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Freeze-Etching of *Azotobacter vinelandii*: Examination of Wall, Exine, and Vesicles

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The structure of the cell wall, the arrangement of the cyst exine, and the origin and distribution of intine vesicles in *Azotobacter vinelandii* ATCC 12837 were examined by freeze-etching and conventional electron microscopic techniques. In the vegetative organism the cell wall appears to have a woven texture which disappears during cyst formation. The exine is composed of two different types of material: the outer layer is a fibrous, amorphous layer, and the numerous inner layers form the basic hexagonal structures which unite to form the cyst coat. The presence of intine vesicles in the encysting organism was confirmed in frozen-etched cells. The appearance of frozen-etched cells and cysts and the distribution of capsular material indicate that extracellular polysaccharide is an important factor in cyst formation.

The various morphological forms of the *Azotobacteraceae* have been investigated by using a number of different techniques of fixation, all known to introduce some degree of ultrastructural change (7, 11, 18). Wyss et al. (21) employed permanganate fixation to study the development of the cyst in *Azotobacter vinelandii*. Negative staining and the electron microscope have been used to observe structures in both vegetative forms and encysted cells, including microtubules (11), elementary particles comprising the exine (12), and encapsulation (4). Lin and Sadoff (7) and Pope and Wyss (12) have employed carbon replicas to demonstrate that the surface structure of the vegetative cell is smooth and regular whereas the cyst coat appears rough and convoluted. Koo et al. (5) described the surface structure of the cyst as seen by using freeze-etching procedures (2), but they were not able to cleave the cyst through the central body or through the intine. Vela et al. (18) have reported the effects of various pretreatments and fixations on the ultrastructure of the organism and some of the artifacts encountered when these procedures were employed. Hitchins and Sadoff (4) have recently implicated vesicular structures produced during encystment, which they have termed "blebs," as being the unit of exine synthesis. They described the migration of these vesicles from the surface of the encysting cell to a site corresponding to the rudimentary exine. These vesicles have also been observed by other workers (3, 12, 18), and in each case the possibility of these being artifacts of fixation was noted.

In this study, we have used freeze-etching procedures to confirm the presence of intine vesicles in the encysting cell and have studied the structure of the frozen-etched vegetative cell and cyst. The results obtained with these methods are compared to those obtained when conventional techniques of electron microscopy are employed.

MATERIALS AND METHODS

Vegetative cells of *A. vinelandii* ATCC 12837 were grown in Burk's salts (20) supplemented with 1% glucose at 26 C for 18 to 24 hr. Vegetative cells were induced to encyst by transferring them to Burk's salts supplemented with 0.3% *n*-butanol and 2% agar. Cyst formation approached 100% after 5 days of incubation.

Shadowed specimens for electron microscopy were placed on collodion-coated 300-mesh copper grids and shadowed with platinum-palladium (80:20) alloy in a vacuum evaporator at an angle of approximately 40°.

Cysts were lysed with 2.5 mM ethylenediaminetetraacetic acid (EDTA) in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.8.

Cells were suspended at ambient temperature (22 to 26 C) for 1 hr in a solution containing 3.6% glutaraldehyde, 0.15% (w/v) ruthenium red in 0.1 M cacodylate buffer (10). The cells were washed with buffer and suspended for an additional hour at 4 C in a solution of 1% OsO_4, 0.15% (w/v) ruthenium red in 0.1 M cacodylate. After fixation, the bacteria were

washed again with buffer and dehydrated in a graded alcohol series, followed by two rinses in propylene oxide. The cells were then embedded in Epon 812 and polymerized for 24 to 48 hr at 60 C. Ultrathin sections were obtained on a Reichert Om-U2 ultramicrotome fitted with a diamond knife. Sections were post-stained with uranyl acetate for 15 min and lead citrate for 7 to 10 min to improve contrast. Thin sections were collected on 300-mesh copper grids and examined with a Phillips 300 electron microscope at an 80-kv accelerating voltage.

Cells and cysts were prepared for freeze-etching by centrifuging them into a pellet, or by removing the bacteria directly from the surface of plates. The cells were then placed on a copper disc (3 mm) that had been scratched to aid in adherance of the material, quickly frozen by immersion in Freon 22, held at liquid nitrogen temperature, and then transferred to liquid nitrogen. The copper disc was removed from the liquid nitrogen and placed on a precooled stage (−100 C) in a Balzer's apparatus (model BA360M) and frozen-etched (8). After etching, the disc was removed and dipped in distilled water until the replica floated free. The replica was treated for 1 hr with 70% H_2SO_4, rinsed several times in distilled water, treated for an additional hour with Eau de Javell (13), and finally rinsed again with distilled water. The replica was collected on an uncoated 400-mesh copper grid, allowed to dry, and examined with the electron microscope.

RESULTS

Cultures of *A. vinelandii* are normally mucoid, indicating that large amounts of capsular material surround the cells. Figure 1 is a shadow-cast preparation of encysting cells of *A. vinelandii* and is shown for the purpose of comparison. In the upper portion of Fig. 1, fibrous polysaccharide (P) is visible; in the lower portion, the polymer has been removed during preparation of the cells for shadow-casting. Attempts to fix this material adequately for ultrastructural studies have not been totally successful, although improved fixation and staining qualities of capsular material have been achieved when ruthenium red was employed during prefixation and staining (3, 12) (Fig. 2, 3). Figure 2 is an electron micrograph of an encysting vegetative cell fixed in the presence of ruthenium red. Numerous intine vesicles (IV) are apparent along the cell wall of the organism and dispersed along the inner regions of the capsular material. In encysted cells fixed in the presence of ruthenium red (Fig. 3), extra-exinic capsular material (P) appears to extend some distance beyond the exine (Ex), suggesting that cysts, as well as vegetative cells, exist in a floc. Frozen-etched replicas also reveal the capsular matrix, which normally surrounds the organism, more clearly than fixed preparations. Extensive amounts of capsular material (P) encompass each cell (Fig. 4, 5, 7, 8) and extend beyond the individual cells.

The vegetative cell (Fig. 5), the precyst (Fig. 6), and the mature cyst (Fig. 7, 8, 9) comprise the morphogenetic forms through which the organism normally cycles. The ultrastructure of the vegetative organism and the cyst have been previously described (16, 21). Figure 5 is an electron micrograph of a replica cleaved obliquely through a vegetative cell (VC) revealing the cell wall (CW), plasma membrane (PM), and cytoplasmic constituents. Strands of extracellular polysaccharide (P) are observed to connect the vegetative cell to a mature cyst (C) and an early precyst (PC). The adhering capsular material in Fig. 5 masks a large portion of a highly ordered cell wall structure (S), which is visible in Fig. 6 and 6a. The periodicity of the cell wall is due to particles 4 to 5 nm in diameter, with center spacing of approximately 6 to 8 nm. It appears that only vegetative forms possess the sculptured cell wall (S). The presence of poly-β-hydroxybutyric acid indicates that cyst formation is occurring.

Precysts (PC), induced when vegetative cells are placed in media containing carbon sources such as *n*-butanol, are intermediate cyst forms (Fig. 4, 5, 6). In the precystic form in Fig. 5, the nature of the rudimentary exine is observed to be composed primarily of fibrous strands (FM), whereas other precysts (Fig. 6) incorporate cellular constituents, as flagella (F). More mature precystic forms (Fig. 4) possess a slightly ordered surface structure that becomes more defined after 3 to 5 days of incubation on butanol-containing media (Fig. 5, 7). In mature cysts, the basic unit of exine composition is an approximately hexagonal unit (HS) (Fig. 5, 7). Shadow-cast preparations of the exine of lysed cysts (Fig. 7a) also indicate that polyhedronal structures (PS) comprise the sheet-like lamella of the exine (Fig. 3).

The cyst is composed of three anatomical regions, the exine, the intine, and the central body. These structures are observed in perspective in Fig. 8, an electron micrograph of a cyst cleaved tangential to the central body (CB). The outer, or primary, layer of exinous (Ex) material appears fibrous (FM), whereas the secondary lamellae do not possess this fibrous nature. The cyst intine (In) has been characterized as an area which lacks the organization of the exine and is analogous to capsular material (21). In several locations, intine fibrils (IF) can be observed to remain attached to the central body. The cell wall (CW) of the

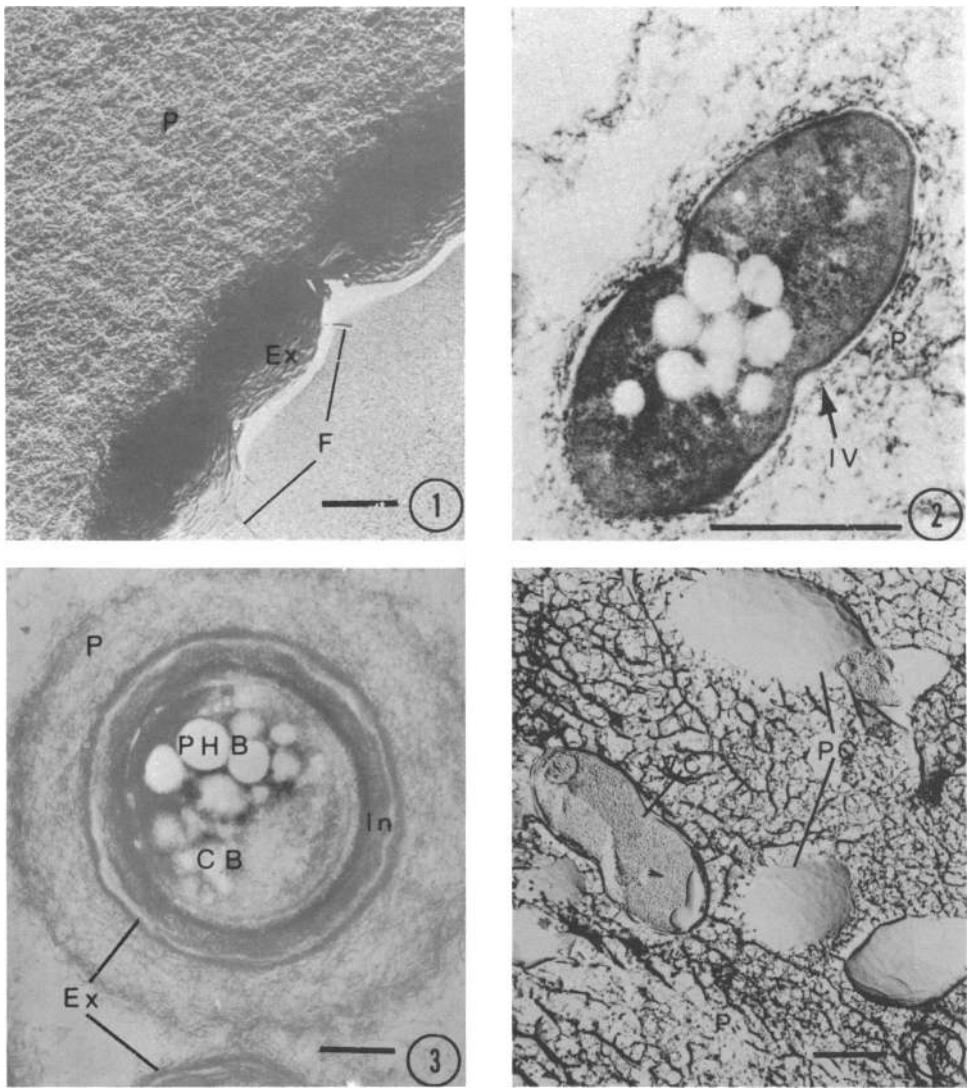


FIG. 1. *Shadow-cast preparation of encysting cells of A. vinelandii covered by a forming exine (Ex) and partially embedded in polysaccharide (P). Several flagella (F) can be seen in the lower portion of this figure. Bar represents 0.5 μm.*

FIG. 2. *Thin section of encysting vegetative cell illustrating the distribution of polymerized capsular material (P) and intine vesicles (IV). Bar represents 1.0 μm.*

FIG. 3. *Electron micrograph of a mature Azotobacter cyst. Extra-exinic polymer (P), exine (Ex), and intine (In) are visible. The central body (CB) contains several deposits of poly-β-hydroxybutyric acid (PHB). Bar represents 0.5 μm.*

FIG. 4. *Frozen-etched preparation of A. vinelandii. A vegetative cell (VC) and several precysts (PC) are embedded in polysaccharide (P). The slightly ordered exine of precysts is apparent. Bar represents 1.0 μm.*

central body has been almost completely removed during cleavage, exposing the plasma membrane (PM). Although the woven texture of the cell wall is absent in the central body of encysted cells, the structure of the plasma membrane appears identical to that of the vegetative and precystic forms (Fig. 5, 6, 6a).

Cysts cleaved through the central body (Fig. 9) exhibit a morphology similar to thin-sectional models (Fig. 3). However, the interpretation of the cyst cleaved in this plane is made more difficult by large amounts of poly-β-hydroxybutyric acid in the central body. Poly-β-hydroxybutyric acid (PHB) remains a plastic

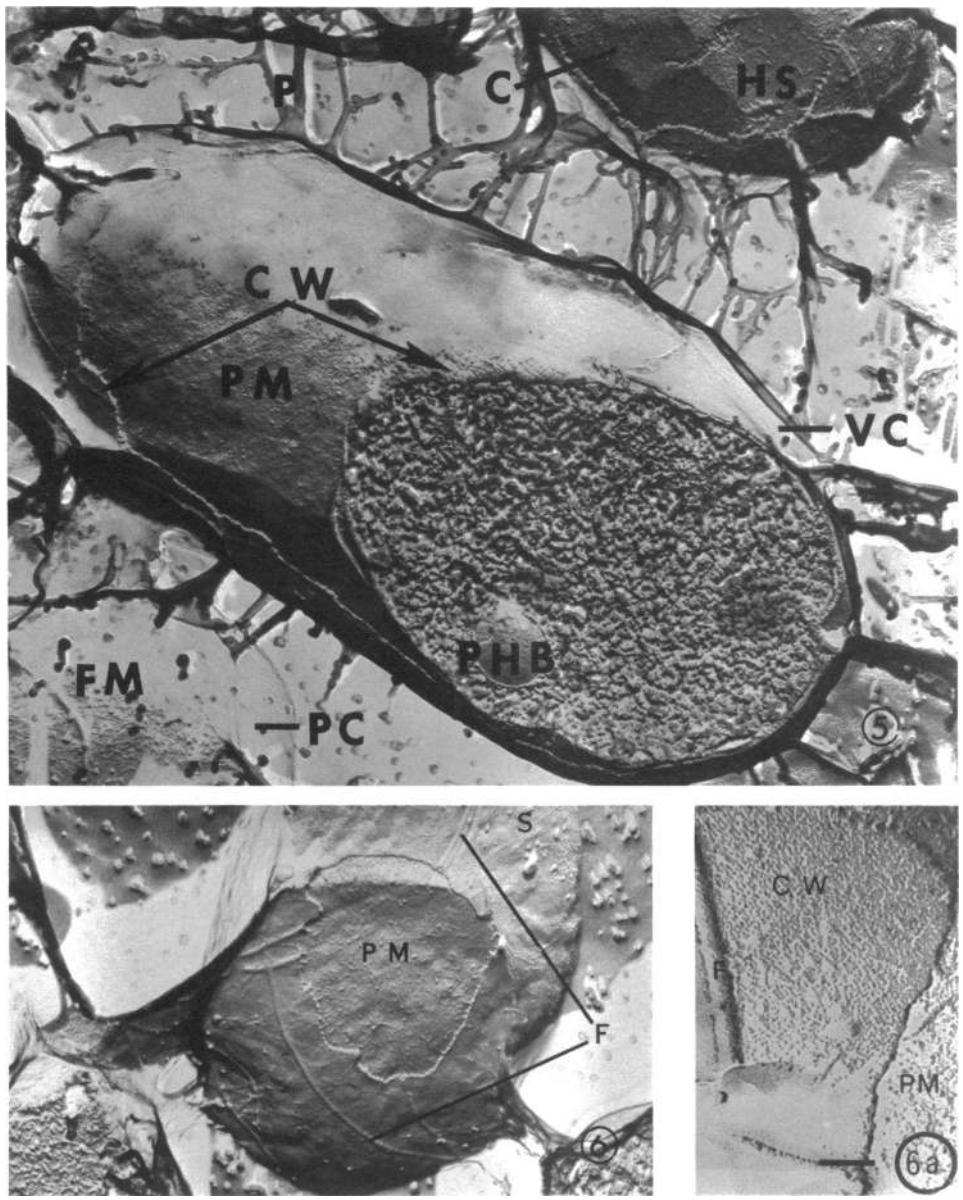
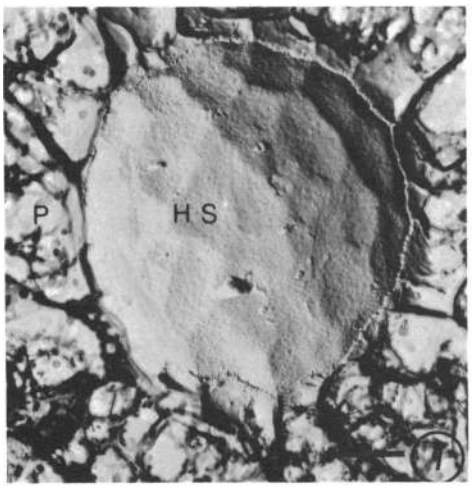


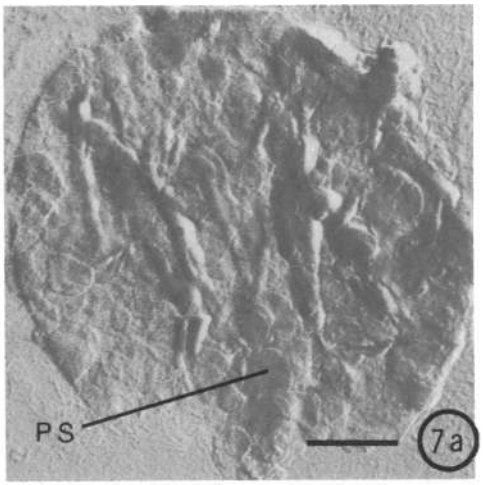
FIG. 5. *Frozen-etched preparation of a mature cyst (C) showing the hexagonal unit structure (HS) of the exine; a vegetative cell (VC) with remnants of the cell wall (CW) to which polysaccharide (P) adheres. Inside the cell wall, the plasma membrane (PM) is covered with particles, and within the cytoplasm a single poly-β-hydroxybutyric acid (PHB) granule is observed. The lower portion of this electron micrograph reveals a precyst (PC) with a forming exine composed of fibrous material (FM). Bar represents 0.1 μm.*

FIG. 6. *Frozen-etched preparation of precystic forms. A single layer of exine has been partially removed exposing a sculptured cell wall (S). Also visible are the plasma membrane (PM), covered with particles, and several flagella (F). Bar represents 0.5 μm.*

FIG. 6a. *Frozen-etched preparation showing the sculptured arrangement of the cell wall (CW), with a portion of the plasma membrane (PM) exposed. Bar represents 0.1 μm.*

material at liquid nitrogen temperatures, and as cleavage occurs the polymer may flow in the direction of the cleave, blurring any structure in that direction. Intine vesicles (IV), previously seen only in thin sections of encysting cells, can be observed along the cell wall of the central body. These vesicles may also be observed along the cell wall of other morpholog-





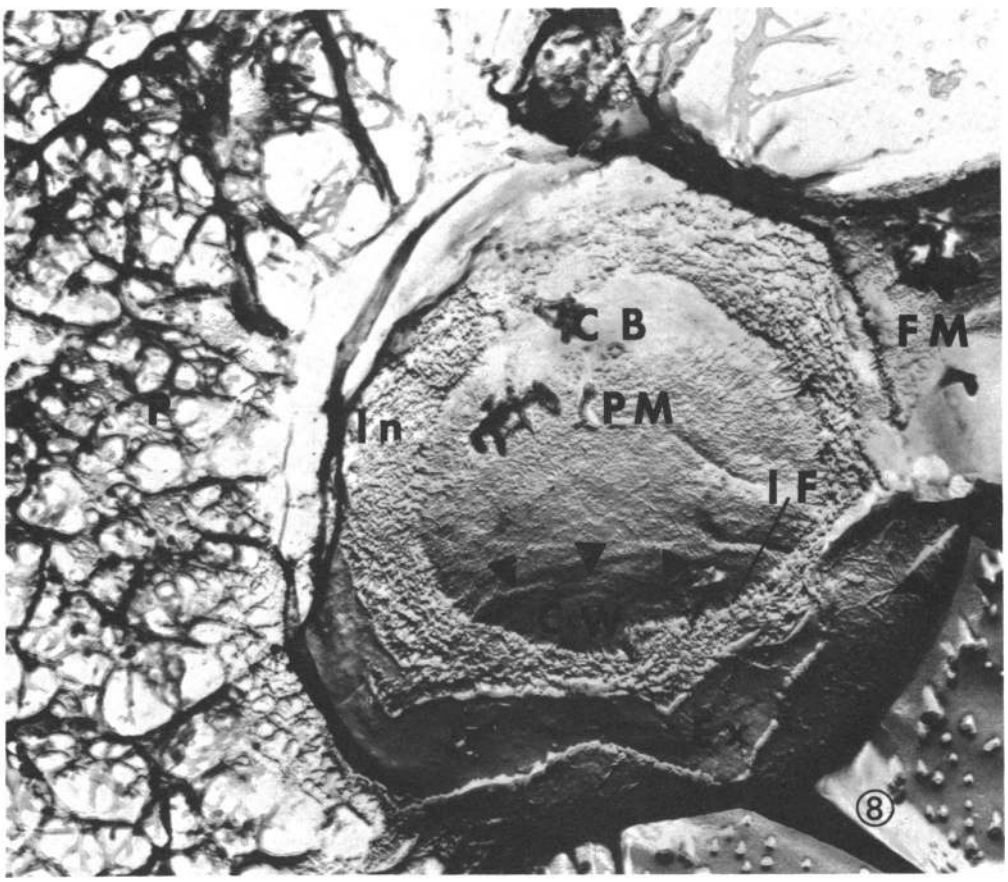


FIG. 7. *Frozen-etched cyst from which the fibrous exinous material has been removed revealing the hexagonal units (HS) of the cyst coat. Bar represents 0.5 μm.*

FIG. 7a. *Shadow-cast preparation of exine isolated from cysts ruptured with EDTA and Tris buffer. Several polyhedronal structures (PS) are apparent. Bar represents 1.0 μm.*

FIG. 8. *Frozen-etched cyst cleaved tangential to the central body (CB), revealing the exine (Ex), intine (In), exposed plasma membrane (PM), and a small portion of the cell wall (CW). A single layer of fibrous material (FM) covers the lamellae of the exine, while intine fibrils (IF) adhere to the central body. Fibrous polysaccharide (P) surrounds the cyst. Bar represents 0.1 μm.*

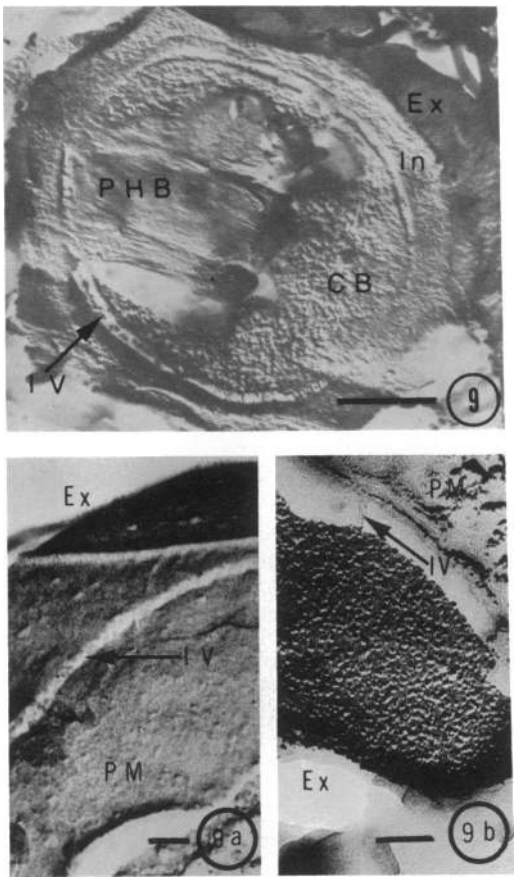


FIG. 9. *Frozen-etched cyst, cleaved through the central body showing the exine (Ex), intine (In), and central body (CB), with large amounts of PHB. Intine vesicles (IV) are observed between the central body and the forming exine. Bar represents 0.5 μm.*

FIG. 9a. *Electron micrograph of an encysting vegetative cell which has been frozen-etched to reveal the exine (Ex) and forming intine vesicles (IV), which partially compose it. A portion of the plasma membrane (PM) is also visible. Bar represents 0.1 μm.*

FIG. 9b. *Frozen-etched preparation of a precystic form. Intine vesicles (IV) are apparent between the exine (Ex) and the cytoplasm (Cy). Bar represents 0.1 μm.*

ical forms, as encysting vegetative cells (Fig. 9a), as well as precysts (9b).

DISCUSSION

The cleavage replicas of frozen-etched cells and cysts of *A. vinelandii* are consistent with thin-sectional representations of the internal structure of the cell. Shadow-cast and frozen-etched replicas present the vegetative and encysted *Azotobacter* cell as being surrounded by copious quantities of polysaccharide, not apparent in thin sections (Fig. 2, 3). The surface configuration of shadowed cells is rough and convoluted, whereas frozen-etched replicas of precysts and cysts appear regular, the surface structure being altered only slightly by the angles formed where hexagonal units combine during exine synthesis. When metal is deposited on the surface of capsular material, it creates a convoluted appearance (Fig. 1). The role of capsular material in exine formation also appears to be more important than proposed (4). Loosely aggregated primary exine possesses a fibrous consistency unlike the inner portions of the exine (Fig. 4, 6, 8). The rudimentary exine appears to be composed of capsular material, flagella with the incorporation of intine vesicles (5). The incorporation of materials into the primary exine may explain the difference in resistant properties observed for cysts cultivated on laboratory media and those removed directly from soil (17, 19). The secondary lamellae of the exine do not possess this fibricity and are probably composed only of "blebs" and materials from the intine (5). The possibility of these vesicles being artifacts of fixation has been noted, and similar artifacts have been observed in other organisms prepared for ultrathin sectioning. This type of artifact is not introduced with freeze-etching (1). Therefore, the presence of intine vesicles in encysting vegetative cells (Fig. 9a), in precystic forms (Fig. 9b), and in cysts (Fig. 9) supports the mechanism of exine synthesis proposed by Hitchins and Sadoff (5).

Frozen-etched preparations of the subunits of the exine clearly exhibit a hexagonal structure (Fig. 5, 7). The manner in which these subunits unite may be primarily responsible for the resistance to physical stresses and to other deleterious effects exhibited by the encysted cell (15). The appearance of the intine is in keeping with that seen in thin sections of the cyst. This anatomical region lacks the ordered arrangement of the exine; however, intine fibrils may serve to anchor or buoy the central body, as several of these structures are observed attached to it (Fig. 8).

The cell wall which appears to be composed of interwoven strands of wall constituents has been previously observed in other organisms (6, 14). It is of interest that this woven cell wall appearance is found only in the vegetative form, not in the encysted cell. Previous observations have noted that during encystment concomitant decreases in cell wall material occur. If this is the case, it would explain the lack of structure in the cell wall of the central

body (Fig. 8), and the near-spheroplast nature of it (9).

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