system in liquid culture to study the possible relationship between these two factors. To facilitate this study, various parameters associated with the cultivation of the organism in liquid culture, both with and without $CaCO_3$, were measured.

Glucose utilization, growth, and nitrogen fixation by the organism are illustrated in Fig. 16. Essentially identical data were obtained when the organism was grown in a medium supplemented with $CaCO_3$ and in a medium in which the pH was controlled by the periodic addition of 0.1 M KOH. The measurements indicate that both the log-phase of growth and nitrogen fixation started about 8 hr after inoculation and reached a maximum after about 24 hr of growth. Nitrogen fixation apparently ceased simultaneously with the termination of cell division. The glucose used as carbon source by the organism was rapidly utilized and after 24 hr of growth none could be detected in the medium.

Figure 17 illustrates the relationships observed between growth, PHB accumulation, encystment, and viscosity development when the cells

Fig. 16. The relationships observed between glucose utilization, growth, and nitrogen fixation by the cells of Azotobacter vinelandii 12837. Essentially identical data were obtained when the organism was grown in a medium supplemented with CaCO_3 and in a medium in which the pH was controlled by the periodic addition of 0.1 M KOH.

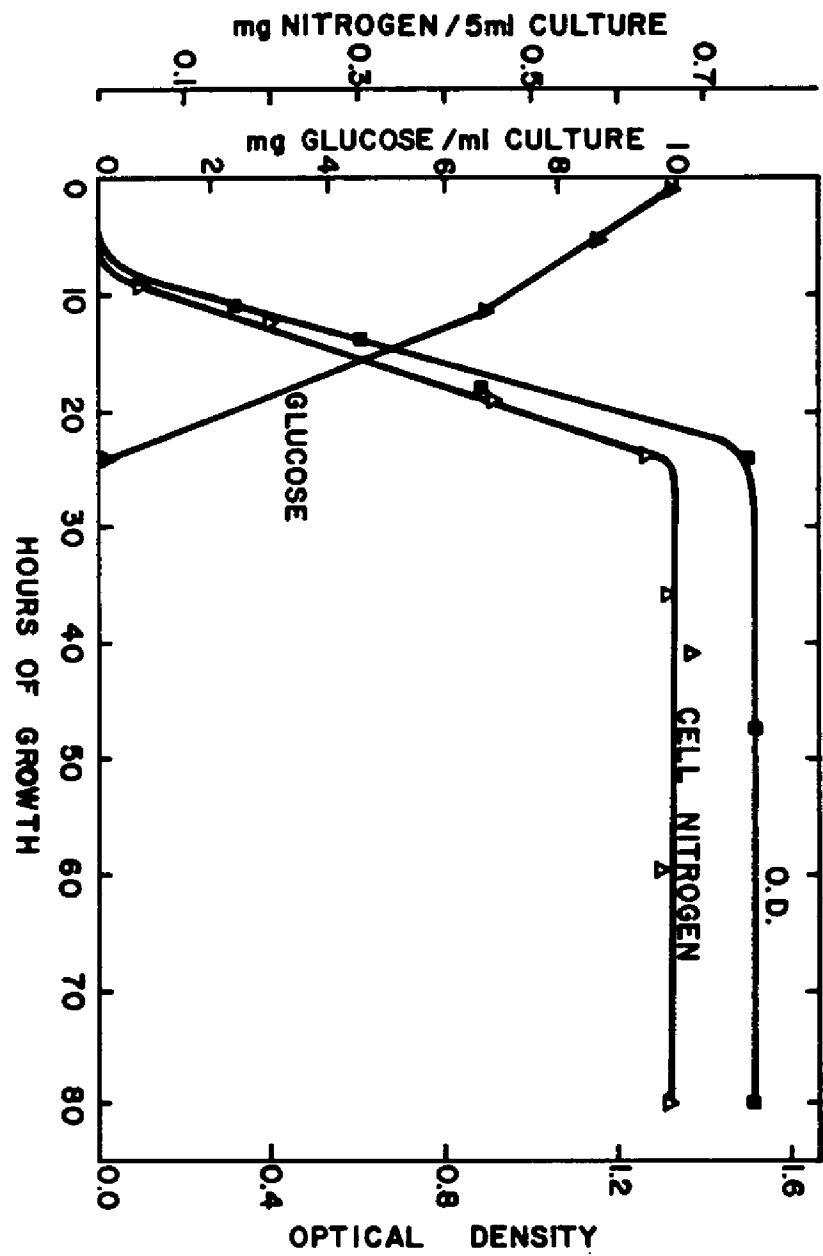
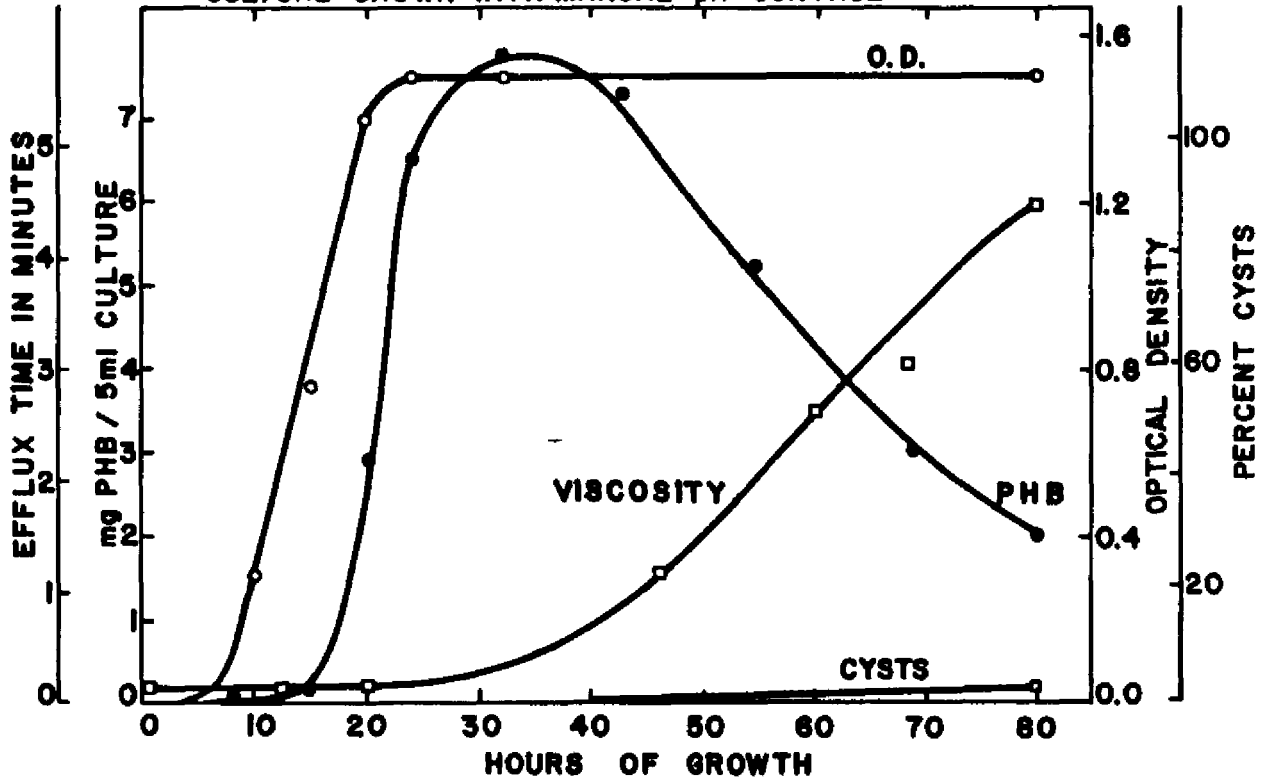


Fig. 17. Relationships observed between growth, PHB accumulation, encystment, and viscosity when cells of Azotobacter vinelandii 12837 were cultivated in a liquid medium in which the pH was adjusted by the periodic addition of 0.1 M KOH. Growth is reflected in the increase in the OD of the medium; polymer is recorded as the mg PHB/5 ml of culture; encystment is presented as the percent cysts; and the viscosity measurements represent the efflux time of the culture supernatant using a number 200 Cannon-Fenske viscometer.

CULTURE GROWN WITH MANUAL pH CONTROL



were cultivated in a medium in which the pH was adjusted periodically by KOH addition. The OD measurements indicate the log-phase started about 8 hr after inoculation and reached a maximum after about 24 hr of growth. PHB accumulation continued during the early stationary phase, reached a maximum after 30 hr of growth, and subsequently declined. The percentage of encystment remained below 10% throughout the experimental period and the viscosity of the medium increased concomitantly with degradation of the polymer reserve.

The relationships observed between these same parameters when the cells were cultivated in a CaCO_3 supplemented medium are illustrated in Fig. 18. The patterns of growth and PHB metabolism are about the same as shown previously. The viscosity of the medium did not increase during the growth cycle. The percentage of cysts in the culture remained very low during the first 20 hr of growth; however, after 35 hr, there was an increase in the percentage of cysts to about 18. After 60 hr of growth, the percent encystment had increased to practically 100. It should be noted that the rise in the percentage of cysts occurs concurrently with the degradation of the polymer reserve.

The effect of varying glucose concentrations on the extent of polymer accumulation and encystment of *A. vinelandii* is illustrated in Fig. 19. The ratio of mg cellular PHB to mg cellular nitrogen was measured at the time of maximum polymer accumulation and the extent of encystment was determined after 4 days of cultivation. Cellular nitrogen was considered to be a convenient reference for

Fig. 18. Relationships observed between growth, PHB accumulation, encystment, and viscosity when cells of Azotobacter vinelandii 12837 were cultivated in a liquid medium supplemented with CaCO_3 . Growth is shown as the OD of the medium; polymer is recorded as the mg PHB/5 ml culture; encystment is presented as the percent cysts; and the viscosity measurements represent the efflux time of the medium using a number 200 Cannon-Fenske viscometer.

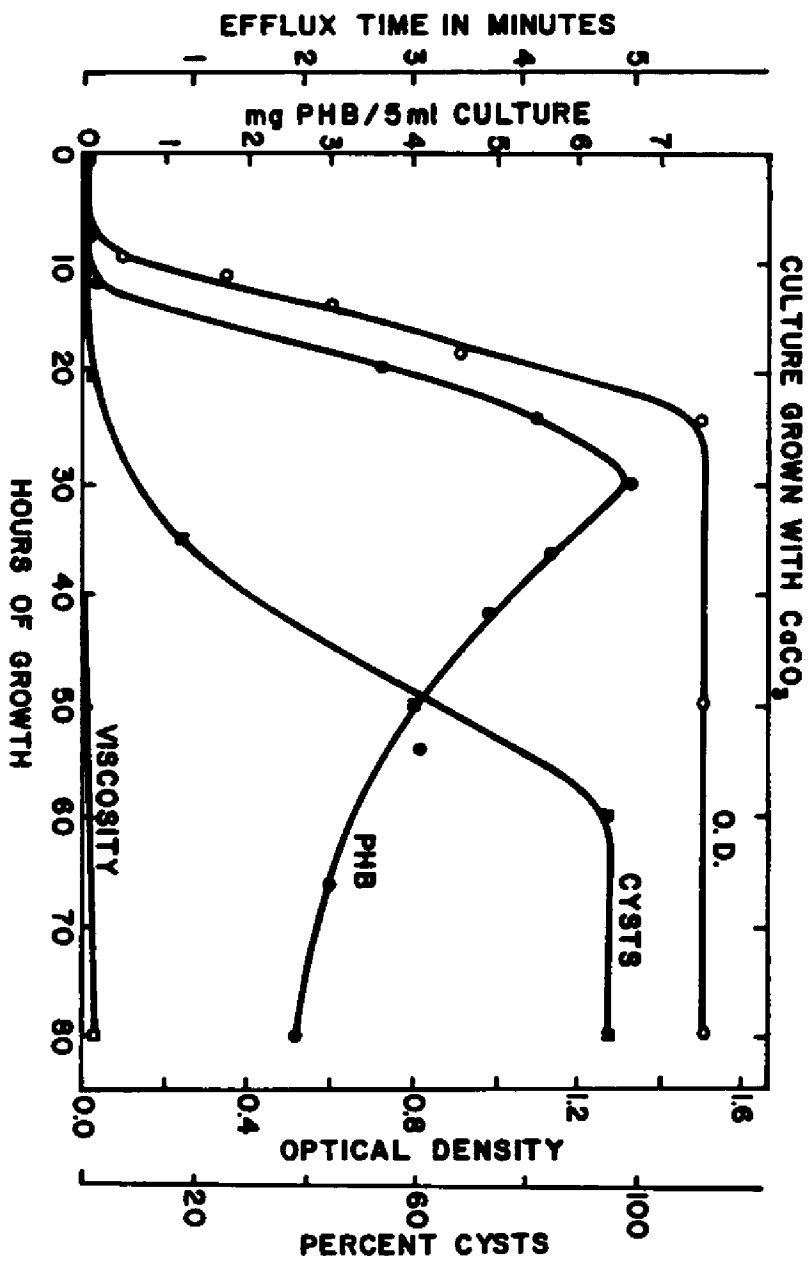
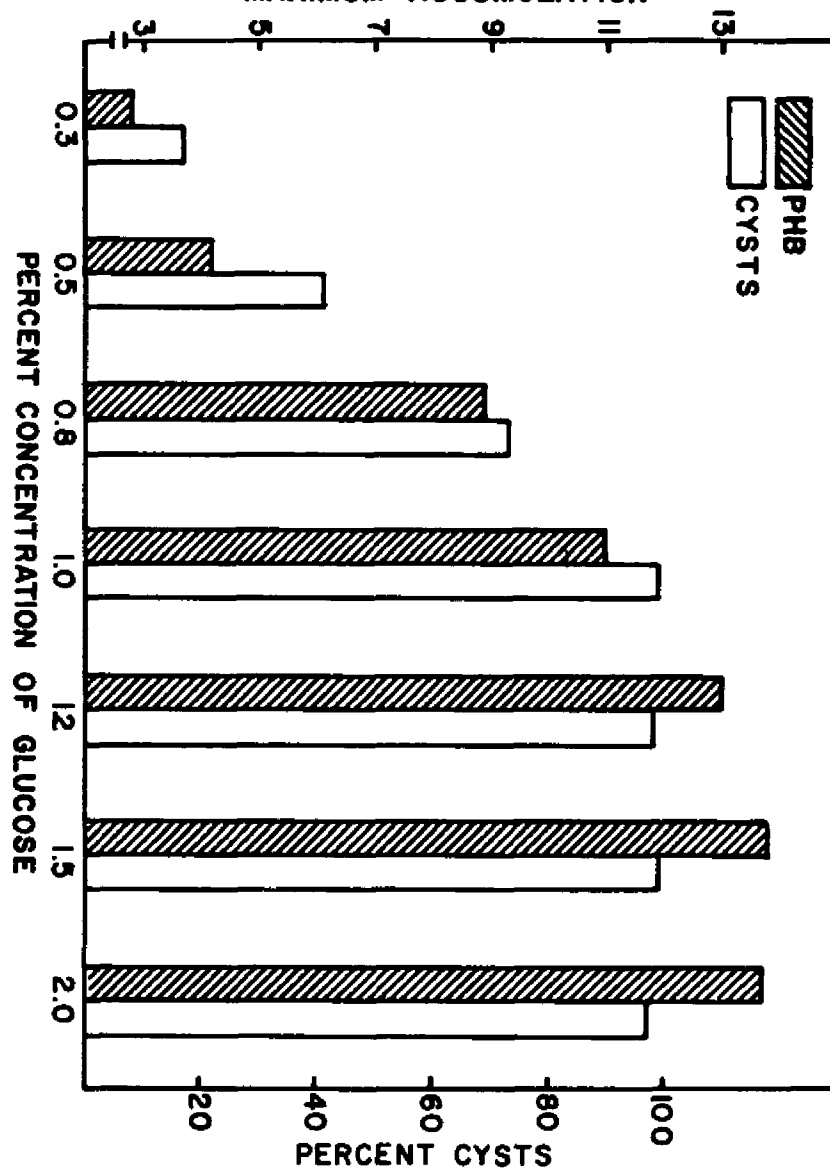


Fig. 19. Effect of varying glucose concentrations on PHB accumulation and the extent of encystment by cells of Azotobacter vinelandii 12837 grown in a liquid medium supplemented with 0.6% CaCO_3 . The polymer is reported as the PHB:nitrogen ratio at the time of maximum accumulation. The extent of encystment is reported as the percent cysts determined after 4 days of growth.

PHB/NITROGEN RATIO AT TIME OF
MAXIMUM ACCUMULATION

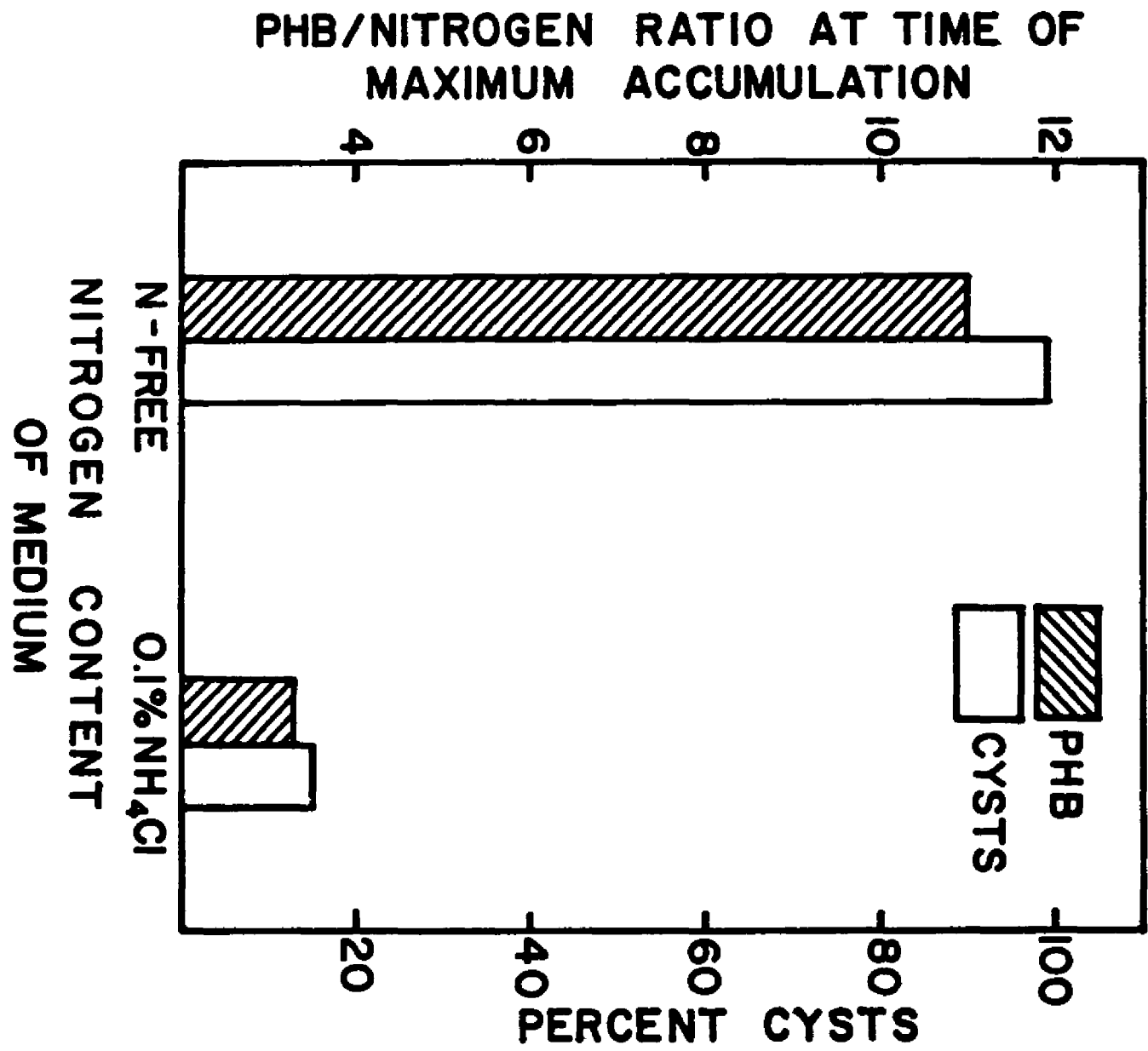


comparing the polymer measurements from the different cultures. An increase was observed in both the maximum PHB:nitrogen ratio in the cells and the extent of encystment as the glucose concentration was varied from 0.3 to 1.0%. At a concentration of 1% glucose, the extent of encystment reached a maximum. A maximum PHB: nitrogen ratio of about 14:1 was obtained when the cells were cultivated utilizing a glucose concentration of 1.5%.

Since Azotobacter will utilize fixed nitrogen in preference to atmospheric nitrogen (111), the effect of an exogenous reduced nitrogen source on PHB accumulation and encystment may be determined. Figure 20 illustrates the alterations observed in the extent of cyst formation and maximum PHB accumulation in cells of Azotobacter when 0.1% NH_4Cl was added to the medium. It is apparent that the cells grown in a medium supplemented with the ammonium ion accumulated significantly less polymer and encysted to a lesser degree. The cultures grown with external control of pH by the periodic additions of 0.1 M KOH did not produce the viscous polysaccharide-like material when the medium was supplemented with ammonium chloride; however, there was an increase in the number of cells in the culture and an increase in cell size.

Since the degree of encystment appeared to be dependent on the extent of PHB accumulation (Fig. 19, 20) and since the percentage of cysts in the culture increased concurrently with degradation of the polymer (Fig. 18), PHB metabolism appeared to be related to cyst formation in some manner. In order to further examine this apparent

Fig. 20. Accumulation of PHB and cyst formation by cells of Azotobacter vinelandii 12837 grown in a glucose and CaCO₃ supplemented liquid medium both with and without 0.1% ammonium chloride. The polymer is reported as the PHB:nitrogen ratio at the time of maximum accumulation. The extent of encystment is reported as the percent cysts determined after 4 days of growth.



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relationship, experiments were undertaken to selectively label the PHB during the period of polymer accumulation and to examine directly the pattern of isotope distribution following cyst development. The isotope used as sodium acetate-2-C¹⁴.

An initial set of experiments was conducted to determine if the acetate would be taken up by log-phase cells and to examine the rate of uptake, if any. The results, illustrated in Fig. 21, indicate that the radioactive acetate was rapidly incorporated into the cells. About 80% of the total activity added to the culture was localized within the cells 2 min after the addition of the isotope to the culture. After 10 min of exposure, greater than 95% of the total activity was present in the cells. Similar results were obtained with cultures grown with or without CaCO₃.

Another set of experiments was undertaken to determine the time of isotope addition to the culture which would yield maximum acetate uptake into the polymer fraction of the cells. The results are illustrated in Fig. 22. The growth of the organism is recorded as the OD of the culture and is shown by the line portion of the graph. The vertical bars indicate the percentage of isotope uptake into the whole cells and into the PHB fractions when the isotope was added to the culture after 6, 13, and 18 hr of incubation. The activities were measured 20 min after addition of the isotope to each culture. The sodium acetate-2-C¹⁴ was taken up by the cells regardless of the pre-incubation time. However, the label was selectively incorporated into the PHB fraction only if the acetate were added during the late

Fig. 21. Uptake of sodium acetate-2-C¹⁴ by log-phase cells of Azotobacter vinelandii 12837.

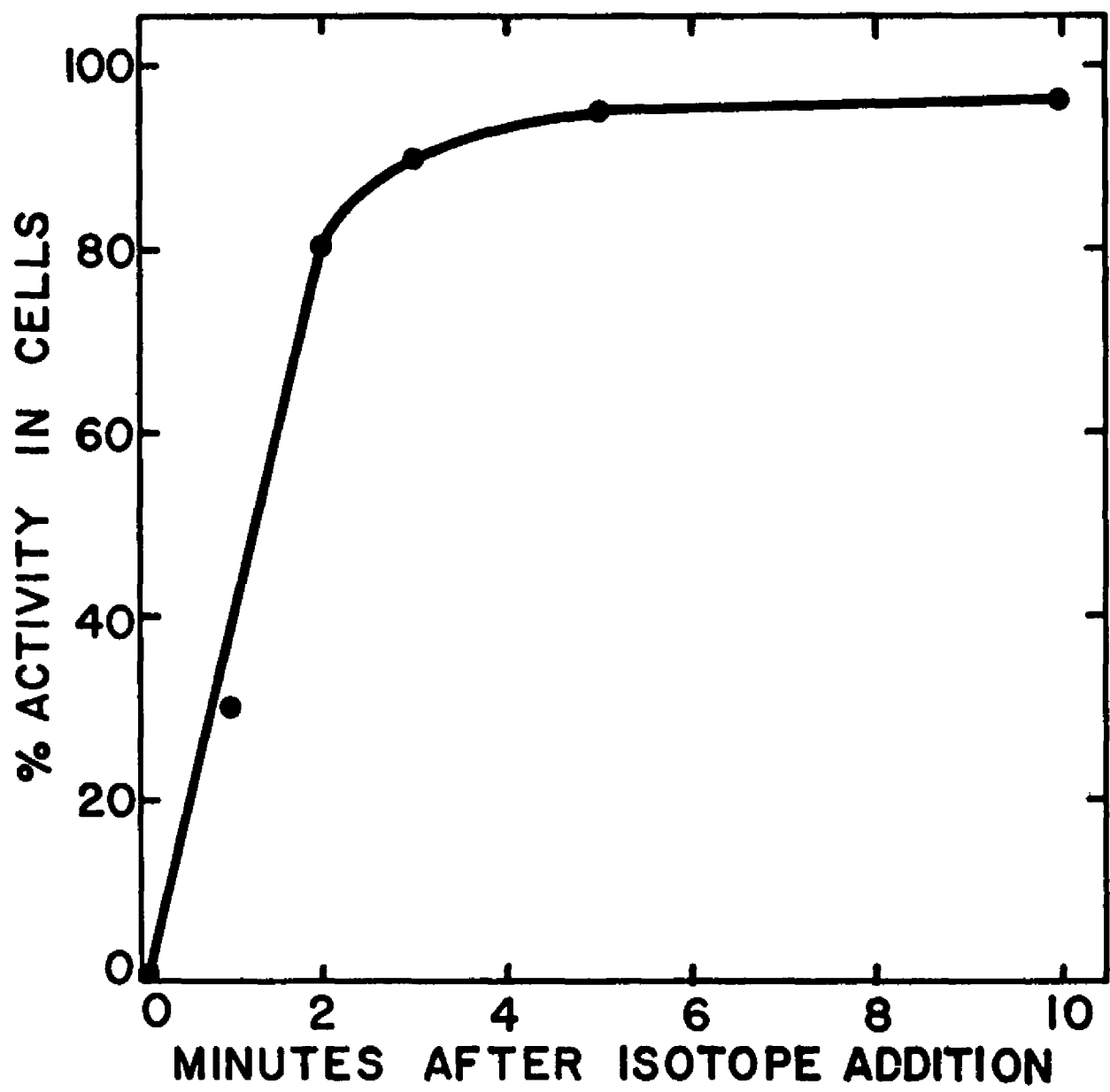
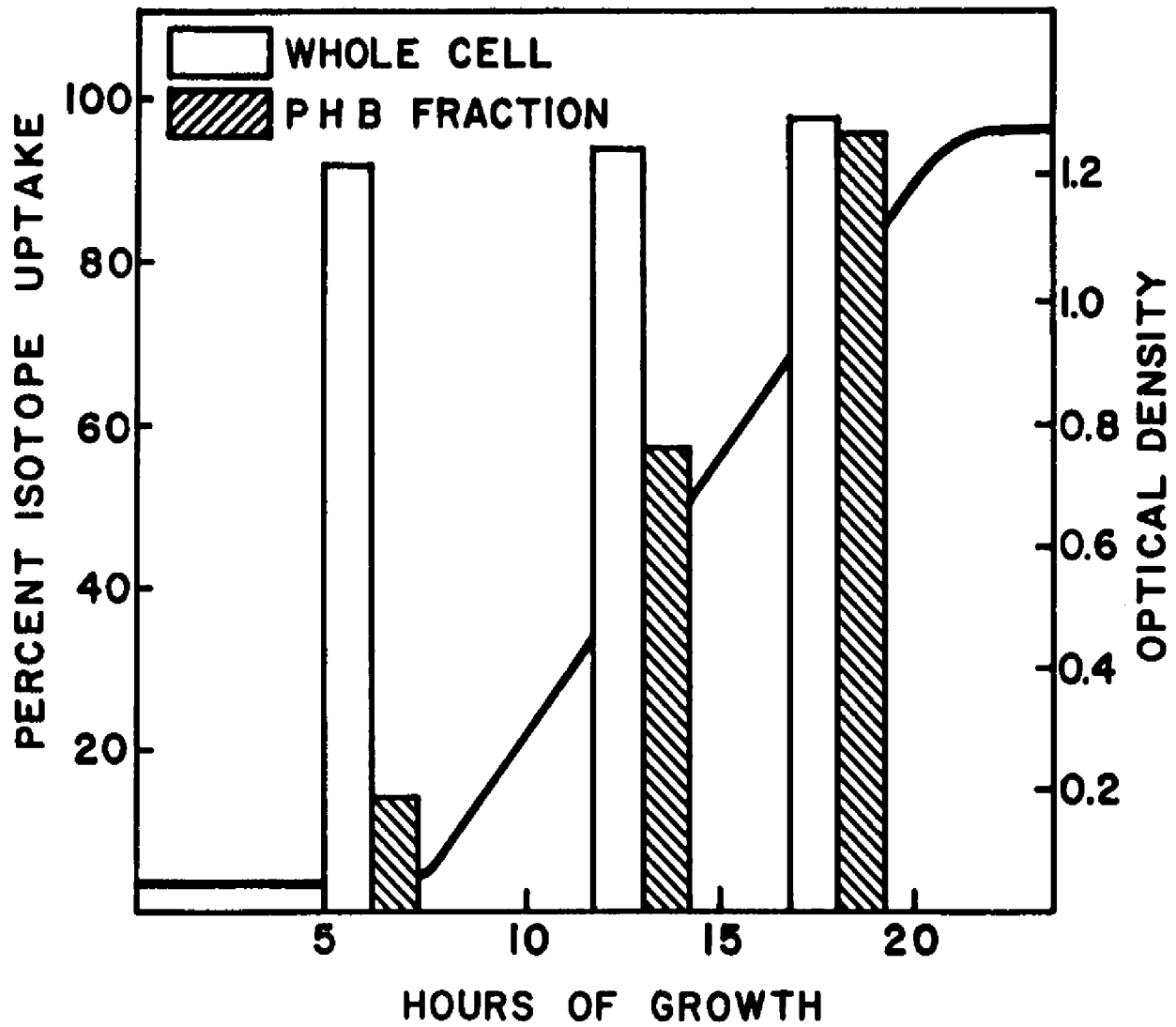


Fig. 22. Percentage of incorporation of sodium acetate-2-C¹⁴ into cells of Azotobacter vinelandii 12837 cultured for 6, 13, and 18 hr. The growth of the cells is recorded as the optical density of the culture and is shown by the line portion of the graph. The vertical bars indicate the percentage of isotope uptake into whole cells and into the PHB fractions.



log-phase. When the C^{14} -acetate was added to a 6-hr culture, about 95% of the total activity was incorporated in the whole cells; however, only a small portion of the cell activity was present in the PHB fraction. On the other hand, when the C^{14} -acetate was added to an 18-hr culture, about 95% of the total activity was present in the whole cells, and almost all of the activity was localized within the PHB fraction. Similar results were obtained when the activities of various fractions were measured at the time of maximum PHB accumulation (after 30 hr of growth) or in cultures grown with or without $CaCO_3$.

Following the degradation of the PHB which had been selectively labeled by the addition of C^{14} -acetate to an 18-hr culture, the activities of various fractions were determined. The results of a representative experiment are shown in Table 4. The activities are represented as the counts per min based on 1 ml of culture. Twenty min after addition of the sodium acetate-2- C^{14} to an 18-hr culture about 95% of the whole cell activity was localized in the PHB fractions in cultures grown with or without $CaCO_3$. Following polymer degradation, the encysted cells grown in a $CaCO_3$ supplemented medium retained the major part of the radioactivity. However, in the cultures grown with external pH adjustment, a significant amount of the radioactivity originally present in the PHB fraction was located in the extracellular polysaccharide-like slime material produced by the cells.

Some of the total cyst activity could be released by treating the cysts with EDTA. The cysts were exposed to the EDTA following

Table 4

Distribution of sodium acetate-2-C¹⁴ in cells of Azotobacter vinelandii 12837

Time of Sampling	Sample	cpm/ml culture	
		without CaCO ₃ ¹	with CaCO ₃
20 min after addition of isotope to 18-hr culture	whole cells	3,616 ² .	4,272 ³ .
	PHB	3,493	3,980
After 4 days of growth	whole cells	1,520	3,340
	PHB	878	962
	polysaccharide-like material	1,260	0
	CO ₂	486 ⁴ .	602 ⁴ .

1. The pH was adjusted by the periodic addition of 0.1 M KOH.
2. Figure represents 95% of total cpm/ml added to culture.
3. Figure represents 97% of total cpm/ml added to culture.
4. Figures represent total cpm/ml culture obtained over the entire experimental period by frequently recharging CO₂ trap with fresh reagent.

Table 5

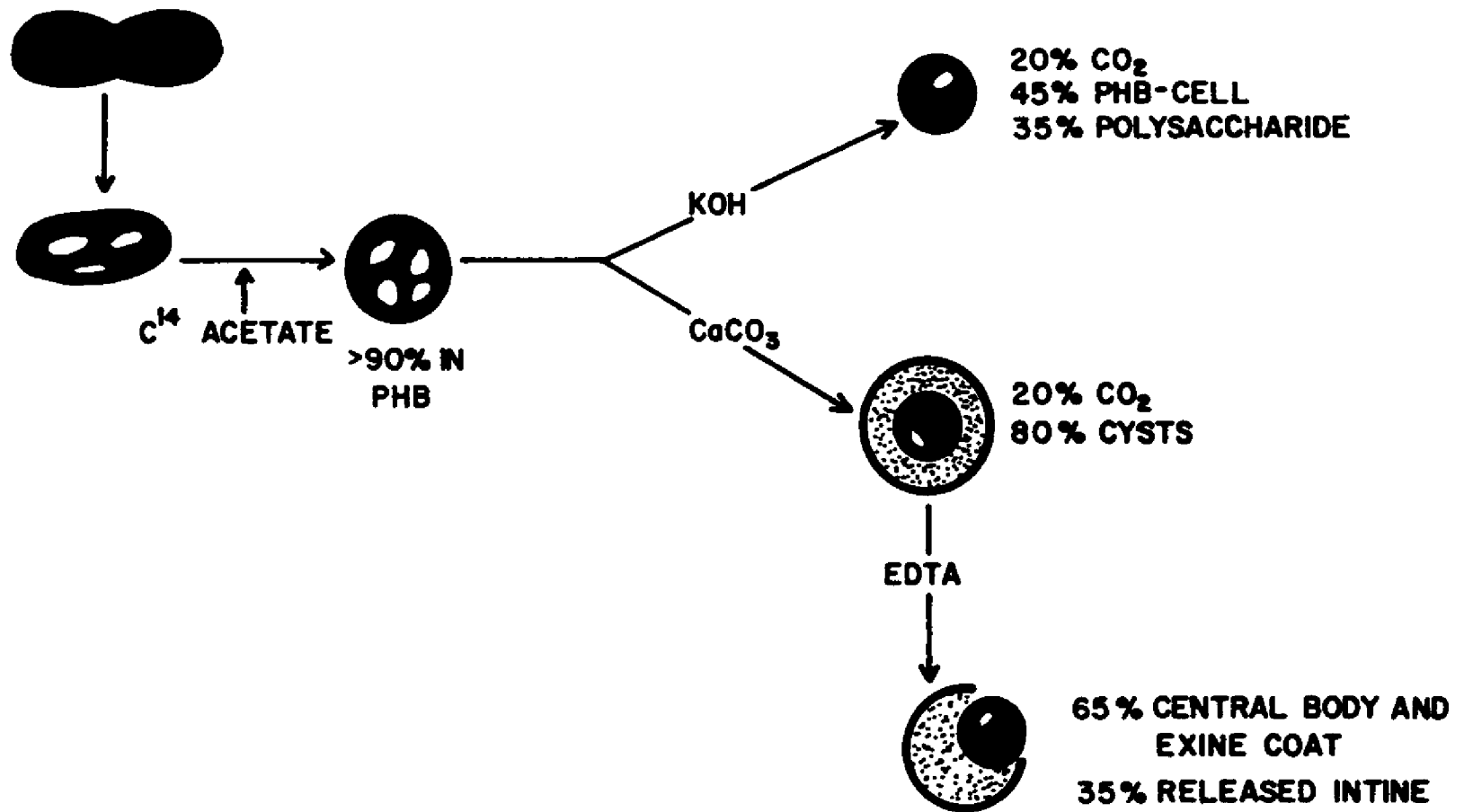
Release of C¹⁴ from cysts after rupture with EDTA

Sample	cpm/ml
Untreated cysts	521
Activity released from cysts after rupture with EDTA	227

the method of Socolofsky and Wyss (88). Following cyst rupture the lysate was centrifuged at 70,000 x g for 2 hr. The central bodies and exine coats were removed from the suspension and the material remaining in solution can be precipitated by the addition of CaCl_2 and is thought to be composed primarily of intine components. The results of one such experiment are seen in Table 5. About 45% of the total cyst activity was released into the supernatant when the cysts were ruptured.

The fate of the C^{14} following its addition to late log-phase cells is illustrated schematically in Fig. 23. The percentages given are the average values obtained from many experiments utilizing varying amounts of the isotope. Shortly after the addition of the sodium acetate-2- C^{14} to the late log-phase cells, greater than 90% of the activity was localized in the PHB fraction. Following polymer degradation in a medium in which the pH was adjusted by the periodic addition of 0.1 M KOH, 20% of the activity was lost as carbon dioxide, 35% was recovered in the polysaccharide-like material from the viscous supernatants, and 45% remained as non-utilized PHB and other cellular components. When polymer degradation was allowed to proceed in an encystment medium supplemented with CaCO_3 , 20% of the activity was lost as CO_2 and the remainder was associated with the intact cyst. Of the total cyst activity, 20% was present in residual PHB and about 35% was present in the intine components which were released when the cysts were subjected to EDTA rupture.

Fig. 23. Schematic diagram illustrating the uptake of sodium acetate-2-C¹⁴ into the cellular PHB fraction and the subsequent distribution of the radioactivity following polymer degradation in an encystment and non-encystment medium. The sodium acetate-2-C¹⁴ was added to the cultures at the end of the log-phase of growth and the distribution of the label was determined after 4 days of cultivation.



DISCUSSION

Typical Azotobacter cysts can be produced in liquid culture by the incorporation of sufficient calcium ions into the medium. The cysts harvested from a liquid medium are identical to cysts grown on a solid surface in respect to their cytological, lytic, and resistance properties. The results reported here are not in agreement with those of Layne and Johnson (50, 51). The latter investigators described the formation of resistant cells which were induced by deletion of certain ions, including Ca^{++} , normally found in Burk's medium. In the present study it was observed that the medium must be supplemented with additional calcium ions for encystment to occur. Stewart et al. (93) have also disputed the results of Layne and Johnson. They reported that both growth and encystment were dependent upon sufficient copper and magnesium ion concentrations in the medium. The cells produced in this investigation by cultivation in a liquid medium with external pH adjustment were similar to those described by Layne and Johnson in that they lacked any coat components, however they were not resistant to several deleterious conditions. They are considered to be cytologically and physically analogous to the central bodies isolated and described by Parker and Socolofsky (72). This further strengthens the belief that the coat material is primarily responsible for the resistance exhibited by the encysted cells (72).

The nature of the acidic material produced by the cells is not known; however, based on previous reports, there are several possibilities.

Jensen (42) reported that several strains of Azotobacter produce acidic intermediates by the incomplete oxidation of carbohydrates. Cohen and Johnstone (14) found that acid production was associated with slime formation and suggested that slime-forming strains of A. vinelandii possessed a specific polymerase which combined the partially oxidized intermediates into an acidic extracellular polysaccharide. Cohen and Johnstone (15) have subsequently reported that the extracellular polysaccharide of A. vinelandii contains large amounts of galacturonic acid and has an overall negative charge. Romanow (77) reported that cyst-forming strains of A. chroococcum produced small amounts of indoleacetic acid.

Cohen and Johnstone (14) suggested that CaCO_3 in azotobacter culture medium served to either neutralize the acidic products formed or inhibit the ability of the cell to synthesize the acidic slime. The work reported in this dissertation indicates that the CaCO_3 used to supplement the encystment medium serves the dual function of acid neutralization and calcium ion source. The neutralization function is important because the acid conditions produced are very detrimental to the cells. The calcium ions fulfill an important structural role in the organization of the polysaccharide-like material into a rigid cyst coat around the central body. Several authors have indicated that divalent metal ions have an important structural role in bacterial cell walls (2, 38, 52). Rogers (76) has proposed that divalent cations such as Ca^{++} and Mg^{++} are involved in the organization

of the outer layers of bacteria through the formation of ionic bonds between negatively charged macromolecules. Since the extracellular polysaccharide of A. vinelandii has been reported to contain large amounts of galacturonic acid, it is conceivable that the cyst coat components also have an appreciable amount of galacturonic acid which would undergo ionic bonding with calcium ions. If adequate ions are not available, abortive encystment takes place. The cyst coat components are produced by the organism; however, they are not held adjacent to the cell and are lost into the surrounding medium. This results in an increase in culture viscosity.

Calcium carbonate is considered to be the best calcium ion source for the encystment medium because it apparently supplies sufficient ions in a continuing supply. Moreover, the concentration of calcium ions in the medium is never high enough to cause severe alterations in the composition of the salts solution as occurs with CaCl_2 concentrations above 2×10^{-3} M. The small amounts of other divalent metal ion salts found as impurities in commercial preparations of CaCO_3 may also contribute significantly to the stability of the cyst coat.

Since the addition of magnesium and manganese ions to the medium results in a lowering of the viscosity of the supernates after growth, these ions may also be of importance in cyst coat formation; however, they cannot completely substitute for calcium ions. Goldschmidt and Wyss (32) have implicated Mg^{++} in coat formation. In view of the marked ability to coagulate the viscous supernatant fluids, cupric, nickel, and zinc ions may also be of importance in trace amounts

found as impurities in the chemicals used in Burk's medium or in the agar when solid media are employed. However, because of their toxicity at concentrations above 10^{-5} M, they cannot be used as substitutes for calcium ions. Stewart et al. (93) have also reported that cupric ions are toxic to Azotobacter at higher concentrations.

The discrepancy between the amount of supplemental calcium ions needed for encystment to occur when the organism is grown utilizing solid and liquid media cannot be fully explained at the present time. However, it is suggested that cells growing on a solid surface do not need as much Ca^{++} for the initial stabilization of the coat as is needed in liquid culture. Additionally, the possible presence of these ions as impurities in the agar may compensate for the lack of calcium ions in the salts solution.

The accumulation of large amounts of PHB by A. vinelandii apparently reflects an unbalanced growth condition. Macrae and Wilkinson (62) have demonstrated that the accumulation of the polymer by B. megaterium is dependent on the composition of the growth medium. They found that as the ratio of carbon source to the nitrogen source in the medium increased, and as the medium became nitrogen-deficient instead of carbon-deficient, there was an increase in the amount of PHB per cell. Unlike non-nitrogen-fixing species of bacteria, which depend on a source of combined nitrogen, the amount of nitrogen available for the growth of Azotobacter is dependent on the rate of nitrogen fixation. Growth of this organism under conditions in which atmospheric nitrogen is utilized apparently resulted in a general

nitrogen deficiency. This was indicated by the accumulation of PHB which is a non-nitrogenous material. Cultivation of the organism utilizing increasing amounts of glucose served to accentuate the nitrogen deficiency and increased amounts of PHB were deposited. When a reduced nitrogen source was added to the medium, the organism rapidly converted the assimilated carbon into nitrogenous cell components and did not accumulate a significant amount of the polymer. Supplementing the growth medium with increasing amounts of glucose or with a fixed nitrogen source also resulted in an increase in the cell yield in each of the cultures.

Polymer deposition within the cells was followed by endogenous degradation of the reserve. The PHB apparently was not utilized for continued growth of the organism since no increase in cellular nitrogen was noted during the degradation phase. The importance of PHB in the endogenous respiration of bacteria is well recognized (17), and Sobek et al. (86) reported that the polymer was the only major cellular material utilized during the endogenous respiration of Azotobacter.

The accumulation of large amounts of PHB is apparently essential to the encystment process. The extent of polymer accumulation and the percent encystment in the liquid medium supplemented with CaCO_3 were observed to be directly proportional. When either the glucose concentration of the medium was reduced or ammonium chloride was added, the cells failed to accumulate significant amounts of the polymer and likewise failed to encyst.

The apparent association between the two parameters is also indicated by the correlation noted between degradation of the PHB and the development of the cysts or an increase in viscosity. During the endogenous utilization of the PHB reserve there was a concomitant increase in the number of cysts when the medium was supplemented with CaCO_3 and a similar rise in the viscosity of the culture medium when a neutral pH was maintained by the periodic addition of 0.1 M KOH.

The direct involvement of the polymer in cyst development is shown by the experiments utilizing C^{14} to determine the fate of the reserve material following the degradation phase. After utilization of PHB which had been tagged with sodium acetate-2- C^{14} , a substantial portion of the radioactivity was recovered in the cyst coat components which were released by EDTA rupture. Considerable activity was also observed in the polysaccharide-like material produced in cultures which lacked sufficient calcium ions for extensive coat development.

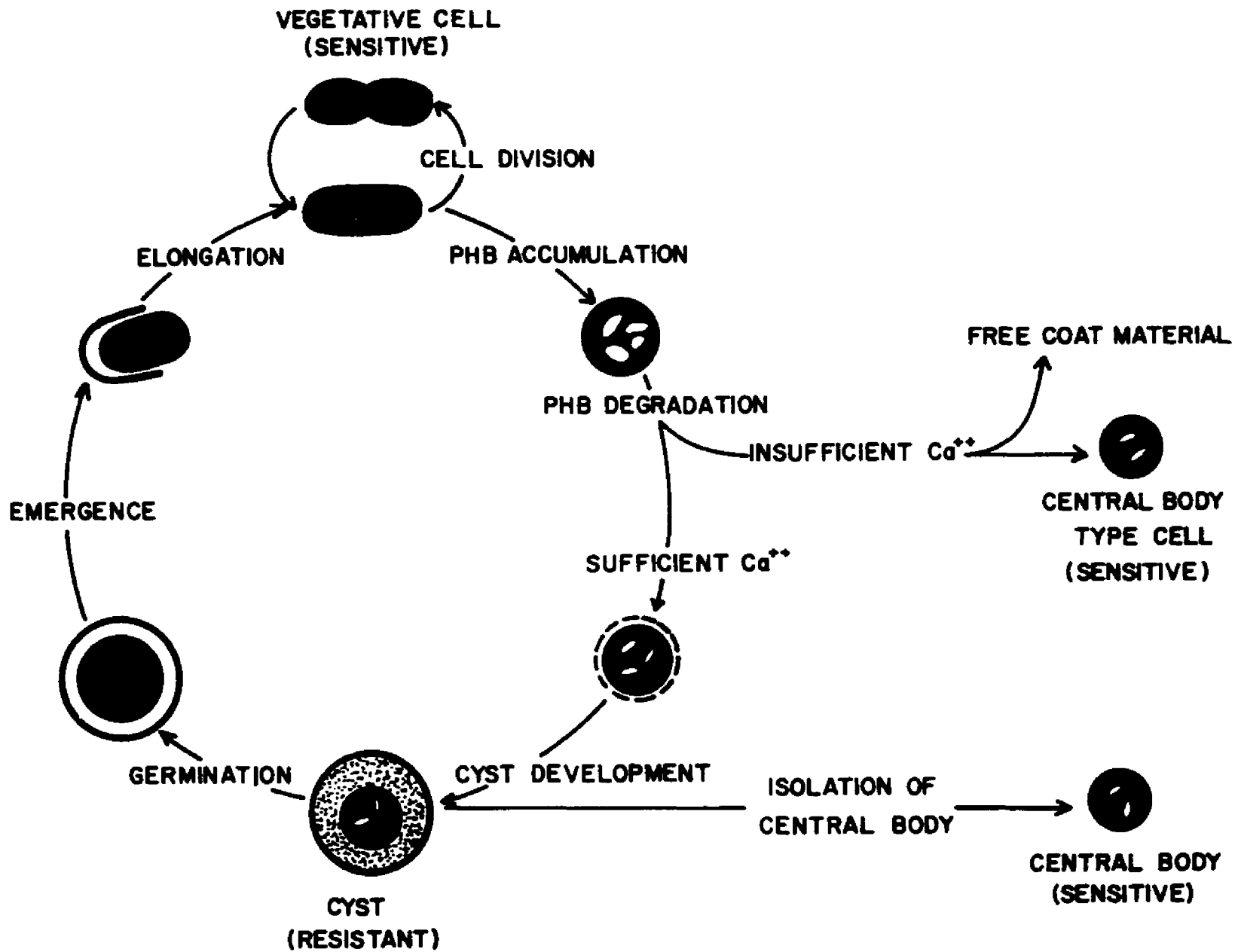
The failure of 6-hr old cells to incorporate the radioactive acetate into the PHB fraction and the selective incorporation of the acetate into the polymer reserve by 18-hr old cells, which is illustrated in Fig. 22, is thought to reflect differences in the level of PHB synthetase activity in the cells after various periods of cultivation. The polymer synthesizing system is not very active in the 6-hr old cells and the acetate is incorporated into other cellular components. After 13 hr of growth, PHB synthetase activity has increased and the cells are growing at a maximum rate, and the acetate added to the culture is incorporated into both the PHB and other

cellular material. Polymer accumulation is proceeding at a maximum rate while cell growth has started to slow down after 18 hr of cultivation. At this time almost all of the acetate added to the culture is rapidly taken up by the PHB synthetase system and converted to polymer while little of the acetate is incorporated into other cellular components.

Cohen and Johnstone (14) reported that certain strains of A. vinelandii failed to produce the acidic slime material in liquid culture and suggested that these strains had no polymerase for synthesizing the extracellular slime. Eklund et al. (26) noted that nonencapsulated mutants of A. vinelandii failed to form cysts and proposed that capsule formation was related to encystment. During the course of this investigation these two phenomena were also noted. When rough, nonencapsulated strains of the experimental organism were cultivated in the liquid medium supplemented with CaCO_3 no cysts developed. Moreover, when external control of pH was effected by the periodic addition of KOH, the culture did not become viscous. However, one additional factor has been noted. The rough strains failed to accumulate significant amounts of PHB. This suggests the possibility that the rough strains may have the enzymatic capacity for capsule formation, but because of the apparent lack of an active PHB polymerase system, the cells do not have an intracellular reserve capable of serving as a carbon and energy source for capsule development.

In conclusion a diagrammatic sketch of the formation and germination of the Azotobacter cyst is shown in Fig. 24. The initial step in cyst development involves the accumulation of intracellular deposits of poly- β -hydroxybutyric acid. This is followed by an endogenous utilization of the polymer reserve as a carbon and energy source for the encystment process. During the polymer degradation phase there is a concurrent production of cyst coat components. If sufficient calcium ions are not available in the environment for the coordination of the coat material into a rigid cyst coat, abortive encystment takes place and the coat material is lost into the surrounding medium. This results in an increase in the viscosity of the culture supernatant and the production of small vegetative cells which are believed to be analogous to the central bodies isolated by Parker and Socolofsky (72). On the other hand, if sufficient calcium ions are available, the coat materials are coordinated around the periphery of the developing central body. This results in the formation of mature cysts which display the cytological, lytic, and resistance properties reported to be characteristic of Azotobacter cysts.

Fig. 24. Diagrammatic sketch of the formation and germination of Azotobacter cysts.



LITERATURE CITED

1. Alper, R., D. G. Lundgren, R. H. Marchessault, and W. A. Cote. 1963. Properties of poly-B-hydroxybutyrate. 1. General considerations concerning the naturally occurring polymer. *Biopolymers* 1:545-556.
2. Asbell, M. A., and R. G. Eagon. 1966. The role of multivalent cations in the organization and structure of bacterial cell walls. *Biochem. Biophys. Res. Commun.* 22:664-671.
3. Batchinskaya, A. A. 1935. Sur la structure et le developpement de l'Azotobacter. *Bull. Inst. Microbiol. Agr.* 6:1-47.
4. Beijerinck, M. W. 1901. Nitrogen-fixing bacteria. *Zentr. Bakteriolog. Abt. II* 7:561-582. (Cited by Kyle and Eisenstark, 47)
5. Bisset, K. A. 1962. *Bacteria*. 2nd ed. E. and S. Livingstone, Ltd., Edinburgh and London.
6. Bisset, K. A., A. C. Baird-Parker, C. M. F. Hale, M. H. Jaynes, and J. C. Lawrence. 1957. The production of gram-positive variants by Azotobacter chroococcum. *J. Gen. Microbiol.* 16:572-575.
7. Bisset, K. A., and C. M. F. Hale. 1953. The cytology and life cycle of Azotobacter chroococcum. *J. Gen. Microbiol.* 8:442-448.
8. Boatman, E. S. 1964. Observations on the fine structure of Rhodospirillum rubrum. *J. Cell Biol.* 20:297-311.
9. Bradley, D. E., and D. J. Williams. 1957. An electron microscope study of the spores of some species of the genus Bacillus using carbon replicas. *J. Gen. Microbiol.* 17:75-79.
10. Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. *Bergey's manual of determinative bacteriology*. 7th ed. The Williams and Wilkins Co., Baltimore, Maryland.
11. Carr, N. G. 1966. The occurrence of poly-B-hydroxybutyrate in the blue-green alga, Chlorocytium fritschii. *Biochim. Biophys. Acta.* 120:308-310.
12. Carr, N. G., and J. Lascelles. 1961. Some enzymic reactions concerned in the metabolism of acetoacetic-coenzyme A in *Athiorhodaceae*. *Biochem. J.* 80:70-77.
13. Chowdhury, A. A. 1963. Poly-B-hydroxybuttersaure abbauende Bakterien und Exoenzym. *Arch. Mikrobiol.* 47:167-200.

14. Cohen, G. H., and D. B. Johnstone. 1963. Acid production by Azotobacter vinelandii. Nature 198:211.
15. Cohen, G. H., and D. B. Johnstone. 1964. Extracellular polysaccharides of Azotobacter vinelandii. J. Bacteriol. 88:329-338.
16. Cohen, G. H., and D. B. Johnstone. 1964. Capsular polysaccharide of Azotobacter agilis. J. Bacteriol. 88:1695-1699.
17. Dawes, E. A., and D. W. Ribbons. 1964. Some aspects of the endogenous metabolism of bacteria. Bacteriol. Rev. 28:126-149.
18. Delafield, F. P., K. Cooksey, and M. Doudoroff. 1965. B-hydroxybutyrate dehydrogenase and dimer esterase of Pseudomonas lemoignei. J. Biol. Chem. 240:4023-4028.
19. Delafield, F. P., M. Doudoroff, N. J. Palleroni, C. J. Lusty, and R. Contopoulos. 1965. Decomposition of poly-B-hydroxybutyrate by pseudomonads. J. Bacteriol. 90:1455-1466.
20. Doudoroff, M., and R. Y. Stanier. 1959. Role of poly-B-hydroxybutyric acid in the assimilation of organic carbon by bacteria. Nature 183:1440-1442.
21. Dworkin, M. 1966. Biology of the myxobacteria. Ann. Rev. Microbiol. 20:75-106.
22. Eagon, R. G., and K. J. Carson. 1965. Lysis of cell walls and intact cells of Pseudomonas aeruginosa by ethylenediamine tetraacetic acid and by lysozyme. Can. J. Microbiol. 11:193-201.
23. Eagon, R. G., G. P. Simmons, and K. J. Carson. 1965. Evidence for the presence of ash and divalent metals in the cell wall of Pseudomonas aeruginosa. Can. J. Microbiol. 11:1041-1042.
24. Eisenstark, A., K. G. McMahon, and R. Eisenstark. 1950. A cytological study of a pleomorphic strain of Azotobacter agile with the electron and phase microscopes and the Robinow nuclear staining technique. J. Bacteriol. 59:75-81.
25. Eisenstark, A., C. B. Ward, and T. S. Kyle. 1950. A study of large bodies in Azotobacter agile. J. Bacteriol. 60:525-531.
26. Eklund, C., L. M. Pope, and O. Wyss. 1966. Relationship of encapsulation and encystment in Azotobacter. J. Bacteriol. 92:1828-1830.
27. Eklund, C., and O. Wyss. 1962. Enzyme associated with bacteriophage infection. J. Bacteriol. 84:1209-1215.

28. Foerster, H. F., and J. W. Foster. 1966. Endotrophic calcium, strontium, and barium spores of Bacillus megaterium and Bacillus cereus. J. Bacteriol. 91:1333-1345.
29. Forsyth, W. G. C., A. C. Hayward, and J. B. Roberts. 1958. Occurrence of poly-B-hydroxybutyric acid in aerobic gram-negative bacteria. Nature 182:800-801.
30. Freeman, J. A. and B. O. Spurlock. 1962. A new epoxy embedment for electron microscopy. J. Cell. Biol. 13:437-443.
31. Garbosky, A. J., and N. Giambiagi. 1963. Thermoresistance chez les Azotobacteriaceae. Ann. Inst. Pasteur. 105:202-208.
32. Goldschmidt, M. C., and O. Wyss. 1966. Chelation effects on Azotobacter cells and cysts. J. Bacteriol. 91:120-124.
33. Gottschalk, G. 1964. Die biosynthese der Poly-B-hydroxybuttersaure durch Knallgasbakterien. Arch. fur Mikrobiol. 47:225-229.
34. Grelet, N. 1952. Le determinisme de la sporulation of Bacillus megaterium. IV. Constituants minéraux du milieu synthétique necessaires a la sporulation. Ann. Inst. Pasteur 83:71-79.
35. Hayward, A. C. 1959. Poly-B-hydroxybutyrate inclusions in the classifications of aerobic gram-negative bacteria. J. Gen. Microbiol. 21:2-3.
36. Hayward, A. C., W. G. C. Forsyth, and J. B. Roberts. 1959. Synthesis and breakdown of poly-B-hydroxybutyric acid by bacteria. J. Gen. Microbiol. 20:510-518.
37. Hiller, A., J. Plazin, and D. Van Slyke. 1948. A study of conditions for Kjeldahl determination of nitrogen in proteins. J. Biol. Chem. 176:1401-1420.
38. Humphrey, B., and J. M. Vincent. 1962. Calcium in cell walls of Rhizobium trifolii. J. Gen. Microbiol. 29:557-561.
39. Jeffrey, H., and J. Alvarez. 1961. Liquid scintillation counting of carbon-14. Use of ethanolamine-ethyleneglycol monomethyl ether-toluene. Anal. Chem. 33:612-615.
40. Jensen, H. L. 1954. The Azotobacteriaceae. Bacteriol. Rev. 18:195-214.
41. Jensen, H. L. 1955. Azotobacter macrocytogenes n. sp., a nitrogen-fixing bacterium resistant to acid reaction. Acta Agric. Scand. 5:280-294.

42. Jensen, V. 1959. Production of acid from arabinose and xylose by strains of Azotobacter. Nature 198:211.
43. Jones, D. H. 1920. Further studies on the growth cycle of Azotobacter. J. Bacteriol. 5:325-342.
44. Kallio, R. E., and A. Harrington. 1960. Sudanophilic granules and lipid of Pseudomonas methanica. J. Bacteriol. 80:321-324.
45. Keeler, R. F., and M. L. Gray. 1960. Antigenic and related biochemical properties of Listeria monocytogenes. 1. Preparation and composition of cell wall material. J. Bacteriol. 80:683-692.
46. Kramer, M. J. 1966. Characterization of the central body of the Azotobacter cyst. Master's Thesis. Louisiana State University, Baton Rouge.
47. Kyle, T. S. and Eisenstark, A. 1951. The genus Azotobacter. Bull. Oklahoma Agricultural and Mechanical College. 48:1-49.
48. Law, J. H., and R. A. Slepecky. 1961. Assay of poly-B-hydroxybutyric acid. J. Bacteriol. 82:33-36.
49. Lawrence, J. C. 1955. Filterability of the swarmers of Rhizobium and Azotobacter. Nature 176:1033-1034.
50. Layne, J. S., and E. J. Johnson. 1964. Natural factors involved in the induction of cyst formation in Azotobacter. J. Bacteriol. 87:684-689.
51. Layne, J. S. and E. J. Johnson. 1964. Resistant properties of Azotobacter cysts induced in response to mineral deficiencies. J. Bacteriol. 88:956-959.
52. Leive, L. 1965. Release of lipopolysaccharide by EDTA treatment of E. coli. Biochem. Biophys. Res. Commun. 21:290-296.
53. Lemoigne, M. 1927. Etudes sur l'autolyse microbienne. Origine de l'acide B-hydroxybutyrique forme par autolyse. Ann. Inst. Pasteur 41:148.
54. Lemoigne, M., B. Delaporte, and M. Croson. 1944. Contribution a l'etude botanique et biochimique des bacteries du genre Bacillus. 2. Valeur du test des lipides B-hydroxybutyriques pour la caracterisation des especes. Ann. Inst. Pasteur 70:224.
55. Lemoigne, M., and H. Girard. 1943. Reserves lipidiques B-hydroxybutyriques chez Azotobacter chroococcum. Compt. Rend. Acad. Sci. Paris 217:557-558.

56. Lemoigne, M., N. Grelet, and M. Croson. 1950. Sur l'origine des lipides B-hydroxybutyriques formes par processus microbien. Bull. Soc. Chim. Biol., Paris 32:719. (Cited by Macrae and Wilkinson, 62)
57. Levine, H. B., and H. Wolochow. 1960. Occurrence of poly-B-hydroxybutyrate in Pseudomonas pseudomallei. J. Bacteriol. 79:305-306.
58. Lin, L. P. 1966. Germination and outgrowth of the Azotobacter cyst. Doctoral Dissertation. Louisiana State University, Baton Rouge.
59. Löhnis, F., and N. R. Smith. 1923. Studies upon the life cycle of the bacteria. Part II. Life history of Azotobacter. J. Agr. Res. 23:401-432.
60. Lundgren, D. G., R. Alper, C. Schnaitman, and R. H. Marchessault. 1965. Characterization of poly-B-hydroxybutyrate extracted from different bacteria. J. Bacteriol. 89:245-251.
61. Macrae, R. M., and J. F. Wilkinson. 1958. Poly-B-hydroxybutyrate metabolism in washed suspensions of Bacillus cereus and Bacillus megaterium. J. Gen. Microbiol. 19:210-222.
62. Macrae, R. M., and J. F. Wilkinson. 1958. The influence of the cultural conditions on poly-B-hydroxybutyrate synthesis in Bacillus megaterium. Proc. Roy. Soc. Edinburgh 27:73-78.
63. Martinez, R. J. 1963. On the nature of the granules of the genus Spirillum. Arch. Mikrobiol. 44:334-343.
64. Merrick, J. M., and M. Doudoroff. 1961. Enzymatic synthesis of poly-B-hydroxybutyric acid in bacteria. Nature 189:890-892.
65. Merrick, J. M., and M. Doudoroff. 1964. Depolymerization of poly-B-hydroxybutyrate by an intracellular enzyme system. J. Bacteriol. 88:60-71.
66. Merrick, J. M., D. G. Lundgren, and R. M. Pfister. 1965. Morphological changes in poly-B-hydroxybutyrate granules associated with decreased susceptibility to enzymatic hydrolysis. J. Bacteriol. 89:234-239.
67. Morris, M. B., and J. B. Roberts. 1959. A group of pseudomonads able to synthesize poly-B-hydroxybutyric acid. Nature 183:1538-1539.

68. Mortenson, L. E., P. B. Hamilton, and P. W. Wilson. 1955. Dissimilation of 6-phosphogluconate by Azotobacter vinelandii. Biochim. Biophys. Acta 16:238.
69. Mortenson, L. E., and P. W. Wilson. 1954. Initial stages in the breakdown of carbohydrates by Azotobacter vinelandii. Arch. Biochem. Biophys. 53:425-435.
70. Mortenson, L. E., and P. W. Wilson. 1955. Metabolism of ribose-5-phosphate by Azotobacter vinelandii. J. Biol. Chem. 213:713-721.
71. Mulder, E. G., M. H. Deinema, W. L. Van Neen, and L. P. T. M. Zevenhuizen. 1962. Polysaccharides, lipids, and poly- β -hydroxybutyrate in microorganisms. Rec. Trav. Chim. 81:797-809.
72. Parker, L. T., and M. D. Socolofsky. 1966. Central body of the Azotobacter cyst. J. Bacteriol. 91:297-303.
73. Peel, D., and J. R. Quagle. 1961. Microbial growth on C₁ compounds. I. Isolation and characterization of Pseudomonas AM 1. Biochem. J. 81:465-469.
74. Primosigh, J., H. Pelzer, D. Maass, and W. Weidel. 1961. Chemical characterization of mucopeptides released from the E. coli B cell wall by enzymatic action. Biochem. Biophys. Acta 46:68-80.
75. Provost, P. J., and R. N. Doetsch. 1962. An appraisal of Caryophanon latum. J. Gen. Microbiol. 28:547-557.
76. Rogers, H. J. 1955. The outer layers of bacteria: the biosynthesis of structure., p. 186. In M. R. Pollock, and M. H. Richmond (ed.), Function and structure in micro-organisms. The Syndics of the Cambridge University Press, London.
77. Romanow, I. 1965. Recherches sur la synthese de l'acide indole-3-acetique par les Azotobacter. Ann. Inst. Pasteur 109:280-292.
78. Rouf, M. A., and J. L. Stokes. 1962. Isolation and identification of the sudanophilic granules of Sphaerotilus natans. J. Bacteriol. 83:343-347.
79. Salton, M. R. J., and J. M. Ghuyssen. 1960. Acetyl-hexosamine components enzymatically released from Micrococcus lysodeikticus. Biochem. Biophys. Acta 45:455-463.
80. Schlegel, H. G. 1962. Die Speicherstoffe von Chromatium okanii. Arch. Mikrobiol. 42:110-116.

81. Schlegel, H. G., G. Gottschalk, and R. Von Bartha. 1961. Formation and utilization of poly-B-hydroxybutyric acid by Knallgas bacteria (Hydrogenomonas). *Nature* 191:463-465.
82. Sierra, G., and N. E. Gibbons. 1962. Production of poly-B-hydroxybutyric acid granules in Micrococcus halodenitrificans. *Can. J. Microbiol.* 8:249-253.
83. Sierra, G., and N. E. Gibbons. 1962. Role and oxidation pathway of poly-B-hydroxybutyric acid in Micrococcus halodenitrificans. *Can. J. Microbiol.* 8:255-269.
84. Slepecky, R., and J. W. Foster. 1959. Alternations in metal content of spores of Bacillus megaterium and the effect on some spore properties. *J. Bacteriol.* 78:117-123.
85. Slepecky, R. A., and J. H. Law. 1961. Synthesis and degradation of poly-B-hydroxybutyric acid in connection with sporulation of Bacillus megaterium. *J. Bacteriol.* 82:37-42.
86. Sobek, J. M., J. F. Charba, and W. N. Foust. 1966. Endogenous metabolism of Azotobacter agilis. *J. Bacteriol.* 92:687-695.
87. Sobek, J. M., and C. E. Clifton. 1962. Oxidative assimilation and C¹⁴ distribution in Azotobacter agilis. *Proc. Soc. Exp. Biol. Med.* 109:408-411.
88. Socolofsky, M. D., and O. Wyss. 1961. Cysts of Azotobacter. *J. Bacteriol.* 81:946-954.
89. Socolofsky, M. D., and O. Wyss. 1962. Resistance of the Azotobacter cyst. *J. Bacteriol.* 84:119-124.
90. Stanier, R. Y., M. Doudoroff, and E. A. Adelberg. 1963. *The microbial world*. 2nd ed. Prentice-Hall, Inc., Englewood Cliffs.
91. Stanier, R. Y., M. Doudoroff, R. Kunisawa, and R. Contopoulos. 1959. The role of organic substrates in bacterial photosynthesis. *Proc. Natl. Acad. Sci. U. S.* 45:1246-1260.
92. Stevenson, L. H., and M. D. Socolofsky. 1966. Cyst formation and poly-B-hydroxybutyric acid accumulation in Azotobacter. *J. Bacteriol.* 91:304-310.
93. Stewart, J. R., K. E. Olson, and O. Wyss. 1967. Effects of metal ions on growth and encystment of Azotobacter. *Bacteriol. Proc.* p. 43.

94. Still, G. G., and C. H. Wang. 1964. Glucose catabolism in Azotobacter vinelandii. Arch. Biochem. Biophys. 105:126-132.
95. Sugiyama, H. 1951. Studies on factors affecting the heat resistance of spores of Clostridium botulinum. J. Bacteriol. 62:81-96.
96. Sussman, A. S., and H. O. Halvorson. 1966. Spores, their dormancy and germination. Harper and Row, New York.
97. Tchan, Y. T. 1953. Studies on nitrogen-fixing bacteria. III. Azotobacter beijerinckii (Lipman 1903) var. acido-tolerans (Tchan 1952). Proc. Linnean Soc. N. S. Wales 78:83-84.
98. Tchan, Y. T., A. Birch-Andersen, and H. L. Jensen. 1962. The ultrastructure of vegetative cells and cysts of Azotobacter chroococcum. Arch. Mikrobiol. 43:50-66.
99. Tinelli, R. 1955. Etude de la biochimie de la sporulation chez Bacillus megaterium. I. Composition des spores obtenues par carence des differents substrates carbonés. Ann. Inst. Pasteur 88:212-226. (Cited by Wilkinson, 109)
100. Tinelli, R. 1955. Etude de la biochimie de la sporulation chez Bacillus megaterium. II. Modifications biochimiques et échanges gazeux accompagnant la sporulation provoquée par carence de glucose. Ann. Inst. Pasteur 88:364-375. (Cited by Wilkinson, 109)
101. Uppal, B. N., M. K. Patel, and J. A. Daji. 1939. Nitrogen fixation in rice soils. Indian J. Agri. Sci. 9:689-702.
102. Van Schreven, D. A. 1962. Effect of the composition of the growth medium on the morphology and reproduction of Azotobacter chroococcum. Antonie van Leeuwenhoek J. Microbiol. Serol. 28:97-120.
103. Van Schreven, D. A. 1966. Effect of penicillin on the morphology and reproduction of Azotobacter chroococcum. Antonie van Leeuwenhoek J. Microbiol. Serol. 32:67-93.
104. Vela, G. R., and O. Wyss. 1965. Radiation resistance of soil Azotobacter. J. Bacteriol. 89:1280-1285.
105. Vincent, J. M., B. Humphrey, and R. J. North. 1962. Some features of the fine structure and chemical composition of Rhizobium trifolii. J. Gen. Microbiol. 29:551-555.
106. Vogt, J. C., W. C. McDonald, and H. M. Nakata. 1967. Effect of intracellular poly- β -hydroxybutyrate on the ultraviolet sensitivity of Bacillus cereus. Radiation Res. 30:140-147.

107. Waksman, S. A., M. Hotchkiss, and C. L. Casey. 1933. Marine bacteria and their role in the cycle of life in the sea. II. Bacteria concerned in the cycle of nitrogen in the sea. *Biol. Bull.* 65:137-167.
108. Weibull, C. 1953. Characterization of the protoplasmic constituents of Bacillus megaterium. *J. Bacteriol.* 66:696-702.
109. Wilkinson, J. F. 1959. The problem of energy-storage compounds in bacteria. *Exp. Cell Res. Suppl.* 7:111-130.
110. Williamson, D. H., and J. F. Wilkinson. 1958. The isolation and estimation of the poly- β -hydroxybutyrate inclusions in Bacillus species. *J. Gen. Microbiol.* 19:198-209.
111. Wilson, P. W., J. F. Hull, and R. H. Burris. 1943. Competition between free and combined nitrogen in nutrition of Azotobacter. *Proc. Natl. Acad. Sci. U. S.* 29:289-294.
112. Wilson, P. W., and S. G. Knight. 1952. Experiments in bacteriol physiology. Burgess Publishing Co., Minneapolis.
113. Wilson, T. G. G., and P. W. Wilson. 1955. The terminal oxidation system of Azotobacter vinelandii. *J. Bacteriol.* 70:30-34.
114. Winogradsky, S. 1938. Sur la morphologie et l'oecologie des Azotobacter. *Ann. Inst. Pasteur* 60:351-400.
115. Wyss, O., M. G. Neumann, and M. D. Socolofsky. 1961. Development and germination of the Azotobacter cyst. *J. Biophys. Biochem. Cytol.* 10:555-565.

VITA

Luther Harold Stevenson was born March 18, 1940 in Bogalusa, Louisiana. He graduated from Bogalusa High School in 1958 and was married to Elizabeth Ann Parker in January, 1961. They have one daughter, Sheila Terae. He received his Bachelor of Science degree in May, 1962 from Southeastern Louisiana College and the Master of Science degree from Louisiana State University in August, 1964. He is presently a candidate for the Doctor of Philosophy degree in Microbiology at Louisiana State University in Baton Rouge, Louisiana.

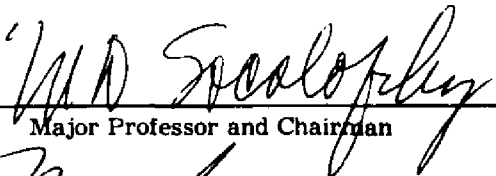
EXAMINATION AND THESIS REPORT


Candidate: Luther Harold Stevenson

Major Field: Microbiology

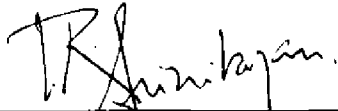
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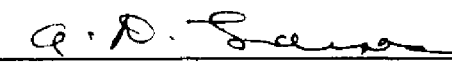

Major Professor and Chairman

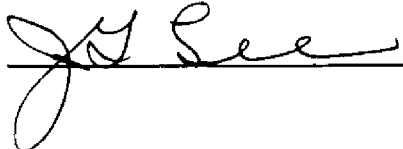

Dean of the Graduate School

EXAMINING COMMITTEE:









Date of Examination:

July 14, 1967