ELECTRON MICROSCOPIC STUDY OF OUTER COAT FORMATION IN AZOTOBACTER VINELANDII CYSTS

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ABSTRACT

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By

Victoria M. Koo

A study was made of the morphological events which occur during the formation of outer coats of *Azotobacter vinelandii* cysts. Cells were grown to late exponential phase in Burk's medium (1% glucose), washed, and induced to encyst in buffered β-hydroxybutyrate (0.2%). Mature cysts were formed within 72 hours. During the course of encystment, samples were prepared for electron microscopy by a modified Kellenberger procedure. At 3-5 hours cells were rounded, non-motile and surrounded by a capsule. At approximately 6 hours, protrusions or "blebs" appeared at the cell surface. These broke off and formed globules which migrated toward the surrounding capsular material. This material appeared to provide a matrix where the globules coalesced, increased in size, flattened and formed membrane-like structures similar to the exine layers. The number of "blebs" per cell increased up to 18-30 hours, when they began to decrease as cysts matured.

The data are consistent with the hypothesis that, upon induction of encystment, synthesis of membranous components and capsule polymer continues in the absence of cell division. The continued deposition of these materials produces the exine.

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INTRODUCTION

The family Azotobacteriaceae consists of the single genus *Azotobacter* and is widely distributed in soil and water. Two characteristics distinguish the six members of the Azotobacteriaceae from other bacteria. These are (1) their relatively large cell size, and (2) their ability to fix atmospheric nitrogen when provided with carbohydrate or some other suitable energy source. Although there are other heterotrophic bacteria which are capable of fixing nitrogen, only the *Azotobacter* species are able to incorporate greater than 10 mg of atmospheric nitrogen / gram of carbohydrate consumed (23).

Azotobacter vinelandii is characterized by its relatively large rod-like appearance with an average cell size of 2.4 μm x 0.5 μm. The cells are Gram-negative, motile by means of peritrichous flagella and are obligate aerobes, usually growing in a film on the surface of non-agitated culture medium.

The *Azotobacter* species are able to undergo cyclic differentiation (27, 33, 37, 41). Upon induction, vegetative cells give rise to specialized resting cells, cysts, which, upon germination develop once more into vegetative cells. The cysts are thick-walled, refractile,

spherical, resting cells. They are more resistant than vegetative cells to the lethal action of ultraviolet light, sonic treatment and dessication (34). Unlike vegetative cells, cysts can be disrupted by the addition of chelating agents in the absence of lysozyme (33). Although cysts and spores are both specialized resting cells of Eubacteria, cysts differ from the endospores in that they do not possess any significant thermal resistance, do not contain dipicolinic acid (33), and the loss of calcium from cysts does not produce a loss of viability nor a decrease in resistance to dessication (32).

Little is known of the initiation of encystment or of the temporal sequence of the biochemical events that take place. Normal butanol (41) or β-nydroxybutyrate (17) will induce this process after exponential growth is completed. Cytological studies of encysting Azotobacter by light microscopy have been reported by Winogradsky (41), Pochan, et al. (27) and Vela and Wyss (38). Electron microscopic observations have suggested that mature cysts are modified vegetative cells with thick coats composed of two layers, the intine and the exine (13, 17, 33, 37, 44).

Recently the morphological changes which occur during encystment have been studied by electron microscopy (43). These authors used $KMnO_4$ as a fixative in preparing their cells. The resolution of their micrography may

have been limited by their choice of fixative. The shortcomings of $KMnO_4$ fixation and the artifacts which are produced have been observed by several investigators. Luft (21) noted that cell membranes appeared as a tracery of thin but very dark lines against a pale background. This was probably due to the extraction of many other cytoplasmic constituents (26, 38). Hake (9) observed that $KMnO_4$ solubilized certain proteins and left the remainder severely altered. Ribosomes were not visible in $KMnO_4$-fixed specimens of animal cells and lipid droplets were poorly preserved (21, 38).

In order to minimize artifacts in the observations of the morphological events leading to the encysted cell, other methods of fixation were considered. Osmium tetroxide is probably the best general purpose fixative known to date. It is used to fix biological materials because it preserves protein structures by cross-linking. Staining of the specimens results from the deposition of the fixative in the vicinity of substances rich in double bonds (9, 14, 36). Glutaraldehyde also preserves fine structure by cross-linking side chains in proteins and hydroxyl groups of polyalcohols. This results in insolubilization of many proteins and gives a relatively undistorted fixation of cellular structures (16, 31). When glutaraldehyde and OsO_4 are used together in

fixation, membranous structures in biological materials can be observed in great detail (31, 38).

Encysting cells of Azotobacter vinelandii were fixed in either OsO_4 or a combination of glutaraldehyde and OsO_4, embedded, sectioned, and their structural development was viewed with an electron microscope. The progressive morphogenesis of Azotobacter vinelandii cysts is presented in this dissertation.

REVIEW OF LITERATURE

Previous interest in the family Azotobacteriaceae was based on their ability to utilize, asymbiotically, molecular nitrogen as their sole nitrogen source. Although other microorganisms including blue-green algae, purple and green bacteria and certain members of the genera Clostridium and Desulfovibrio fix nitrogen, few incorporate as much as the Azotobacter species (15-16 mg nitrogen/gram of glucose, sucrose, or mannitol) (10, 23). Six species have been isolated in pure culture and described in the literature: Azotobacter chroococcum, Azotobacter agilis (agile), Azotobacter vinelandii, Azotobacter beijerinckii, Azotobacter insignis (insigne), and Azotobacter macrocytogenes. They are all Gram-negative and obligate aerobes. Two types of pigments are produced by the Azotobacteriaceae: non-water soluble and water-soluble pigments. Non-water soluble pigments give rise to pigmented colonies whereas water-soluble pigments diffuse into the agar medium (23).

Azotobacter vinelandii was first isolated and described by Lipman in 1904 (20) and is characterized by a relatively large rod or peanut-shaped vegetative cell with an average individual cell size of 2.4 μm x 0.5 μm.

This organism produces a soluble green pigment which fluoresces apple-green under ultraviolet light (23). The bacteria are motile by peritrichous flagella (1) and produce a resting cell known as a cyst.

Certain groups of bacteria are capable of undergoing physiological and morphological changes which give rise to the formation of dormant cells. These resting cells include the endospores which are produced by Gram-positive rods of the genera Bacillus and Clostridium and by the coccus Sporosarcina and cysts, which are produced by the genus Azotobacter. The dormant state may persist over long periods of time but, under suitable conditions, spores and cysts germinate and give rise to new vegetative cells. The morphology, biochemistry and physiology of endospores have been extensively studied, whereas relatively little is known about cysts.

Like the endospores, cysts have a morphology which is distinct from that of their respective vegetative cells. Cysts are thick-walled, spherical bodies that are refractile and produced from rod-shaped vegetative cells (33). Socolofsky and Wyss (34) noted that cysts have different physiological properties than vegetative cells. They are more resistant to dessication, sonic treatment, and ultraviolet irradiation than vegetative cells and endogenous O_2 uptake is not readily detectable. In addition, cysts differ from vegetative cells in that cysts

can be lysed by the chelating agent ethylenediaminetetra-acetic acid (EDTA) in the absence of lysozyme (7, 33).

Upon comparing spores and cysts, it is noted that cysts differ from endospores in many aspects. Cysts do not possess any significantly greater heat resistance than vegetative cells (33). Endospores contain greater quantities of calcium than vegetative cells and have a compound, dipicolinic acid (2,6-pyridinedicarboxylic acid) not found in vegetative cells. Socolofsky and Wyss (33) observed that dipicolinic acid, which is a chelating agent, ruptured cysts. Smith, et al. (32) found that the loss of calcium from cysts did not decrease their viability or decrease their resistance to dessication.

The morphological, biochemical and physiological events of encystment are not well defined. Little is known of its initiation although Winogradsky (40) observed that compounds such as ethanol or butanol, serving as carbon source, would induce encystment.

Layne and Johnson (15) showed that cultures of A. vinelandii, which were grown in limiting concentrations of Ca, Fe, Mg, or Mo in the medium gave rise to atypical cysts. These resting cells did not possess the characteristic exine and intine of cysts which developed from cells grown with butanol as a carbon source. Parker and Wyss (25) noted that the defective cysts were not

dessication-resistant and concluded that the outer coats were involved in the resistance of cysts to lethal agents.

The polymeric ester of D-(-)-β-hydroxybutyric acid, poly-β-hydroxybutyric acid (PHB) is a major storage compound unique to a variety of bacteria genera including Azotobacter, Bacillus, Rhizobium, Actinobacillus, Spirillum, Vibrio, Chronobacterium and Micrococcus (4, 6). Steveson and Socolofsky (35) observed a relationship between the content of PHB in cells and cyst formation in A. vinelandii. Glucose and n-butanol, which promoted the production of PHB in cells, also led to the production of cysts. Lin and Sadoff (17) studied the oxidation products of n-butyl alcohol and found that β-hydroxybutyrate or crotonate would induce encystment. They found that glucose inhibited encystment while glucose plus β-hydroxybutyrate promoted an abortive encystment which was characterized by the appearance of a disorganized exine and the release of a highly viscous polymer into the medium.

The morphology and cytology of Azotobacter species was studied by Winogradsky (41) and Pochon, et al. (27) who noted that only young vegetative cells of Azotobacter vinelandii were rod-shaped and motile, whereas older cells were oval and non-motile. Carr (3) showed by electron microscopy that the vegetative cells in stationary phase were surrounded by a dense fibrous capsule. He

speculated that the synthesis of this material proceeded outwards from the cell wall and was followed by an interwinding of individual layers at the outer limits of the capsule. He did not detect a limiting membrane at the capsule periphery. Proctor (28) regarded the cyst coat as a specialized capsule of greater complexity.

Using the techniques of metal shadowing and negative staining, Baillie, et al. (1) demonstrated that the flagellar array in the Azotobacteriaceae is variable. Azotobacter vinelandii, Azotobacter agilis and Azotobacter chroococcum are peritrichously flagellated. Azotobacter insigne and Azotobacter macrocytogenes both have polar flagellation with one or two flagella. Azotobacter beijerinckii are non-motile and do not have flagella.

The fine structure of Azotobacter species cysts have been studied by means of electron microscopy. Socolofsky and Wyss (33), Wyss, et al. (44), and Lin and Sadoff (17), studying thin sections of $KMnO_4$-fixed Azotobacter vinelandii, suggested that cysts are modified vegetative cells with a thick wall composed of two layers: the exine and the intine. The dense central body of the cyst is surrounded by the electron-transparent material of the intine, which in turn, is surrounded by an electron-dense exine composed of several overlapping layers.

Tchan and co-workers (37) described cyst formation in cells fixed in OsO_4. They observed that the vegetative cells were surrounded by a triple-layered cytoplasmic membrane, which, in turn, was enclosed by a triple-layered cell wall. During early encystment, a loose membrane was produced "which surrounded the vegetative cell like a veil." The exocystorium (exine) was composed of a series of these membranes, each of which was triple-layered and 70-80 Å thick. The intine was a homogeneous matrix between the central body and the exine layers. Numerous loosely packed, randomly oriented triple-layered units similar to those in the outer matrix were found in the intine. They suggested that these units accumulated near the exine and eventually formed the thick multilayered exine.

Employing the method of freeze-cleaving, Koo, et al. (13) demonstrated that the surface of cysts appeared to be composed of several overlapping layers. This view is consistent with that of Lin and Sadoff (19) who observed cysts in thin sections, by negative staining and shadow casting.

Wyss, et al. (43) have reported the morphological events that take place during the process of endogenous encystment of Azotobacter vinelandii. They showed that the vegetative cells became spherical and were surrounded by a fibrous capsule. Fragments of exine were laid down

along the outer edges of the fibrous capsule with a concommitant compaction of the central body. The narrow intine appeared as a space between the central body and the exine.

Successful electron microscopy of thin sections of biological materials requires chemical fixation, dehydration, infiltration of the specimen with plastic monomers, polymerization of the resin, sectioning, and heavy metal staining of the thin sections. Many of the artifacts seen in electron micrographs are due to the improper use or choice of fixatives.

Potassium permanganate was first introduced as a fixative by Luft (21). He demonstrated that the oxidative potential of this fixative could be applied to preserving lipoprotein complexes in membrane systems. Other cellular components are leached out by the fixative so that the membranes appear as thin dark lines against a pale background. Trump and Ericsson (38) observed that the membrane contours of cells often appeared distorted. Lipid droplets and ribosomal particles were not visible and membrane-limited structures, such as mitochondria in animal cells, were swollen and distorted. Hake (9) and Lenard and Singer (16) both noted that $KMnO_4$ solubilized certain proteins and left others markedly changed.

The treatment of biological materials with OsO_4 results in relatively artifact-free fixation. Originally

unbuffered OsO_4 was used, but it was found to produce structural changes which were probably due to acidification of the tissue. Palade in 1952 (24) developed a slightly alkaline buffered osmium tetroxide (acetate-veronal) solution. In 1958 Kellenberger and co-workers (11) developed a method of fixation to study the DNA and heads of phage. This technique, now known as the Ryter-Kellenberger standard procedure, is widely used in bacterial cytology. Cells are fixed in 1% OsO_4 in acetate-veronal buffer of Michaelis and washed in 0.5% uranyl acetate solution. They are dehydrated in acetone and embedded in polyester Vestopal W. The method used in this study was a slight modification of the Kellenberger method as described by Burnham, et al. (2).

Hake (9) and Stoeckenius and Mahr (36) showed that OsO_4 functioned in two ways. It preserved protein and reacted with unsaturated lipids to produce electron staining. One limitation in the use of OsO_4 is that it does not penetrate tissue rapidly.

Sabatini, et al. (31) found that glutaraldehyde was a good cross-linking fixative which reacted rapidly with active hydrogen, amino and imino groups in proteins and hydroxyl groups of polyalcohols. Many proteins became insoluble and the cellular structures remained relatively undistorted (16). Furthermore, the tissue penetration was rapid. Various investigators (31, 38) have

recommended the use of a combination of glutaraldehyde and OsO_4 fixation in order to achieve the best resolution of the membranous structures in biological materials.

EXPERIMENTAL METHODS

The object of the research was to describe the sequential events that take place during the process of encystment by Azotobacter vinelandii employing electron microscopy. The following is a description of the materials and techniques used in sample preparation.

Strain and Cultivation

Azotobacter vinelandii ATCC 12837 was used throughout the experiments and was cultivated in Burk's nitrogen-free medium plus 1% glucose at 30°C on a rotary shaker. The medium, described by Wilson and Knight (40) contains the following per liter: 0.2 g KH_2PO_4, 0.8 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaSO_4 \cdot 2H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$ and 0.25 mg $Na_2MoO_4 \cdot 2H_2O$. The carbon sources were dissolved separately in distilled water and sterilized by autoclaving. Burk's medium without the carbon source is termed Burk's buffer.

Preparation of Cysts

Vegetative cells of A. vinelandii can be induced to encystment by the addition of n-butanol (33) or β-hydroxybutyrate (17) as a source of carbon. Two 500 ml Erlenmeyer flasks, each containing 100 ml of Burk's

buffer with 1% glucose, were inoculated with A. vinelandii from a slant. The vegetative cells were grown at 30°C on a rotary shaker for 18 hours, harvested and resuspended in 100 ml of fresh Burk's buffer containing 0.2% β-hydroxybutyrate. Flasks were then returned to the rotary shaker and incubated at 30°C. At 3-hour intervals during encystment, 10-ml samples were withdrawn, washed, fixed and embedded in Epon. Cysts were also prepared by growth of A. vinelandii on 0.2% β-hydroxybutyrate agar plates.

Fixation, Embedding and Sectioning

The fixation and embedding procedures were those of Burnham, et al. (2) and consisted of the following steps: prefixation with glutaraldehyde, embedding in agar, postfixation with osmium tetroxide, uranyl acetate staining, dehydration and transfer into the embedding resin. The following solutions were used:

1) veronal buffer containing 2.94 g sodium barbital (barbitone sodium), 1.94 g sodium acetate (hydrate), 3.40 g NaCl and distilled water to 100 ml;

2) Kellenberger's buffer having 5 ml veronal buffer, 13 ml distilled water, 0.25 ml 0.1 M $CaCl_2$ and 6.5 to 7.0 ml 0.1 N HCl to adjust the pH to 6.0;

3) Kellenberger's fixative containing 1% (w/v) OsO_4 made by dissolving 0.5 g OsO_4 (Eastman Organic Chemical, Rochester, New York) in 50 ml Kellenberger's buffer;

4) buffered agar made by dissolving 1 g Bacto Noble agar (Difco Laboratories) in 50 ml Kellenberger's buffer and warmed to 50°C prior to use;

5) tryptone medium containing 1 g Bacto tryptone (Difco Laboratories) and 0.5 g NaCl in 10 ml distilled water;

6) uranyl acetate solution consisting of 0.5 g uranyl acetate dissolved in 100 ml Kellenberger's buffer.

The Epon embedding material contained 5 ml Epon 812, 5 ml Epon 815, 16 ml dodecenylsuccinic anhydride (DDSA) and 0.4 ml 2,4,6-tri(dimethyl-aminomethyl) phenol (DMP-30) (Polysciences, Inc., Warrington, Pa.).

In a preliminary fixation step, cells in a 10 ml volume were mixed with 1 ml of Kellenberger's fixative and removed from suspension by centrifugation in an International clinical centrifuge (Model CL) for 5 minutes. Alternatively, cells were prefixed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.3 for 1-48 hours. The supernatant was removed and a few drops of warm buffered agar was added to the pellet, quickly mixed and spread onto a clean glass microscope slide to harden. Small cubes of about 1 mm^3 were cut out and then placed in a test tube along with 2 ml of Kellenberger's fixative and 0.2 ml tryptone medium. The test tube was covered with parafilm and left under a ventilated hood for 24-48 hours.

After fixation was complete, 10 ml of Kellenberger's buffer was added to the test tube, mixed and poured off. This was done to remove excess osmium prior to post-fixation staining. The cubes were washed with uranyl acetate solution, soaked in 5-10 ml of uranyl acetate solution for 2.5 hours, and dehydrated prior to embedding in Epon. The dehydration step consisted of passing the agar embedded cells through a graded concentration series of 70, 90, 95, and 100% (anhydrous) acetone solutions at approximately 3/4 to 1 hour intervals each.

To promote the penetration of the resin into the biological material, the specimens were immersed in increasing concentrations of the Epon with the DMP-30, a tertiary amine accelerator. The cubes were first placed in a 3:1 acetone:Epon mixture for 15 minutes, and then into 1:1 acetone:Epon mixture for 1 hour. Equilibration with the 1:3 acetone:Epon required 2-5 hours after which the cubes were transferred into the undiluted Epon mixture and held overnight, and then in the Epon mixture (with DMP-30) for 12 hours. These operations were carried out in a vacuum dessicator at room temperature. Polyethylene Beem capsules (Polysciences, Inc.) were filled with fresh Epon mixture and the bacterial samples were gently pushed to the bottom of the capsules. These samples were then placed overnight in a 40°C oven and

then moved into a 60°C vacuum dessicator for 3-5 days for polymerization of the Epon resin.

Thin sections of fixed, embedded cells were cut with diamond knives (Ge-Fe-Ri, Frosinone, Italy) on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.). These were mounted on 300 and 400 mesh copper grids (Ted Pella Co., Altadena, Calif.) and post-stained with lead citrate for 5 minutes in the presence of KOH to avoid lead carbonate precipitation. A simplified lead citrate stain described by Fahmy (5) was made by dissolving 1 pellet of NaOH in 50 ml of 3X distilled H_2O followed by the addition of 0.25 g of chemically purified grade lead citrate (Pfaltz and Bauer, Inc., Flushing, New York).

Negative Staining

To study the distribution of flagella of Azotobacter vinelandii, bacterial samples were supported on coated grids which were made from a stock solution of 0.4% parlodion in amyl acetate. Parlodion is the trade name of a nitrocellulose plastic manufactured by the Mallinckrodt Chemical Works, St. Louis, Missouri. The coatings were stabilized by evaporating carbon (45° angle) onto the surface with a Varian vacuum evaporator (Varian Vacuum Division, Palo Alto, Calif.).

A drop of cell suspension was placed carefully on the coated grid for 2-3 minutes and the excess was removed with filter paper. A drop of 1% phosphotungstic acid (PTA) buffered with 1 N KOH to pH 6.8-6.9 was added and immediately withdrawn with paper from the coated grid.

Electron Microscopy

All electron micrographs were taken using the Philips 300 electron microscope with 60 kv acceleration. Kodak electron image plates (3 1/4 x 4") were exposed for 1 second and were then developed for 3 minutes in Kodak HRP developer. Following development, the plates were washed in distilled water, fixed for 5 minutes in Kodak Rapid Fixer, washed for 0.5 hours, and passed through a solution of Kodak Photo-flo. Positive prints were made on Kodak single weight Kodabromide Photographic paper.

RESULTS

The research was undertaken to observe the morphogenesis of Azotobacter vinelandii during the course of encystment. Electron microscopy was the technique used in viewing structural changes. The data, therefore, are presented as a series of electron micrographs which show the sequential development of the cyst outer coat. In these micrographs, there appeared to be no differences between OsO_4 fixation and a combination of glutaraldehyde and OsO_4 fixation. In micrographs obtained by either procedure the encysting cell or central body contained dark polyribosomes and poly-β-hydroxybutyrate granules. The delicate strands of DNA were often obscured by the large lipid granules. The central body was enclosed by a "double-track" membrane, which in turn was surrounded by a "double-track" cell wall.

Vegetative growth occurred for approximately 18-20 hours after inoculating cells of Azotobacter vinelandii into Burk's buffer containing 1% glucose, with no indication that encystment had started. Under the phase contrast microscope, all cells were rod or peanut-shaped, motile and many were in the process of division. Figure 1 is an OsO_4-fixed thin section of a typical Gram-negative

vegetative cell of A. vinelandii. DNA strands and polyribosomal particles can be readily found in the more electron-transparent areas of the cytoplasm while the polyribosomal particles appear as dark, electron-dense granules dispersed throughout the finely granular cytoplasm. Surrounding the cytoplasm is a triple-layered cytoplasmic membrane, which is circumscribed by a triple-layered cell wall. There appears to be a few extrusions or "blebs" which resemble myelin bodies protruding from the cell surface.

Figures 2 and 3 show the progress which occurs in cell division in A. vinelandii. The process involves inward wall growth and constriction of the cell until the organism is literally "pinched-in-two." There is no evidence of septum formation prior to cross wall formation, an event which is common in the division process of Gram-positive bacilli.

Early Encystment Period

Cells were transferred into the encysting medium containing Burk's buffer and 0.2% β-hydroxybutyrate and samples were removed at regular time intervals for viewing under the electron microscope. Synchronously growing cultures of A. vinelandii were not used in this study. However, cytological examination revealed that there was a predominant developmental stage within each time

interval. Although there were overlapping stages within any given sample, a definite trend was observed starting from vegetative cells and ending with mature cysts. The data presented are drawn from three separate experiments in which encystment was observed.

One hour after the induction of encystment, a sample was withdrawn and the cells were negatively-stained with 1% phosphotungstic acid. Figure 4 shows that the cells were still in the vegetative state with typical peritrichous flagella.

The first detectable morphological event in encystment is the conversion of a vegetative cell to a rounded, non-motile cell surrounded by non-structured capsular material (Figures 5, 6, 7, 8). Figures 5 and 6 show cells that were probably in their last division before beginning encystment. Poly-β-hydroxybutyrate granules (PHB), readily observed in these cells, are not encased in a typical "unit" membrane (32). After induction (3-6 hours of encysting) there was an increase in the frequency of the occurrence of protrusions from the cell surface.

As the cells developed toward mature cysts, this budding material or "blebs" extended into the electron-transparent region between the encysting cell and the capsular material. The "blebs" were not extensions of the cytoplasmic membrane but of the cell wall. In these

samples, the protrusions appeared to break free, form globular structures and move outward toward the capsular material. In the course of their migration, globules coalesced, increased in size, and flattened to form triple-layered membranous fragments similar to those which make up the layers of the exine coat (Figures 9, 10). Arrows on the electron micrographs point out the structures described above.

The process of discontinuous layering or "shingling" of the membranous structure in the capsule became more pronounced 12 hours after cells had been transferred into the encystment medium (Figures 11, 12) and continued until encystment was completed. The number of globules and flattened spheroids per cell increased up to 30 hours after which time they decreased in number as the cyst matured. Individual layers of the membranous structure in the exine appeared to be formed at the inner edge of the capsular material. However, after 36 hours the exine layers were noted in the middle or outer edges of the capsule (Figures 13, 14). The central body became more compact and this was timed with a reduction in the number of PHB granules (Figures 15, 16).

Mature Cysts

Sections of mature cysts (4-5 days) are presented in Figures 15, 16, and 17 which reveal the typical

multilayered exine, an electron-transparent area or intine, and a compact, nearly spherical central body. The layers of the exine are arrayed about the outer edges of the capsular material. Intertwined in the capsular matrix are a few membranous-like fragments, each measuring 60-70 Å thick. The intine has no structure which is detectable by electron microscopy. As the cyst matures, the volume occupied by the intine seems to increase. The central body is surrounded by the typical triple-layered wall and triple-layered membrane. Furthermore in mature cysts, polyribosomes and strands of nuclear material were readily observable. In the space between embedded cysts there were fragments of membrane-like structures. These appear to be layers of the exine which peeled from the mature cysts. No structural differences were noted between cysts grown in liquid culture and those grown on agar medium (Figures 15, 16, 17 agar medium; Figure 18 liquid culture).

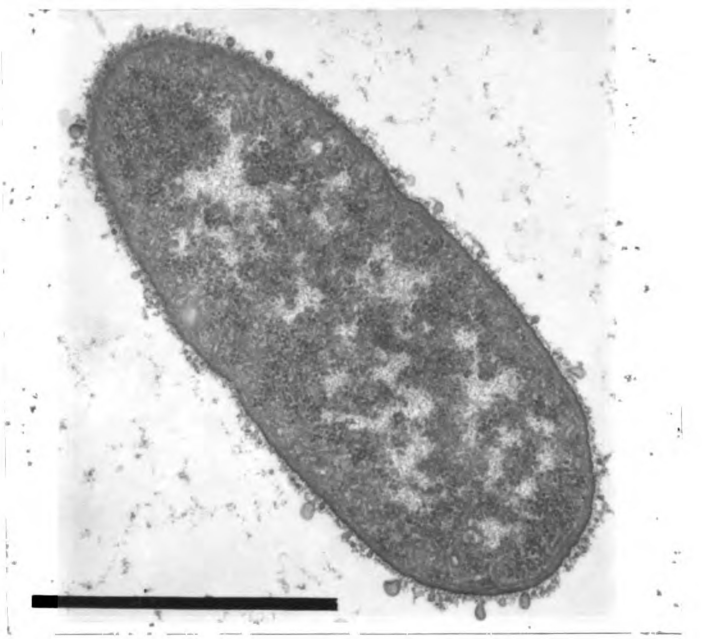

Figure 1.--Thin section of a typical rod-shaped A. vinelandii vegetative cell which has been fixed in OsO_4. Bar indicates 1 μm.

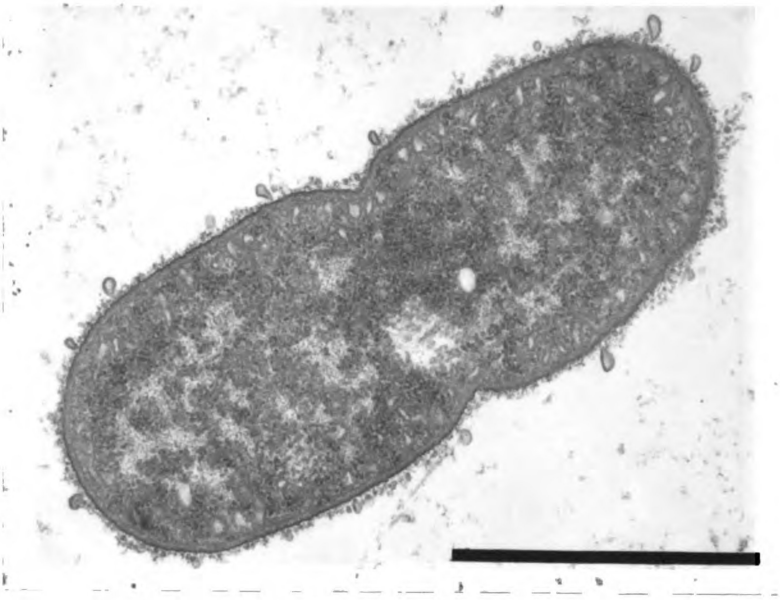

Figure 2.--Thin section of an OsO_4-fixed vegetative cell beginning cell division. Bar indicates 1 μm.

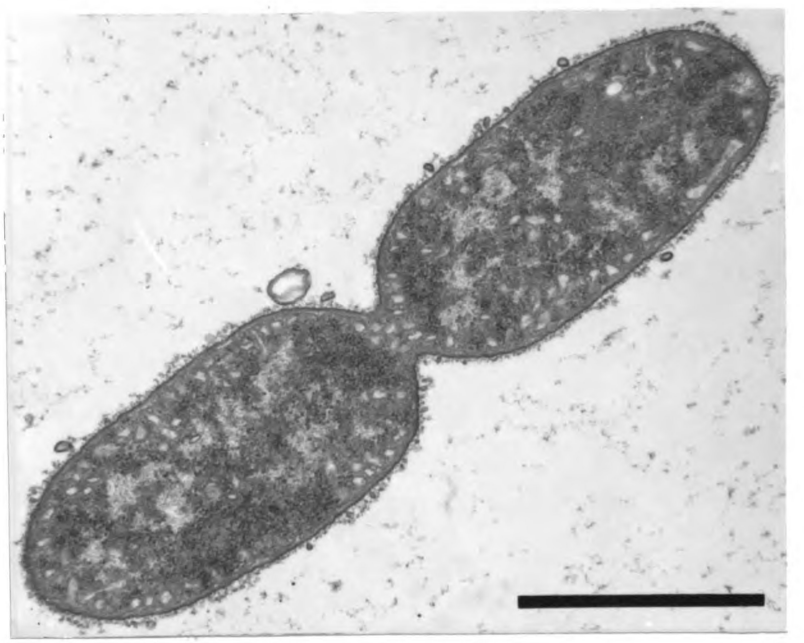

Figure 3.--Thin section of a peanut-shaped, dividing cell of A. vinelandii which has been fixed in OsO_4. Bar indicates 1 μm.

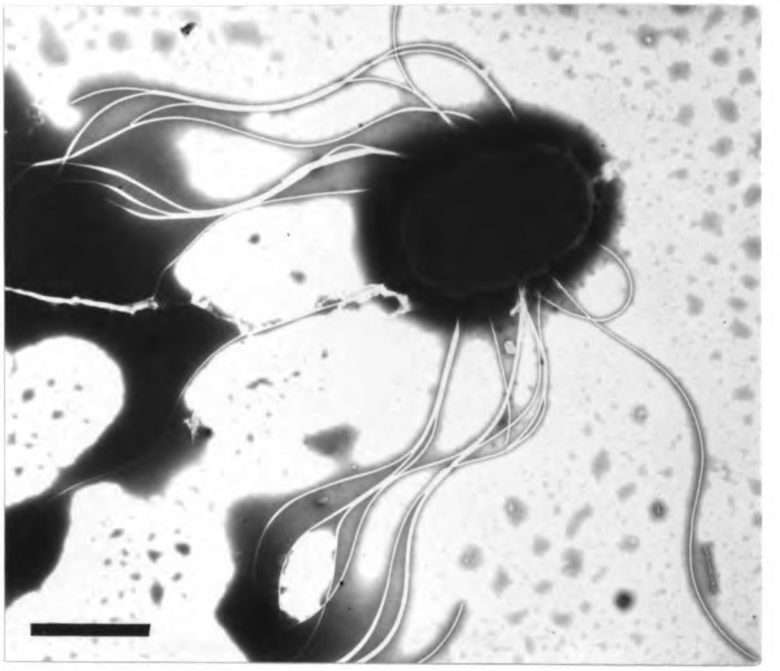

Figure 4.--A negatively stained preparation showing a vegetative cell with typical peritrichous flagella. Bar indicates 1 µm.

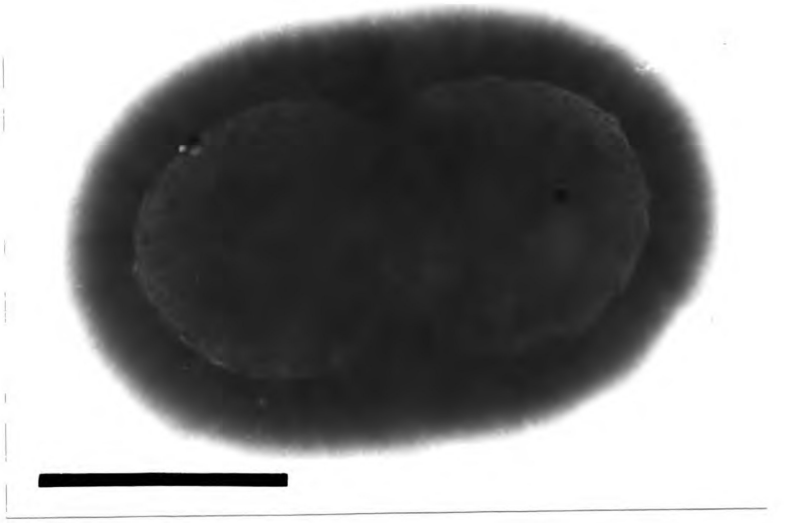

Figure 5.--A negatively stained preparation of a non-motile cell in its last division before encystment. Bar indicates 1 µm.

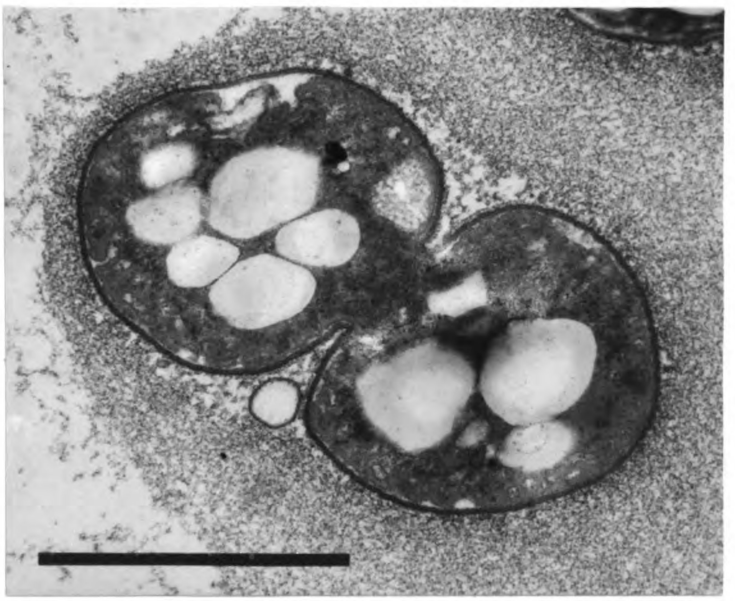

Figure 6.--Thin section showing an OsO_4-fixed dividing cell before encystment. It is surrounded by a non-structured capsule and contains PHB granules. Bar indicates 1 μm.

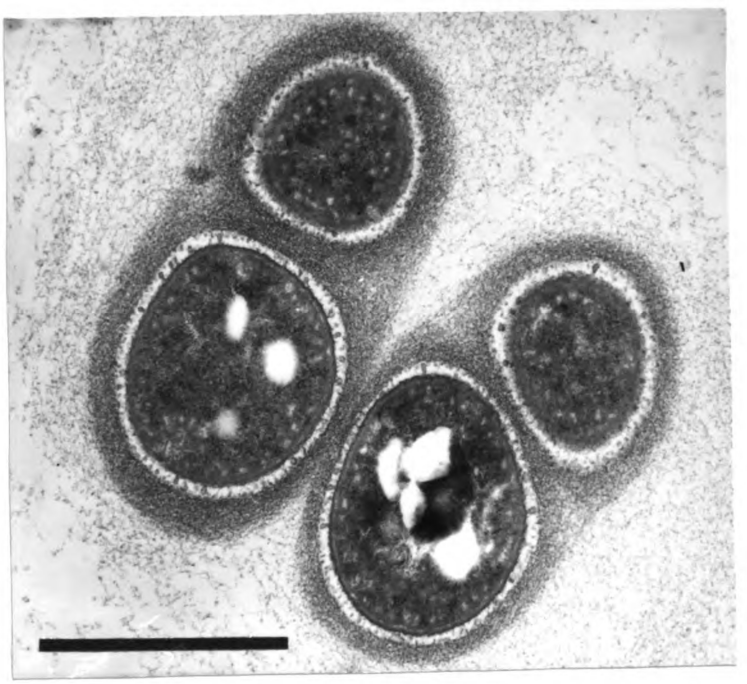

Figure 7.--Thin section of OsO_4-fixed cells following division and surrounded by non-structured capsular material. Bar indicates 1 μm.

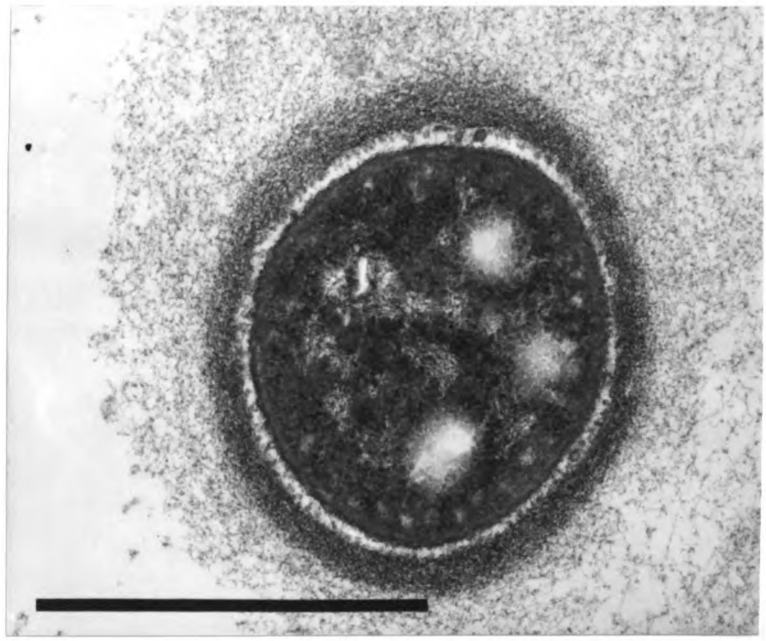

Figure 8.--Thin section of a nearly spherical, non-motile cell which is surrounded by capsule and has been fixed with OsO_4. Bar indicates 1 μm.

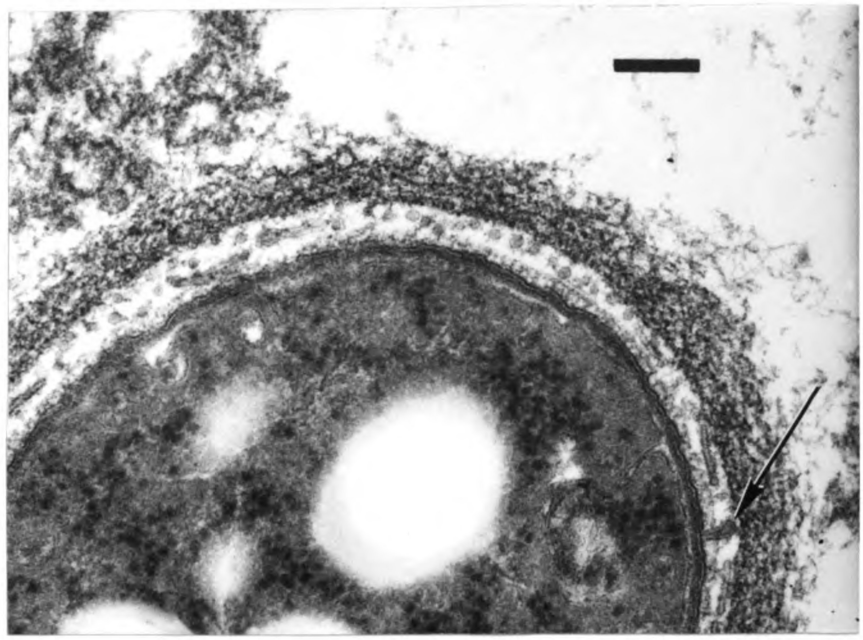

Figure 9.--Thin section of an encysting cell which has been fixed in glutaraldehyde and OsO_4 9 hours after transfer into the encystment medium. "Blebs" (arrow) and capsular material are seen extruding from the cell surface. Bar indicates 0.1 µm.

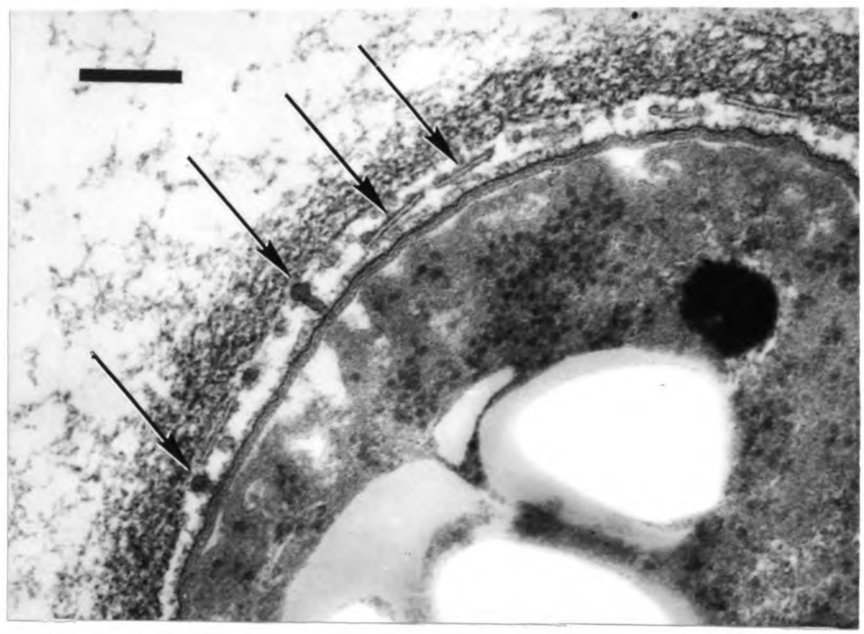

Figure 10.--A glutaraldehyde- and OsO_4-fixed cell showing "blebs" (arrow) and globules coalescing to form flattened membranous fragments (arrow). Bar indicates 0.1 µm.

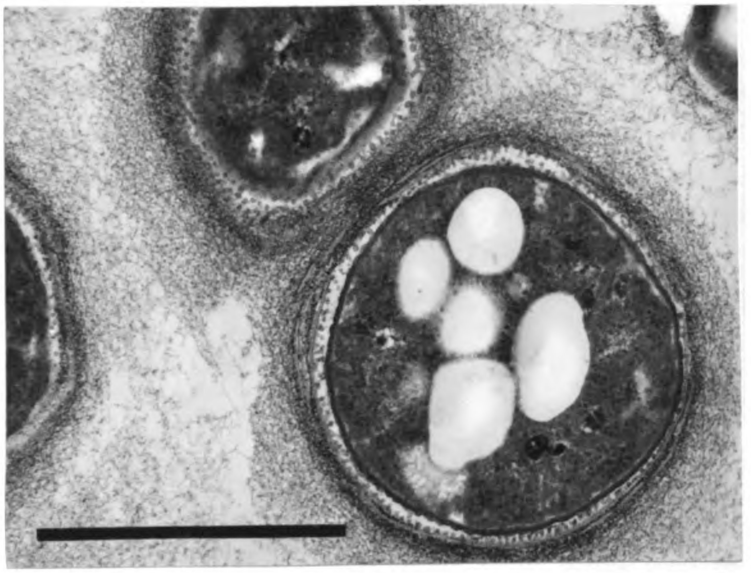

Figure 11.--Thin section of a cell, which was fixed in OsO_4 12 hours after induction of encystment, showing discontinuous layers of membranous components. Bar indicates 1 μm.

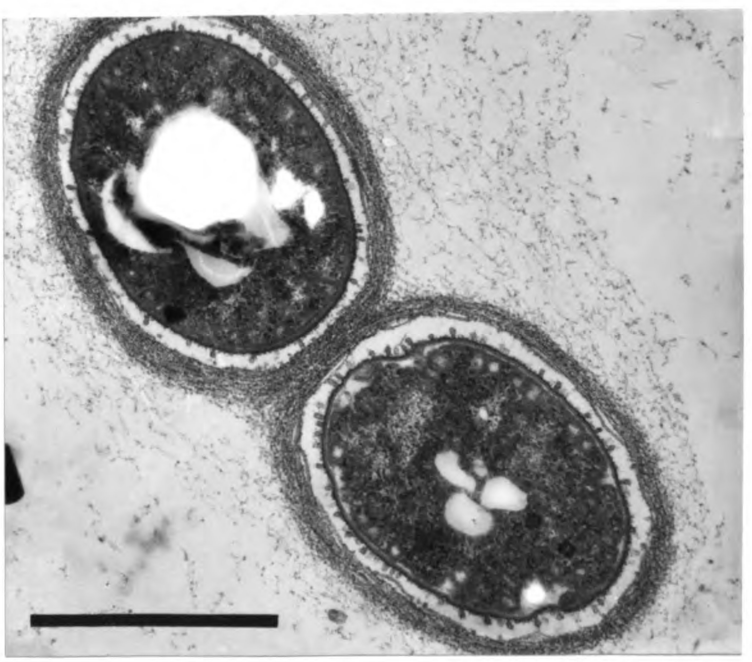

Figure 12.--Thin section of a 12-hour encysting cell, which was fixed in OsO_4, showing "blebs," membrane-like fragments and PHB granules. Bar indicates 1 μm.

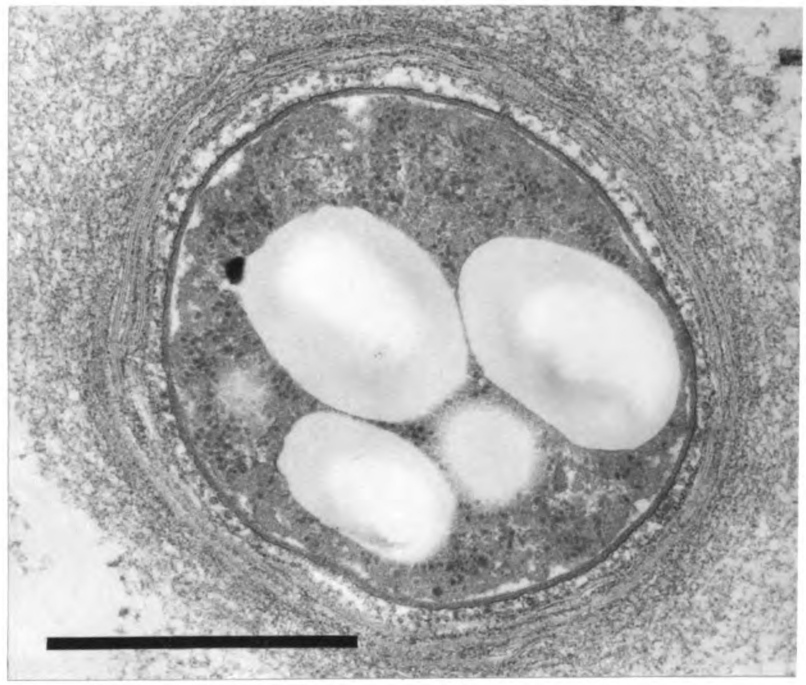

Figure 13.--Thin section of an OsO_4-fixed cell 18 hours after induction of encystment revealing further layering of the exine coat. Bar indicates 1 μm.

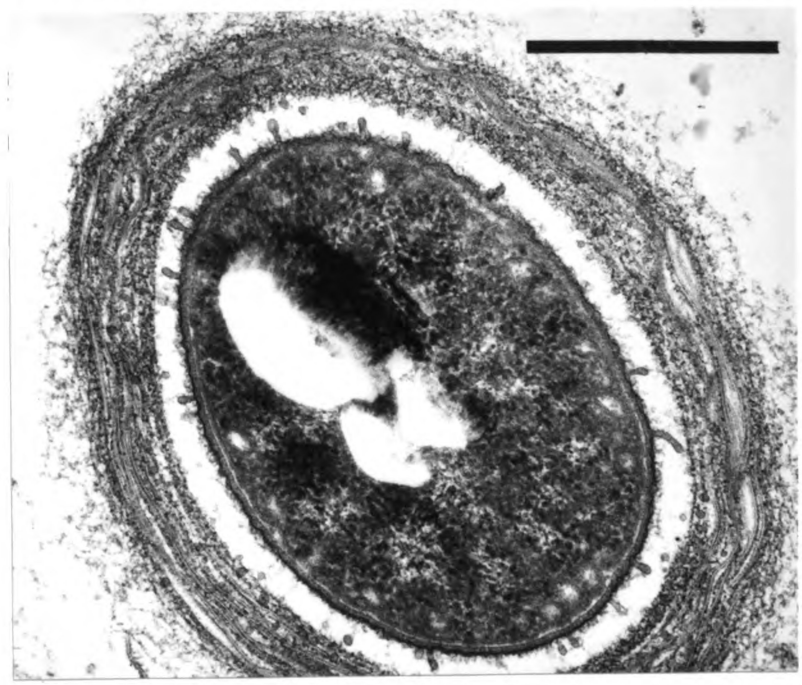

Figure 14.--Thin section of an OsO_4-fixed cell 30 hours after initiation of encystment showing the exine layers in the middle and outer edges of the capsule, and a decrease in the number of flattened spheroids per cell in the intine region. Bar indicates 0.5 μm.

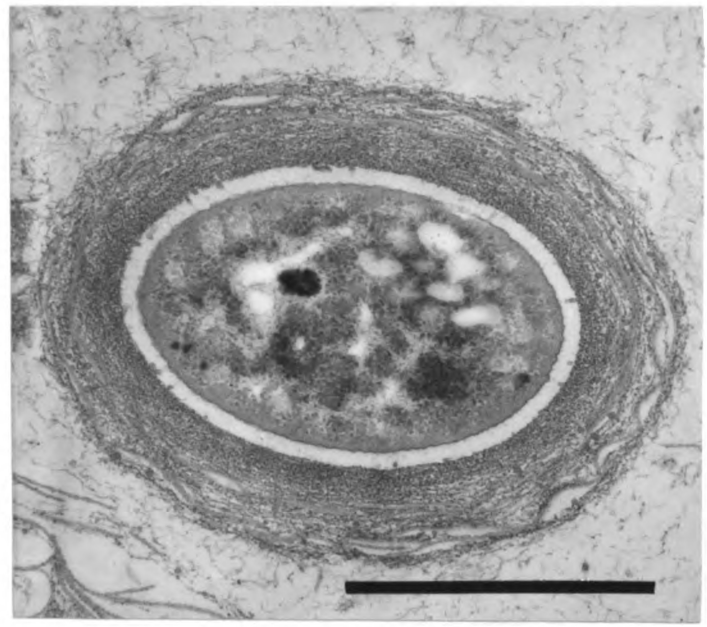

Figure 15.--Thin section of a mature cyst (4-5 days) grown on 0.2% β-hydroxybutyrate agar and fixed with glutaraldehyde and OsO_4. Bar indicates 1 µm.

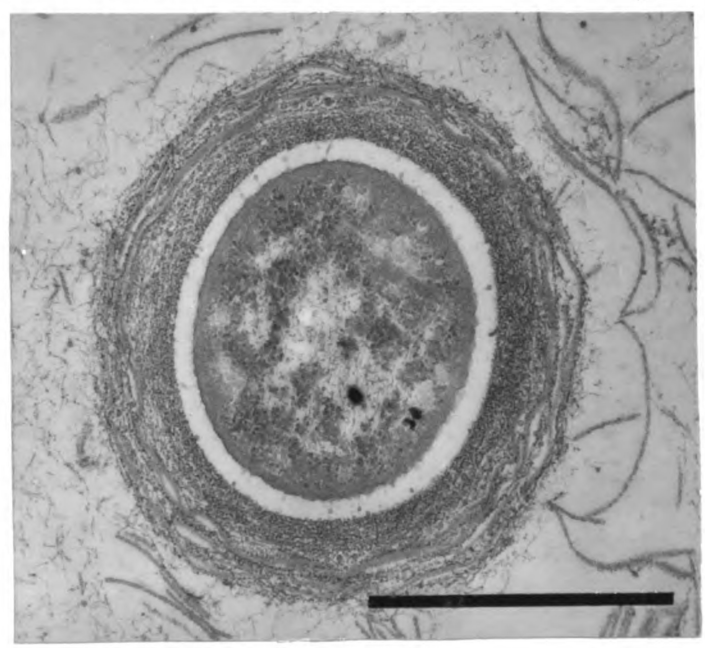

Figure 16.--Thin section of a mature cyst grown on solid medium and fixed in glutaraldehyde and OsO_4. Scattered about the cyst are membrane-like fragments that had peeled away from the exine coat. Bar indicates 1 µm.

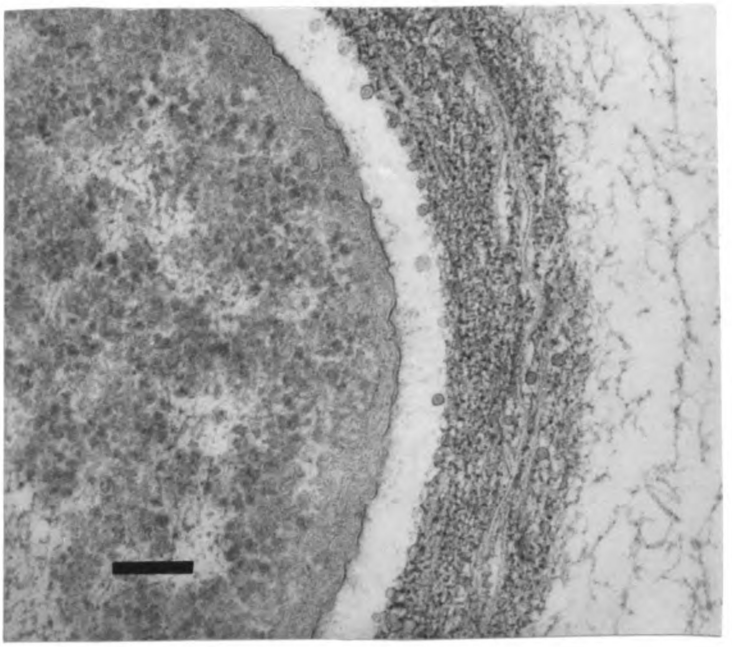

Figure 17.--An enlargement of a thin section of a mature cyst, which was fixed in glutaraldehyde and OsO_4, showing the lack of detectable structures within the intine region. Bar indicates 0.1 μm.

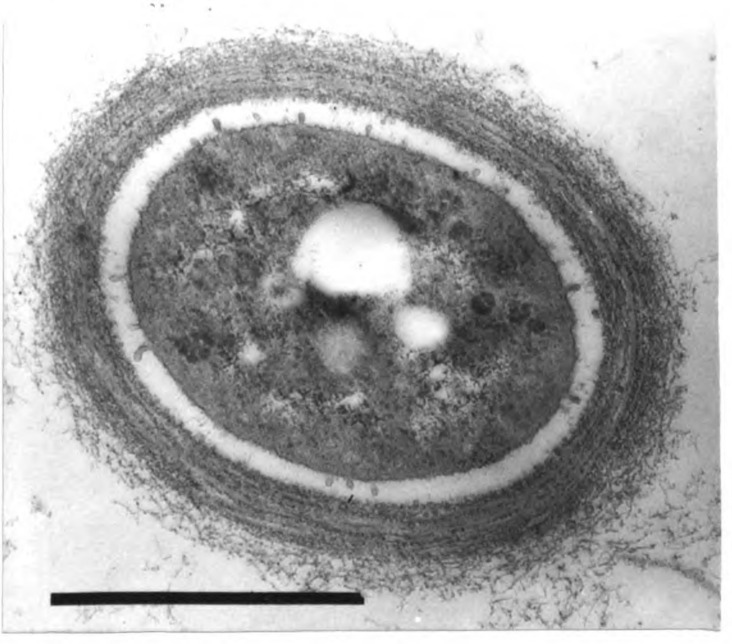

Figure 18.--Thin section of a mature cyst grown in liquid Burk's buffer with 0.2% β-hydroxybutyrate and fixed with OsO_4. Bar indicates 1 μm.

DISCUSSION

Encystment of Azotobacter vinelandii is a differentiation phenomenon which is uncomplicated by sexual processes and provides a means of studying cellular morphogenesis in procaryotic cells. Some of the interrelated events which occur during the production of cysts from vegetative cells or pre-cysts can be followed using the techniques of electron microscopy. In the electron micrographs which were presented, a deliberate selection of medial sections was made in order to view the maximal cell section. This may have restricted the interpretation of the three dimensional array of the cellular structure but enhanced the ability to follow the development of the cyst coat.

The structural details of Azotobacter vinelandii seen in this study correlated well with those of Azotobacter chroococcum (37). The rod or peanut-shaped vegetative cells of both species had a "double-track" cell wall and a "double-track" cytoplasmic membrane. Their cytoplasm was filled with electron-dense polyribosomal particles and delicately-stranded DNA. Dividing cells of both A. vinelandii and A. chroococcum were devoid of septa but division occurred by constriction in the middle

of the cell. The mature cysts of A. chroococcum were surrounded by an exine made up of triple-layered membrane units with each unit of 70-80 Å thickness. Between the exine and the central body, Tchan, et al. (37) noted a homogeneous electron-transparent region, the intine, which contained small "double-track" membranous units. These investigators believed these structures to be the same as those found in the exine wall. Tangential sections showed these fragments to be circular, indicating that in the intact cyst they might be cylinders.

The first morphologically detectable event in encystment was the conversion of vegetative cells into rounded, non-motile forms. These could be readily seen by phase contrast microscopy and could have arisen by a process similar to "spheroplasting" or by a final division of cells in a metabolic shift down. Studies in this laboratory (Berke, unpublished) showed that the latter case was correct. That is, after induction of encystment, A. vinelandii underwent a final cell division resulting in two small, nearly spherical, non-motile cells. These have been called pre-cysts and, undoubtedly, are the progeny of the non-flagellated dividing cell seen in Figure 5.

Data from this study revealed the presence of protrusions or "blebs" extruding from the cell wall of A. vinelandii into the area between the encysting cell and the capsular material. These "blebs" migrated out toward

the capsule, broke free and formed "globules" of varying sizes. The globules coalesced to form larger structures, which then flattened to form membrane-like fragments measuring 1150 to 3550 Å in length and 60-70 Å in width. These are the cross-sectional dimensions of the sheet-like exine elements in mature cysts. In immature cysts, these fragments first appeared intertwined within the inner edge of the capsular material and, as encystment proceeded, these membrane-like fragments were found in the middle and outer edges of the pre-exine capsule. The number of "blebs" and flattened globules per cell increased up to 30-36 hours after induction of encystment and then decreased as the cysts matured and the exine layers became more dense and compact.

The following hypothesis was formulated from the observations made in this research. Upon induction of encystment, there is continued synthesis of capsular material and membranous components in the absence of cell division. The continued deposition of these materials produces the layers of the exine coat.

The protrusions from the cell wall of encysting A. vinelandii appear to play an important role in the formation of outer coats of cysts. The possibility that they are artifacts of fixation must be considered. These structures were seen in developing cysts which had been fixed by either OsO_4 or glutaraldehyde. Furthermore,

the time course for the deposition of these membranous fragments was observed and the end result of the process was a multilayered exine coat similar to those seen in cysts fixed with potassium permanganate, glutaraldehyde, or osmium tetroxide (17, 33, 44).

Extrusions from the cell wall have also been observed in two other Gram-negative organisms: Escherichia coli and Salmonella typhimurium. The characteristics of the extrusions were similar to those seen in A. vinelandii. Work, et al. (42) and Knox, et al. (12) both reported the appearance of "blebs" forming from the outer cell wall layers of a lysine-requiring mutant of E. coli. Thin sections of OsO_4-fixed samples and negatively stained preparations revealed that there was no lysis of the cells but changes in the cell surface had occurred. "Blebs" often appeared continuous with the outer triple-layered envelope of the cell wall. These became detached and were released into the surrounding culture fluid as extracellular globules. The globules and particles surrounding the cells were bounded by triple-layered membranes, and were presumed to have originated from the outer layers of the cell wall of E. coli. These occasionally became arrayed in stacked plates which is characteristic of lipoprotein and lipopolysaccharide.

Rothfield and Horne (29) prepared lipopolysaccharide leaflets from cells of E. coli and S. typhimurium.

Electron micrographs of negatively stained samples revealed that the purified lipopolysaccharide presented a uniform population of hollow spheres and some flattened spheroids. Phosphatidyl ethanolamine appeared as closely packed leaflets in a regular laminated structure. When phosphatidyl ethanolamine and purified lipopolysaccharide were mixed together, negatively-stained preparations showed distinct interaction of these components. The lipopolysaccharide leaflets appeared to penetrate into the phosphatidyl ethanolamine leaflets with continuity between the two leaflets.

Rothfield and Pearlman-Kothencz (30) observed that lipopolysaccharide, phospholipid and protein were excreted from growing E. coli and S. typhimurium. The excreted materials were in the form of buds which were membrane-bound and similar to those described by Knox, et al. (12). The small buds or vesicles appeared to be a membrane complex that was formed from the outer membrane of cell envelopes of growing Gram-negative bacterial cells. These investigators suggested that this may reflect an imbalance between continued growth of the outer membranes in the absence of protein synthesis and the growth of the other cellular structures.

Although electron micrographs do not reveal the biochemical composition of the various cell structures, they do give an indication of the structural differences

between the outer coat layers of the cyst. Thus, the exine, which appears as electron-dense multilayers and the intine, which appears as an electron-transparent region, are most likely biochemically different.

The intine appears as a space between the central body and the exine. However, the technique of metal shadowing demonstrates that the intine layer is not an empty space (19). It is composed of 44% carbohydrate, 9.1% protein and 37% lipid.

Previous chemical studies by Lin and Sadoff (19) have shown that the exine contained 32% carbohydrate, 28% protein, and 30% lipid. The lipid in the exine is primarily bound while in the intine it is mostly free. These data suggest that the exine may be a lipoprotein-lipopolysaccharide complex and are consistent with the hypothesis of exine coat formation which has been presented.

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