FINE STRUCTURE OF THE GRAM-NEGATIVE BACTERIUM *ACETOBACTER SUBOXYDANS*

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ABSTRACT

The morphological features of the cell wall, plasma membrane, protoplasmic constituents, and flagella of *Acetobacter suboxydans* (ATCC 621) were studied by thin sectioning and negative staining. Thin sections of the cell wall demonstrate an outer membrane and an inner, more homogeneous layer. These observations are consistent with those of isolated, gram-negative cell-wall ghosts and the chemical analyses of gram-negative cell walls. Certain functional attributes of the cell-wall inner layer and the structural comparisons of gram-negative and gram-positive cell walls are considered. The plasma membrane is similar in appearance to the membrane of the cell wall and is occasionally found to be folded into the cytoplasm. Certain features of the protoplasm are described and discussed, including the diffuse states of the chromatinic material that appear to be correlated with the length of the cell and a polar differentiation in the area of expected flagellar attachment. Although the flagella appear hollow in thin sections, negative staining of isolated flagella does not substantiate this finding. Severe physical treatment occasionally produces a localized penetration into the central region of the flagellum, the diameter of which is much smaller then that expected from sections. A possible explanation of this apparent discrepancy is discussed.

INTRODUCTION

The gram-negative cell wall has previously been studied by sectioning, ghost isolation, and chemical analysis. Sections of *Escherichia coli* (4, 22, 30, 42, 52) and other gram-negative genera (3, 39, 40) have demonstrated a membrane that lies outside of the plasma membrane, is 6 to 10 mμ thick, and is usually implied to be the cell wall of the bacterium. "Macromolecular spheres" have been observed to lie within the smooth outer layer of shadowed, isolated, gram-negative cell walls (23, 49, 59); however, sectioned bacteria generally have not shown a comparable structure between the membrane of the cell wall and the plasma membrane. Chemical analyses of isolated cell walls generally yield about 20 per cent lipid, a large percentage of protein, and some mono- and polysaccharides in addition to a mucopeptide complex (49); such analyses suggest that the cell wall thickness is about 25 mμ (38) and, therefore, is structurally more complex than a single membrane.

The possibility of flagellar hollowness was proposed as early as 1941 (44). Proposed models of bacterial flagella based on x-ray diffraction pattern analysis (2, 6), and on ultracentrifuge analysis and electron microscopy (32), illustrate a hollow central region of the fibril. Although observations of sections containing bacterial flagella

indicate hollowness (32), observations with negatively stained flagella are possible only after rigorous physical treatment.

In order to further explore the fine structure of the gram-negative cell wall and the flagella, the polarly flagellated, pleomorphic bacterium *Acetobacter suboxydans* was selected. The carbohydrate metabolism of this bacterium has been extensively studied (9), but its fine structure has so far been neglected. This report deals primarily with morphological features of the cell wall and plasma membrane, the protoplasmic constituents, and the flagella, as observed by thin sectioning and negative staining in the electron microscope. Certain metabolic studies are presently being carried out and will be reported elsewhere.

MATERIALS AND METHODS

CELL GROWTH: Stock cultures of *Acetobacter suboxydans* (ATCC 621) were maintained on Acetobacter agar slants containing 0.1 per cent each of yeast extract and $CaCO_3$, 0.15 per cent agar, and 0.03 per cent glucose under conditions employed by the American Type Culture Collection (51). A medium containing 0.2 per cent each of glucose and yeast extract and 0.3 per cent peptone was used to subculture the bacteria at 30°C for 24 hours with slight agitation. Five ml of the subculture were used as an inoculum for Roux flasks containing 150 ml of Acetobacter agar. Growth was accomplished at 30°C for 24 hours to produce cells for sectioning, or at 20°C for 120 hours to produce flagellated cells for flagellar isolation (38).

FIXATION: The cells were prepared for fixation by a technique similar to the plasma clot method described by Glauert *et al.* (19). Cells were scraped from the agar surface with a glass rod and suspended in enough freshly drawn chicken plasma to make a eurbid suspension. One part of fresh chick embryo txtract was added to 4 parts of the plasma-bacteria suspension, and this mixture was spread thinly on a glass surface and allowed to clot. The clot was placed in an osmium tetroxide–tryptone medium solution which was buffered at pH 6.1 with acetate-veronal buffer (48) and left at room temperature for 15 minutes. The clot was removed, cut into cubes (*ca.* 1 mm^3), and returned to the fixation solution for 16 hours. The cubes were then placed in two 15-minute changes of 0.5 per cent uranyl acetate solution maintained at pH 5.2 with acetate-veronal buffer (48), and in a third change of this solution for 2 hours.

DEHYDRATION AND EMBEDDING: Dehydration was performed by 20-minute changes of 50, 75, and 95 per cent ethanol with two 20-minute changes in absolute ethanol. The methacrylate used was a mixture of 2 parts ethyl and 3 parts *n*-butyl methacrylate with 1 per cent benzoyl peroxide; this mixture was dried immediately before use by filtering through anhydrous, powdered Na_2SO_4. Infiltration was accomplished with 3 changes of the methacrylate mixture (20 and 45 minutes and 16 hours at room temperature). Each cube was transferred to a separate gelatin capsule and covered with methacrylate which was polymerized at 60°C.

SECTIONING AND STAINING: Sections were cut on LKB or Porter-Blum microtomes, mounted on 400-mesh grids covered with a parlodion membrane, and overlaid with a methacrylate membrane; however, a few of the sections were mounted on methacrylate membranes and overlaid with carbon membranes. Sections were stained with uranyl acetate (16) at a concentration of 0.5 per cent for 2 hours at pH 4.5 (19) before the overlay was added, or for 1 minute with a 1/100 dilution of a stock lead hydroxide stain (29), or for 1 hour with 1 per cent potassium permanganate (34).

FLAGELLA ISOLATION: Two Roux flasks containing 5-day-old cultures of *A. suboxydans* grown at 20°C were each washed with 15 ml of distilled water. The resulting cell suspension was placed in a stoppered flask and shaken vigorously for 5 minutes. The bacterial cells were removed by centrifugation with a Servall superspeed angle centrifuge model SS-1 at 14,000 *g* for 15 minutes, and the flagella were removed from the supernate by centrifugation with a Spinco model L preparative ultracentrifuge at 75,000 *g* (average) for 45 minutes. The pellet was resuspended in distilled water and centrifuged at 25,000 *g* for 15 seconds in order to remove excess contaminating material (28); no visible pellet was produced. After further centrifugation at 75,000 *g* for 45 minutes, a pellet was formed and was resuspended in 0.8 ml of distilled water. This flagellar suspension was stored at 4°C and used without further dilution.

FLAGELLA DISRUPTION: The isolated flagella were partially disrupted by ultrasonic vibration (Schoeller ultrasonic spray dispenser, Brinkmann Co., Great Neck, New York). An Erlenmeyer flask containing the suspension was lowered to the bottom of the treatment chamber which was partially filled with ice water. Samples of the suspension were taken after treatments of 30, 60, 120, and 300 seconds. After each of these time intervals, the 1 megacycle transmitter was turned off, the aliquot removed, the surrounding water replaced with ice water, and the treatment continued.

NEGATIVE STAINING: A stock solution of 2 per cent phosphotungstic acid was adjusted with 1 N KOH (5) to pH 5.3. Equal volumes of the potassium phosphotungstate and the flagella or flagella–Tobacco Mosaic Virus (TMV) suspensions were

mixed and a droplet placed on a supporting membrane where the particle-phosphotungstate suspension dried in 20 to 30 minutes. The flagella suspension was used either without dilution or was mixed with an equal volume of TMV suspension containing 10^{10} particles/ml.

Supporting membranes of parlodion with small holes were formed in high humidity atmosphere and lightly filmed with carbon before the specimen-phosphotungstate suspension was added, in order to increase contrast of the holes. The presence of the holes in the membrane served as a focusing aid and a constant check for astigmatism.

ELECTRON MICROSCOPY: An RCA EMU 3F with a standard single condenser lens was operated at 100 kv with a 60-μ objective aperture. Negatives were produced at magnifications up to 20,000 times. Higher instrumental magnifications of 37,000 to 49,000 were obtained by inserting a smaller bore pole piece in the intermediate lens (RCA high magnification kit). The high magnification kit was used (with a 30-μ objective aperture) primarily for the observation of negatively stained flagella.

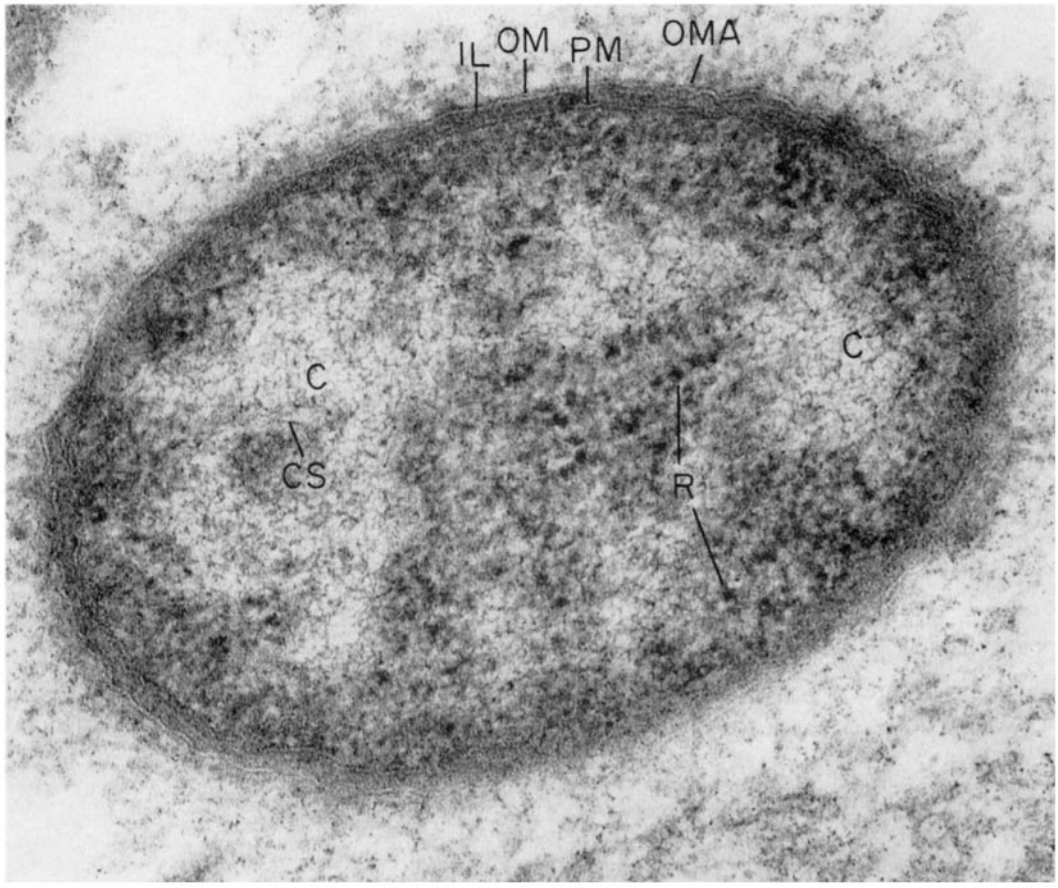


FIGURE 1 Relatively short cell stained with uranyl acetate, demonstrating aggregated chromatin areas (*C*) with chromatin strands (*CS*) and ribosome-like particles (*R*). The protoplasm is surrounded by an outer membrane (*OM*), an intermediate layer (*IL*), and the plasma membrane (*PM*). A frequently observed anomaly occurring in the outer membrane is shown (*OMA*). × 162,000.

OBSERVATIONS

Cell Wall and Plasma Membrane

Three separable structures are observed between the capsular material and the protoplasm of *Acetobacter suboxydans*. In cross-section, the outermost structure appears as two layers of high electron scattering, each 3 mμ thick, separated by a space

about 2.5 mμ thick (Figs. 1, 2 *a* and *b*, *OM*). This structure has the appearance of a unit membrane and will be referred to as the outer membrane. Another similar membrane, located beneath the outer membrane and having slightly thinner separation of layers, appears to surround the protoplasm and is referred to as the plasma membrane (Figs. 1, 2 *a* and *b*, *PM*). Between these membranes is a layer of material that appears homogeneous, is less dense than the high electron-scattering layers of the membranes, and is 5 to 10 mμ thick; this is referred to as the intermediate layer (Figs. 1, 2 *a* and *b*, *IL*).

The outer membrane usually appears more obvious than the plasma membrane because it has a thicker electron-transparent layer and is bound only on one side by cellular material. Frequently, where the protoplasm seems well preserved, the outer membrane appears serrated, although still unbroken. Although the outer membrane usually appears as a continuous envelope, several anomalies have been observed: (*a*) the outer membrane appears to branch toward the region of the plasma membrane, (*b*) perpendicular "bridges" or striations appear between the outer membrane and the plasma membrane or protoplasm (Fig. 7), and (*c*) the outer membrane appears to branch and rejoin giving the appearance of an additional membrane (Fig. 1, *OMA*).

The region of the intermediate layer is also subject to some structural variation. This layer often appears to be thicker at the polar region of a cell (Fig. 2 *a*, *IL*) without any difference in density regardless of the stains used. However, two types of cells lack the intermediate layer and may have increased separation of the two membranes: (*a*) those in which the shape of the cell and the appearance of the protoplasm seem normal (Fig. 4), and (*b*) those in which the cell is more spherical and the protoplasmic constituents are largely missing (Fig. 5 *a*). In both instances, the inner layer of the outer membrane has a greater thickness which appears to be due to the adherence of material of the same density as the intermediate layer (Figs. 4, 5 *a* and *b*, *A*).

Since both the outer membrane and the intermediate layer are outside the plasma membrane and both appear to be distinct, separable layers, the term cell wall will be used to include both of these layers.

The plasma membrane occasionally appears to be folded into the cytoplasm. In general, these intracytoplasmic membranes are found in higher concentrations at the ends of the cells, either as single membranes (Figs. 7 *M*, and 9, arrows) or as whorls (Figs. 11 and 12, *W*); however, such material can occasionally be seen along the sides of the cells in regions other than where the plasma membrane would be expected to invaginate during the initial stages of division (Figs. 6 and 10, *M*).

A layer that varies in thickness and appears as a loose aggregation of undifferentiated material is present outside the cell wall (Figs. 1 through 14) and is thought to be the slime layer or capsule.

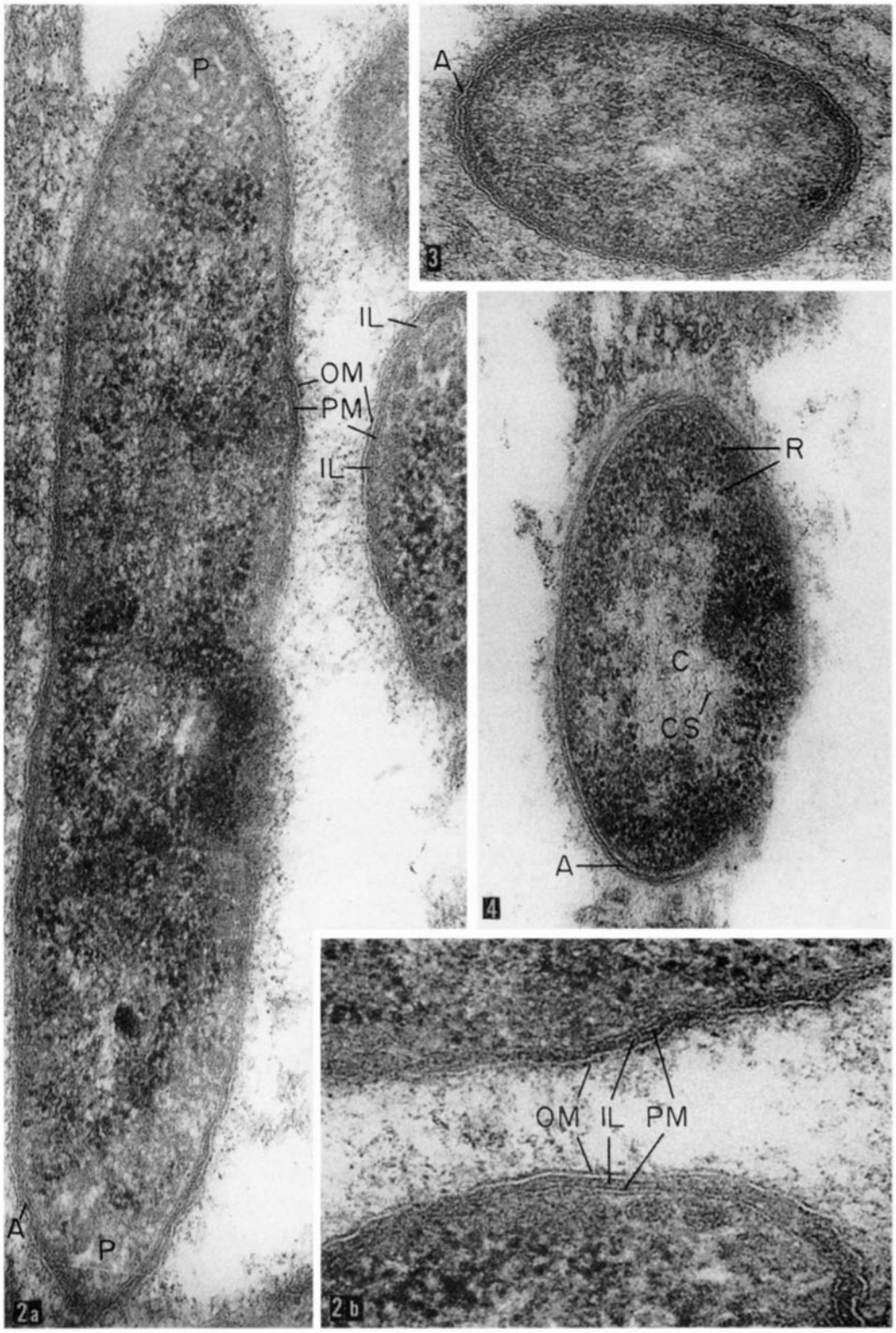
Protoplasmic Constituents

Structures appearing almost spherical with a diameter of *ca.* 14 mμ were found in great numbers distributed randomly throughout the cyto-

FIGURE 2 *a*. Section stained with lead hydroxide demonstrating the outer membrane (*OM*), plasma membrane (*PM*), and the varying thickness of the intermediate layer (*IL*). Polar regions (*P*) of the elongated cell lack ribosome-like particles. × 84,500.
b. Enlargement of adjacent cells in Figure 2 *a*, again demonstrating the outer membrane (*OM*) and intermediate layer (*IL*) of the cell wall, and the plasma membrane (*PM*). × 148,000.

FIGURE 3 Permanganate-stained section demonstrating the cell wall, the plasma membrane, and a structural anomaly (*A*) in the outer membrane. Distinct ribosome-like particles are not demonstrated. × 73,500.

FIGURE 4 An atypical cell demonstrating an absence of most of the intermediate layer; that which remains appears as a thickening of the inner layer of the membrane (*A*). The protoplasm of the cell is normal, showing aggregated chromatin (*C*) with chromatin strands (*CS*) and ribosome-like particles (*R*). × 79,000.



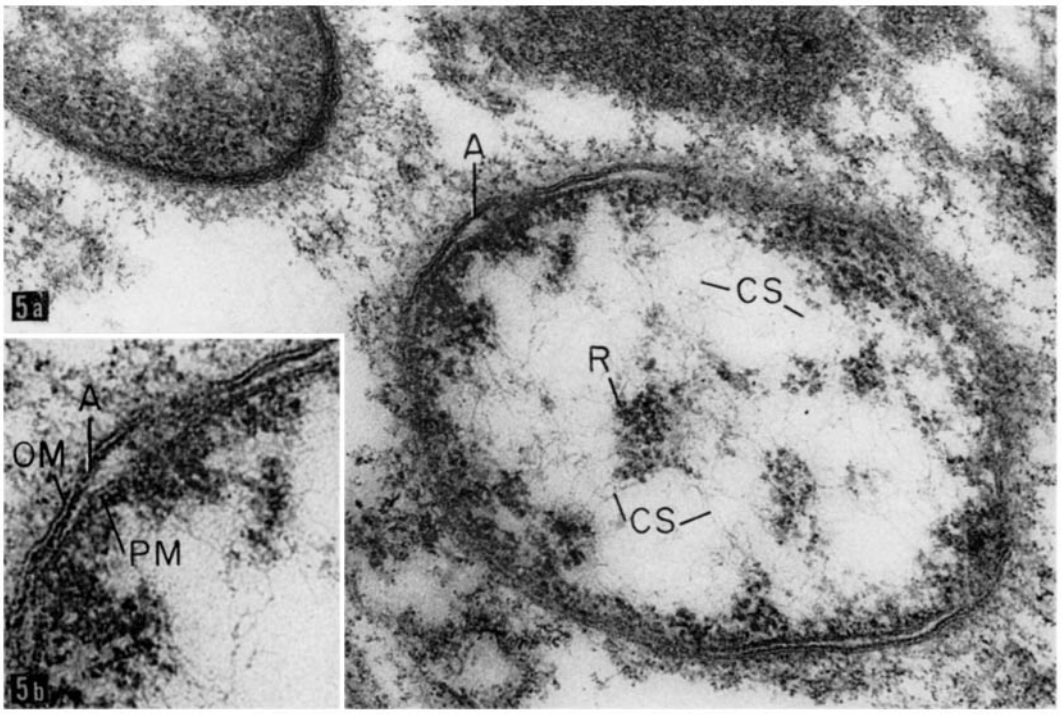


FIGURE 5 *a*. Cell has atypical shape and a protoplasmic area that contains only chromatin strands (*CS*) and a few ribosome-like particles (*R*). The absence of most of the intermediate layer of the cell wall is demonstrated; that which remains (*A*) appears as a thickening of the inner layer of the outer membrane (*OM*), yet separated from the plasma membrane (*PM*). The adjacent cell appears typical. × 83,000.
b. Enlargement of Fig. 5 *a*, demonstrating atypical appearance of the intermediate layer (*A*). × 147,000.

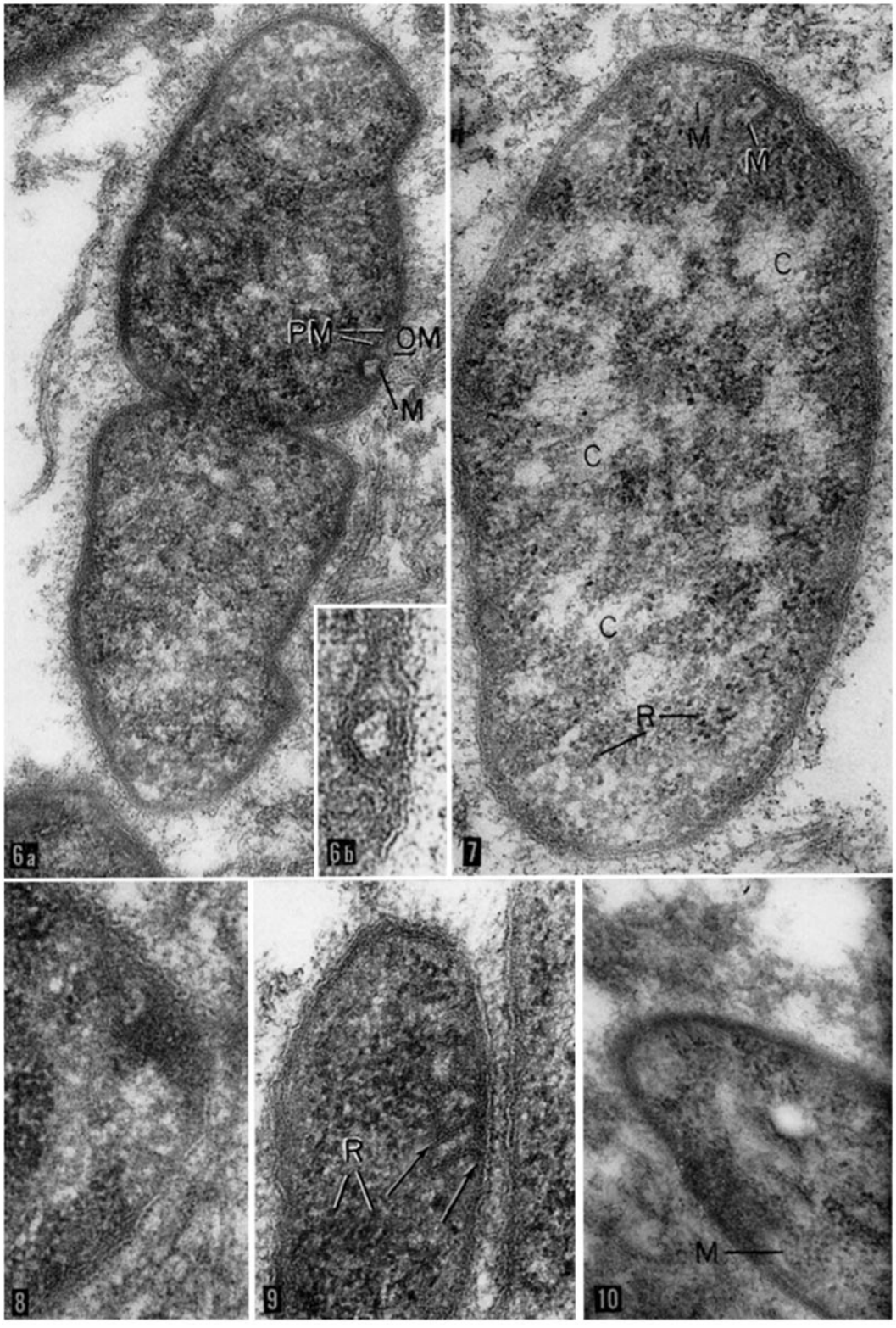
plasm. A slight accumulation of these particles occasionally occurred at the polar region of elongated cells (Fig. 13, *R*). The contrast between these particles and the remainder of the cytoplasm was greatly improved by staining with uranyl acetate (Figs. 1, 4, 7, 11, and 13, *R*). Such particles were also observed in sections stained with lead hydroxide, but the cytoplasm was also stained

FIGURE 6 *a*. Dividing cell which demonstrates the invagination of plasma membrane. (*PM*) and the extramembranous material (*M*) between the plasma membrane and the outer membrane (*OM*). × 62,000.
b. Enlargement of the extramembranous area of Fig. 6 *a*. × 149,000.

FIGURE 7 Demonstration of membranous invaginations (*M*) at the polar end of uranyl acetate-stained cell. Chromatin (*C*) is partially dispersed, and the ribosome-like particles (*R*) are distinct. × 87,000.

FIGURE 8 Differentiation of polar region demonstrates electron-transparent area, electron-opaque parallel lines, and the lack of ribosome-like particles. Lead stained. × 108,000.

FIGURES 9 and 10 The plasma membrane is occasionally observed to invaginate into the cytoplasm (Fig. 9, arrows). Membrane-like inclusions of the cytoplasm which cannot be traced to the plasma membrane are also observed (Fig. 10, *M*). Fig. 9, lead hydroxide stained, × 110,000; Fig. 10, uranyl acetate stained, × 62,000.



so that the particles contrasted more poorly with their surroundings (Figs. 9 and 11, *R*). In cell sections stained with permanganate, the particles were almost unobservable (Fig. 3). In their staining affinity and structural appearance, these particles are characteristically similar to ribosome-like particles found in other cells.

Uranyl acetate–staining strands measuring about 1.8 mμ in diameter are seen in electron-transparent localizations varying in number and size. These strand-containing areas are assumed to represent the chromatin of the cells and will be thus denoted, although definitive proof is not yet available. The relative distribution of the chromatin material seems to correspond with the length of the cells: the chromatin of short cells (*ca.* 1 μ) appears aggregated (Figs. 1, 4, and 17, *C*); that in cells of intermediate length (*ca.* 1.5 μ) demonstrates a more randomly dispersed appearance (Fig. 7, *C*); and that in elongated cells (*ca.* 2 μ) or cells in the process of division seems most dispersed (Figs. 6 *a* and 11). In cells stained with uranyl acetate the contrast between the chromatin and the cytoplasm is greater (Figs. 1 and 4, *C*) than it is in cells stained with permanganate (Fig. 3) or lead hydroxide (short cell, Fig. 2). The 1.8 mμ strands of the chromatin area (Figs. 1 and 4, *CS*) are more readily apparent in sections stained with uranyl acetate. Lead hydroxide occasionally demonstrates the intrachromatin strands (Fig. 12, *CS*), whereas with permanganate staining the stranding seems virtually unobservable (Figs. 3 and 17). Strands of the same diameter are readily visible in uranyl acetate-stained cells that are devoid of most of their protoplasm (Fig. 5 *a*, *CS*).

The polar regions of most elongated cells show a specialized low density region. In uranyl acetate–stained longitudinal sections, this area appears as a cluster of non-membrane–limited spheres which demonstrate a diameter of *ca.* 7 mμ (Figs. 11 and 13, *P*). A few distinctly separated spherical structures were occasionally found at the polar region of cells presumably cut at a more oblique angle. Virtually all uranyl acetate–stained sections showing cell elongation or division and dispersed chromatin material also demonstrate the polar cluster of spheres (Figs. 11 and 13, *P*), whereas sections through relatively short cells demonstrating accumulated chromatin do not (Figs. 1 and 4). The non-membrane–limited spheres (or the cluster) differ from the remainder of the cytoplasm in that they are free of 14-mμ particles; they also differ from the chromatin material by their location within the cell and the absence of 1.8-mμ strands (Figs. 11 and 13). When sections are stained with lead hydroxide, the polar region seems to be distinct only in that (*a*) it lacks the 1.8-mμ strands and the 14-mμ electron-opaque particles, and (*b*) its electron scattering is often similar to that of the chromatin material (Figs. 2 and 12, *P*).

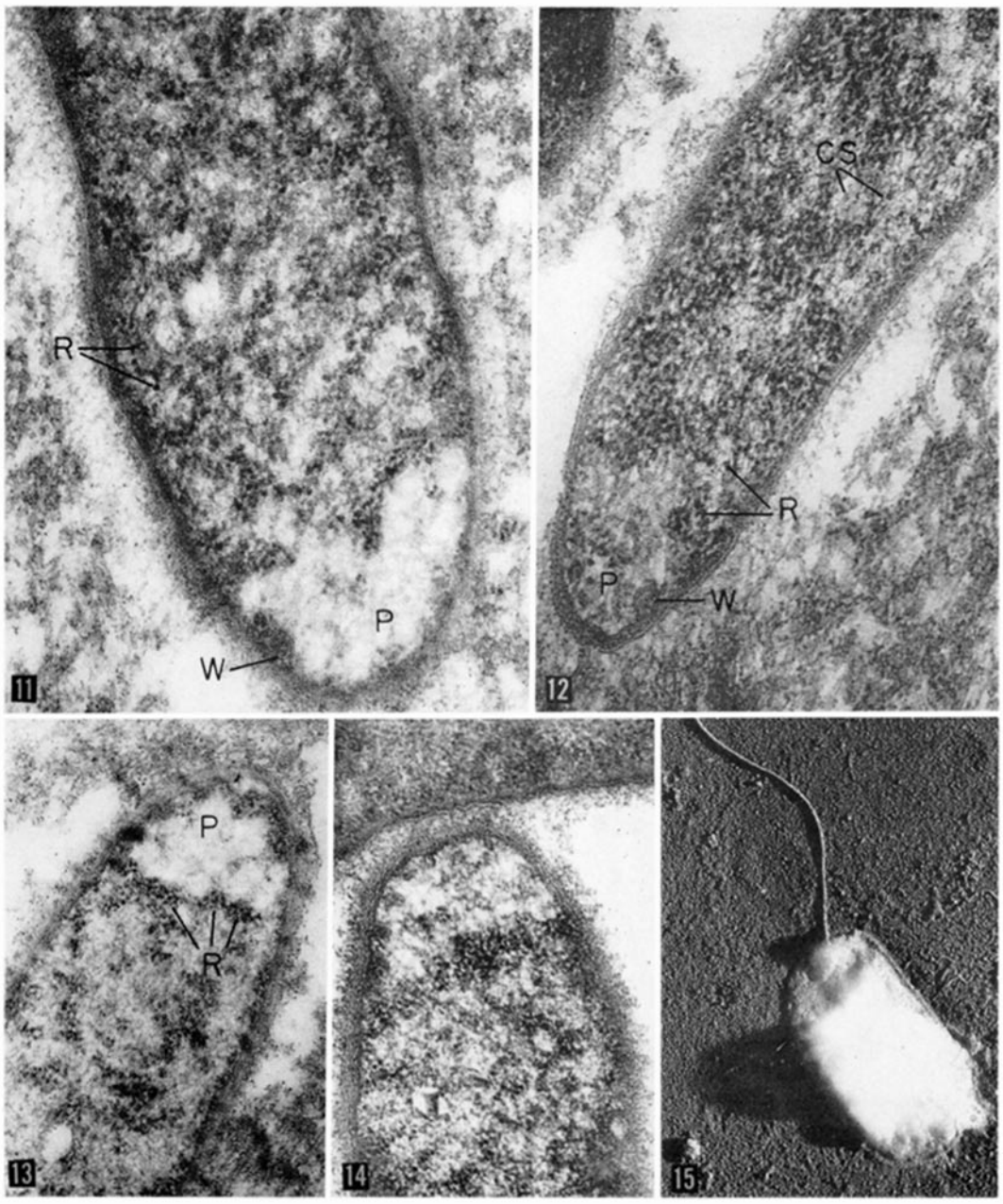
When whole cells are partially plasmolyzed, dried on grids, and shadowed with palladium, a basal granule or blepharoplast-like enlargement can be seen at the polar region of the cell near the area of attachment of the flagellum (Fig. 15).

Flagella

Cell preparations stained for light microscopy according to Leifson (35) demonstrate up to 4 polar flagella and aggregations of detached flagella. The present studies demonstrate that formalin-fixed cells shadowed with *ca.* 15 A of palladium at an angle of 10° and observed in the electron microscope usually show no more than one flagellum *ca.* 12 to 15-mμ diameter without visible periodicity (Fig. 15). Cells fixed with formalin and negatively stained with potassium phosphotungstate have multiple polar flagella with the same diameter and without periodicity. Staining for the light microscope and both shadowing and negative staining for the electron microscope show that the flagella have a wave length of 1.4 μ and an amplitude of 0.3 μ, which are in agreement with the observations of Leifson (36).

In sections, flagella are occasionally observed in high concentrations that are believed to be aggregates of detached flagella similar to those observed in preparations stained for the light microscope. Longitudinally sectioned flagella appear as two parallel dense lines, each 3.5 mμ thick, separated by a space of lesser scattering that is *ca.* 4.5 mμ thick, so that the total thickness is *ca.* 12 mμ (Figs. 16 and 21); oval or circular profiles that are oblique or cross-sections are noted less frequently (Figs. 16 and 17). When sections are observed which are cut through areas of the plasma clot not containing bacterial cells, these filamentous structures are almost never seen, while the cell-containing sections almost invariably demonstrate evidence of the 12-mμ filaments.

Permanganate (Figs. 16 and 17) and lead-hydroxide (Fig. 18) staining increase the electron

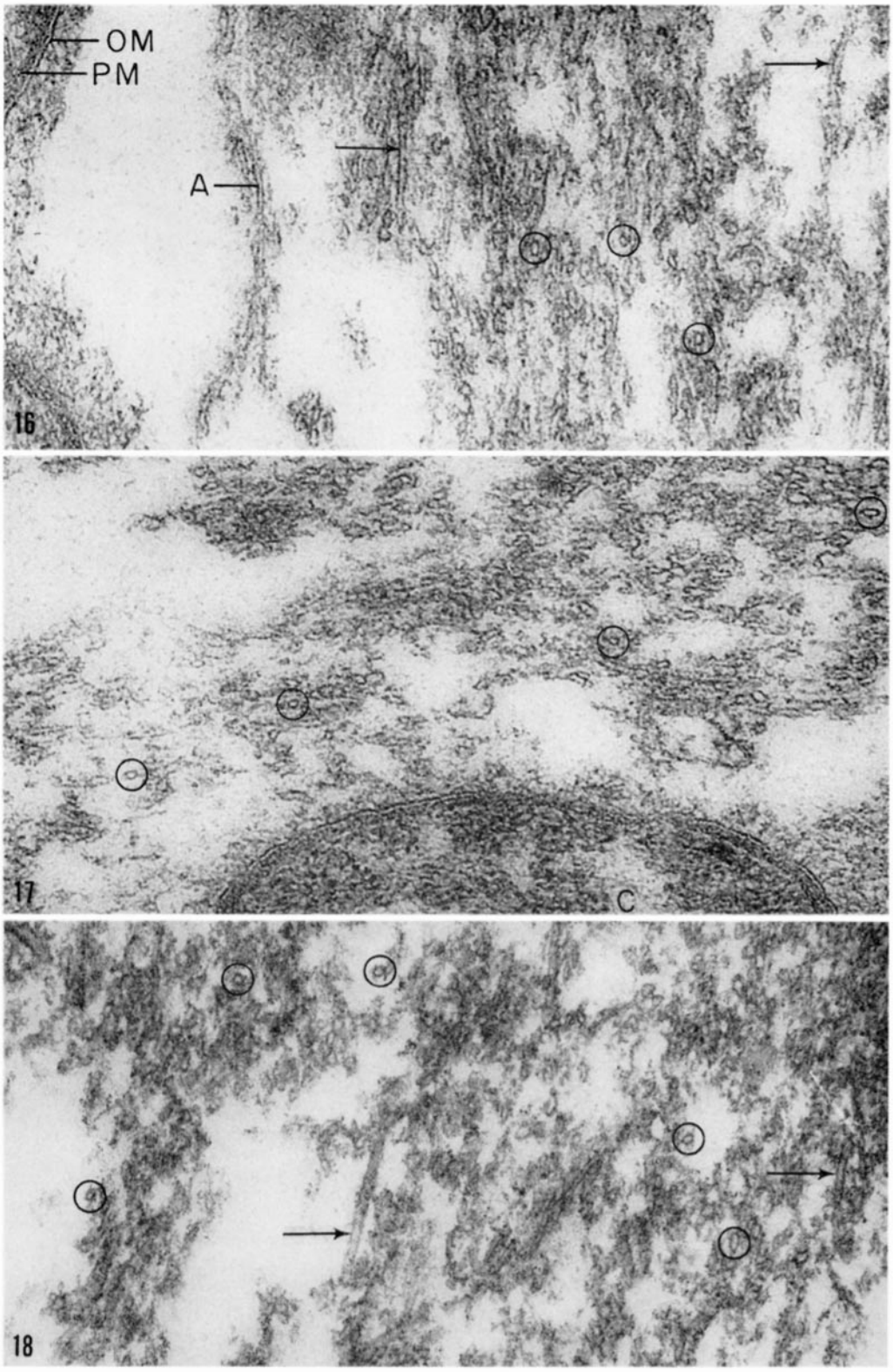


FIGURES 11 and 12 A characteristic polar appearance (*P*) as found with uranyl acetate staining (Fig. 11) and lead staining (Fig. 12). Occasionally observed whorl of membrane material (*W*) and ribosome-like particles (*R*) are demonstrated by both figures, but chromatin strands (*CS*) are more apparent in Fig. 12. Fig. 11, × 74,500; Fig. 12, × 78,000.

FIGURE 13 Concentration of ribosome-like particles (*R*) around polar area (*P*), demonstrated with uranyl acetate–stained section. × 44,500.

FIGURE 14 Polar region as demonstrated by lead hydroxide staining. × 92,000.

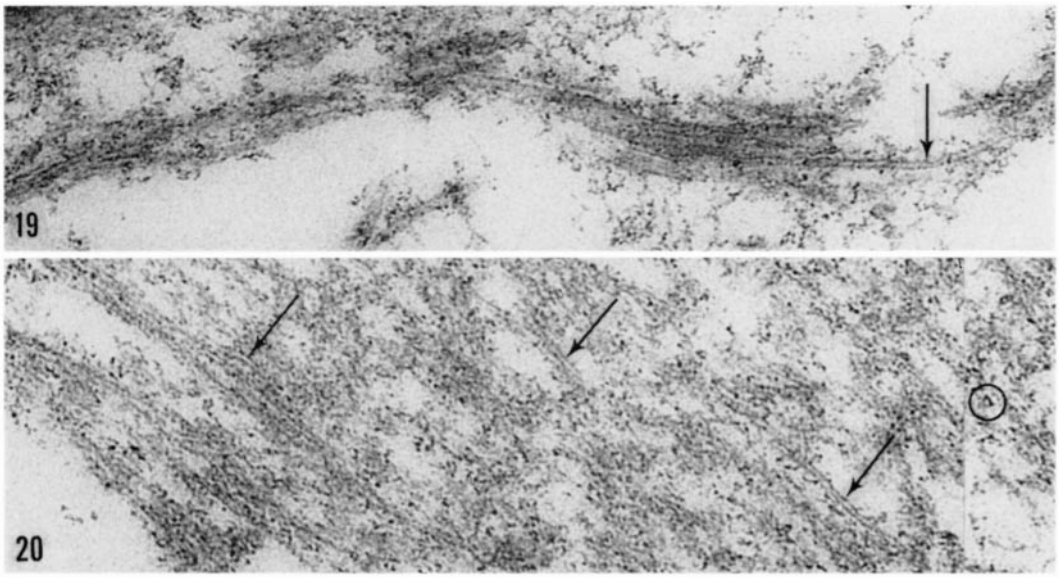
FIGURE 15 Palladium-shadowed, plasmolyzed cell demonstrating protruding body at the polar end of the cell near the base of the flagellum. × 20,700.



scattering of the outer portion of the flagellum; permanganate staining appears to result in more contrast between the outer and inner regions of the flagellum. Uranyl acetate appears to cause no increase in electron scattering (Figs. 19 and 20). The diameter of 12 mμ is not strictly consistent; occasionally 14- to 15-mμ oblique and cross-sections and 10- to 16-mμ longitudinal sections are observed.

Negatively stained, isolated flagella were studied after having been dried on carbon-coated support membranes containing small holes. TMV particles are added to the flagellar suspensions in order to aid the focusing and to provide a particle with a similar diameter and a known central core (*ca.* 4 mμ). When the darkened central core of the TMV particle is best demonstrated, the supporting membrane appears slightly underfocus. In order to be assured that optimum focus is achieved in preparations, either the TMV or the width of the underfocus fringe around the membrane hole can be utilized.

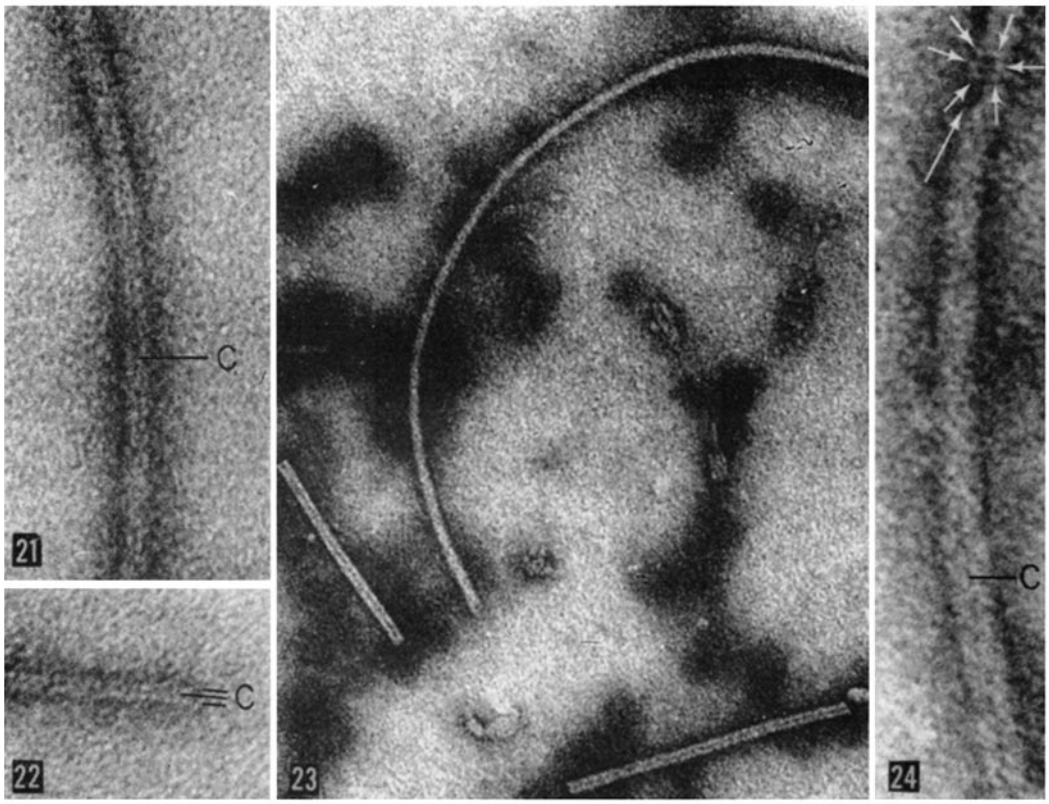
Negatively stained, isolated, but otherwise untreated, flagella do not show a darkened central region like that of TMV (Fig. 23). After ultrasonic treatments of 30 and 60 seconds, the flagella are shorter, occasionally demonstrate a subtle central darkening (Figs. 21, 22, and 24), and have a more irregular appearance along their length. The darkened central region is not apparent in all treated flagella and, when apparent, it can only be detected along short portions of the flagellum. The width of this central region is about 1.5 mμ. Occasionally, a region giving the



FIGURES 19 and 20 Appearance of longitudinal (arrows) and oblique or cross-sectioned (circles) flagella in an unstained section (Fig. 19) and in one stained with uranyl acetate (Fig. 20). Fig. 19, × 91,000; Fig. 20, × 85,000.

FIGURES 16 and 17 Longitudinal (arrows) and oblique or cross-sections (circles) of flagella as demonstrated by permanganate staining. The structure labeled (*A*) demonstrates an atypically small dimension. Aggregated chromatin (*C*) may be observed in adjacent cell-sections. Fig. 16, × 110,000; Fig. 17, × 107,000.

FIGURE 18 Lead-stained section demonstrating longitudinal (arrows) and oblique or cross-sections (circles) of flagella. × 97,000.



FIGURES 21 and 22 Flagella negatively stained with phosphotungstate after treatment with ultrasonic vibrations for 30 and 60 seconds (Figs. 21 and 22, respectively). Central darkening is apparent (*C*) in Fig. 21; Fig. 22 demonstrates two parallel rows of subunits (outer lines) separated by a darkened central region (*C*). Fig. 21, × 366,000; Fig. 22, × 334,000.

FIGURE 23 Flagellum, not previously treated with ultrasonic virbration, and two particles of Tobacco Mosaic Virus, the latter demonstrating central darkening due to hollowness. Negatively stained with phosphotungstate. × 134,000.

FIGURE 24 Flagellum, previously treated for 60 seconds with ultrasonic vibration, demonstrating darkened central region along part of its length (*C*), apparent void (large arrow), and separate subunits (small arrows). Negatively stained with phosphotungstate. × 435,000.

impression of partial degradation of the flagellum appears as a void in the structure (Fig. 24, large arrow). Flagella treated for 120 or 300 seconds are severely altered so that they appear as only slight darkenings of the supporting membrane.

DISCUSSION

Cell Wall and Plasma Membrane

A detailed concept of the gram-negative cell wall and plasma membrane structure has arisen from various studies. Prior to 1959, little evidence (57) had been presented to support the occurrence of membranes which are structurally similar to the "unit membrane" described by Robertson (45). Since Glauert and Hopwood's convincing demonstration of a multilayered plasma membrane in hyphae of *Streptomyces coelicolor* (20) similar evidence has been given for gram-negative and gram-positive bacteria which seem to vary only in the thickness of the electron-transparent layer (11, 15, 22, 25, 27, 40, 42, 43, 46, 54). A similar additional membrane has been observed outside the plasma membrane of certain gram-

negative bacteria (3, 22, 30, 40, 42, 53). The clearest observations of the cell-wall membrane and the plasma membrane have come from studies of phage-infected and induced spheroplasts of gram-negative bacteria (22, 31, 40, 53) where two membranes are present but separated by an apparently empty space. When gram-negative cell-wall fragments are observed in the electron microscope, "macromolecular" spheres having diameters of 8 to 12 mμ (49) have been observed to lie within, or occasionally outside, a smooth layer (40, 41, 49, 59); such a "macromolecular" layer in sectioned gram-negative bacteria has been conspicuous only by its absence, as noted by Chapman (7). Cohen-Bazire and Kunisawa (10) have recently indicated that the cell wall of *Rhodospirillum rubrum* may consist of two layers. Ogura (43) has also very recently described a cell wall consisting of two layers; however, the lack of consistent demonstration of a plasma membrane leaves the observer without a point of reference with which to interpret the whole cell envelope. Murray (41) has recently described a gram-negative cell wall containing a thin layer between the cell-wall membrane and the plasma membrane; "macromolecular" spheres were described on the outside of the cell-wall membrane.

The sectioned cell wall of *A. suboxydans* has been found to consist of more than an outer membrane. An inner, more homogeneous layer having a thickness similar to that described for the layer of "macromolecular" spheres is also present. The plasma membrane of *A. suboxydans* is structurally similar to the cell-wall membrane and separates the cell-wall inner layer from the protoplasm.

A structural concept of the gram-negative cell wall and plasma membrane can be formulated on the basis of this and the several other studies cited above. Fig. 25 is a diagrammatic presentation of the structure of the cell envelope found in *A. suboxydans* and is thought to be representative of the morphological features generally expected in gram-negative bacteria.

The chemical composition of gram-negative cell walls also indicates that they consist of more than a single membrane. The cell walls are reported (49) to contain about 20 per cent lipid, a large percentage of protein, and some mono- and polysaccharides in addition to a mucopeptide complex. From such studies, it has been estimated that the gram-negative cell wall has a thickness of *ca.* 25 mμ (38). This estimate corresponds closely with the combined thickness of the cell-wall membrane and inner layer of *A. suboxydans*. A difference in the number of carbon atoms in the fatty-acid chains of the electron-transparent lipid layer of membranes may account for the variable thicknesses reported in bacterial membranes (25); such a difference may also account for the slight variation noted in electron-transparent layers of the cell-wall membrane and plasma membrane of this bacterium. Chemical studies also indicate that the cell walls consist of a thin network of

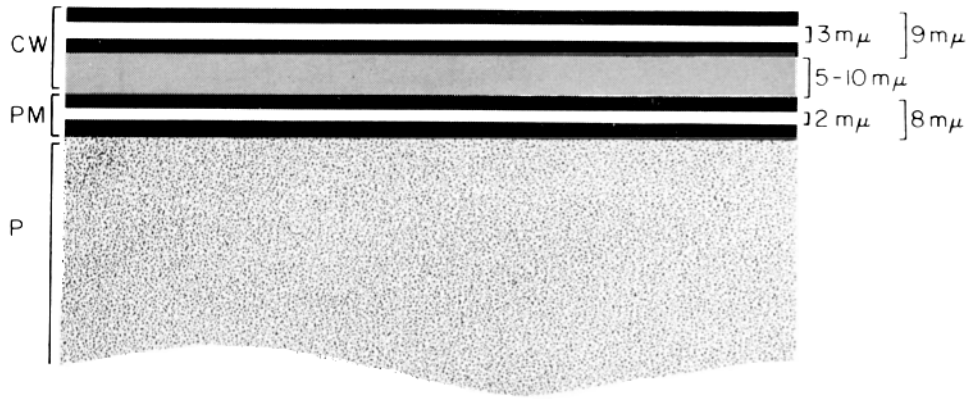


FIGURE 25 Diagrammatic representation of the sectioned cell wall (*CW*), consisting of a membrane covering a more homogeneous inner layer, and the plasma membrane (*PM*). This structural concept of the sectioned cell wall and plasma membrane, as found surrounding the protoplasm (*P*) of *Acetobacter suboxydans*, is thought to be characteristic of gram-negative bacteria in general.

"mucocomplex" (25), similar in composition to the cell wall of the gram-positive organisms, and that this complex is covered on the outside by a plastic film of lipoprotein (38, 49, 63). Such a concept is in keeping with the previous observations of isolated cell walls and the appearance of sectioned cell walls in *A. suboxydans*.

Chemical studies suggest that part of the "mucocomplex" portion is responsible for the structural rigidity of the cell wall (49, 63). The gram-negative cell-wall membrane and the plasma membrane of phage-infected cells and induced spheroplasts are separated by an empty space (22, 31, 40, 53). Furthermore, evidence (63) suggests that phage infection enzymatically destroys the material necessary for the structural rigidity of the cell wall, and that phage have affinity for the "macromolecular" spheres isolated from cell walls of *E. coli* (60). The morphological evidence of abnormal cells in this study (Figs. 4 and 5 *a*) similarly suggest that the lack of the cell-wall inner layer is correlated with the loss of structural rigidity.

Thus, the structural evidence that the cell wall in sections of *A. suboxydans* consists of an outer membrane and an inner, homogeneous layer agrees with the information from chemical and ghost isolation studies of the gram-negative cell wall. In addition, the inner homogeneous layer of the cell wall probably corresponds structurally to the "mucocomplex" portion that, in part, is functionally vital to the structural rigidity of the cell wall.

One possible explanation for the previous lack of preservation of the inner layer in sections of gram-negative cell walls may be that which Weidel *et al.* (58) recently suggested for the erroneous interpretations in physical and chemical studies of cell wall ghosts, that is, the extremely rapid action of the autolytic enzymes which destroy the continuous rigid mucopolymer layer in cell walls of *Salmonella gallinarum*. The use of a plasma clot suspension in this study may have better preserved the conditions necessary for cell maintenance and thereby greatly decreased the time during which the cells were subjected to nonphysiological conditions prior to inhibition of the autolytic enzymes.

Although the chemical composition of the "mucocomplex" varies, gram-negative and gram-positive bacteria have mucopeptide constituents in common (49, 63), a fact which has led to the idea of a "basal" chemical structure being present in all bacterial cell walls (62). Sectioned gram-positive cell walls appear as a non-differentiated layer (7, 14) similar to the cell-wall inner layer of *A. suboxydans*. From the chemical considerations and from the electron microscope studies, it can be postulated that the cell-wall inner layer of gram-negative bacteria is morphologically similar to the complete cell wall of gram-positive bacteria.

Protoplasmic Constituents

When bacteria are fixed by various methods (7, 48) and sectioned, the chromatin material appears as a non-membrane–limited, relatively electron-transparent region that contains fibrillar material in an unorganized (31) or organized (17, 28) mass; the fibrillar material in the chromatin of *A. suboxydans* appears unorganized. Under the experimental conditions of the studies reported here, the chromatinic material of short cells is much more aggregated than that of elongated cells, which would suggest a possible correlation between chromatin dispersion and cell growth. These observations are not in keeping with those on other bacteria in which continuously aggregated chromatin is observed (7, 8, 11, 37); however, Glauert (18) has reported similar findings in *Clostridium welchii* and *Lactobacillus acidophilus*.

A differentiation in the polar region is typically present in *A. suboxydans*. Although its chemical nature or function is not known, the region was not observed in permanganate-stained sections and was not stained by uranyl acetate or lead hydroxide (see 26, 55). Typically, elongated cells with highly dispersed chromatin best demonstrate these polar structures, whereas short cells do not show them with certainty. Similar inclusions have been reported in the polar region of *E. coli* and *B. megaterium* (39, 40) and have been described as polysaccharide accumulations; however, these inclusions are less specifically localized and are fewer in number than those reported here.

Leifson (36) characterizes *A. suboxydans* as having polar multitrichous flagella. In the present study, shadowed preparations of plasmolyzed cells have demonstrated the appearance of a protruding body near the base of the flagellum, while sections show the polar differentiations described above and occasionally some intracytoplasmic membranous material. Recently, the presence of extramembraneous material and the lack of ribosome-like particles in the region of

flagellar insertion of *Spirillum serpens* has been reported (41). The possibility that the polar differentiation found in *A. suboxydans* is structurally or functionally correlated with the flagella seems possible, but is not established.

Flagella

Since their description by Cohn in 1872 (see 24), bacterial flagella have been described as uniform filaments (56) having diameters of 12 mμ (46), although diameters of from 12 to 19 mμ (1, 32, 52) have been reported. Helical subfibrils have been reported upon occasion in the sheathed and unsheathed flagella (12, 21, 33, 56, 61). Studies of the chemical nature of flagella indicate that they consist almost entirely of proteinaceous material of the keratin-myosin-elastin-fibrinogen group (56). The flagellin (2) macromolecular subunit has been reported by Erlander, Koffler, and Foster (13) to have a molecular weight of 20,000, while other workers have suggested a molecular weight of from 30,000 to 40,000 (32).

The examples of structures presented as flagella in Figs. 16 through 20 are believed to be the flagella of *A. suboxydans* for the following reasons. First, the diameter of the linear, circular, or oval structures is the same as the flagella diameter determined by shadowing or negative staining; the fact that the over-all diameter is greater than that of either the outer membrane of the cell wall or the plasma membrane eliminates the slight possibility that these structures are membranous debris. Secondly, the finding of scattered areas of high concentration of flagella in sections correlates well with the light microscope observation of their detachment and aggregation; the presence of little or none of these structures in sections of the plasma clot devoid of cells strongly indicates they are not components of the clot. Finally, the appearance of these structures closely resembles the appearance in sections of isolated flagella from *S. typhimurium* (32). Thus, from sections of *A. suboxydans* the structure of flagella appears to consist of a dark outer region surrounding a region which is either hollow or filled with material having an electron-scattering property similar to that of methacrylate.

Negative staining of isolated flagella does not readily demonstrate hollowness. This fact is accentuated when negatively stained, isolated flagella are compared with TMV particles (Fig. 23). Since the diameter of the central region of the flagellum as observed in sections is similar to the known luminal diameter of the TMV particle, one would expect the flagellum to also show the penetration of phosphotungstate if this central region is hollow. Rigorous physical treatment of the flagellum appears necessary for such penetration into the central region, and even then the diameter of this region is very small.

The second flagellar model suggested by Burge (6), which consists of 6 subunits surrounding a central subunit, seems unlikely in this case, since the phosphotungstate penetration, when seen, appears centrally located. Erlander *et al.* (13) report that sedimentation values for one form of flagellin indicate that it is ellipsoid in shape. An arrangement of such subunits would result in a much smaller central diameter than would an arrangement of essentially spherical subunits suggested by Kerridge *et al.* (32). Correlation of the present negative staining and sectioning results along with the chemical and physical studies on other flagella suggests the hypothesis that the large protein molecules are polymerized in such an orientation that osmium can react only at the periphery of the molecule, while the remainder of these macromolecules, surrounding a nearly closed central region, is left unaffected.

Similar filaments, though slightly larger in diameter, are observed in sections of the mitotic apparatus, cilia, centrioles, and the protozoan infraciliature, as recently reviewed (47). Regarding the function of such filaments, Slautterback (50) has emphasized their "tubular" nature by suggesting that they are utilized for fluid passage and transport. The present study suggests, however, that the luminal diameter demonstrable from sections may also be much smaller for these filaments.

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Note Added in Proof

The generalized concept of the gram-negative cell wall structure as presented here has been strength-

ened by the recently published micrographs of the genus *Bacteroides* (BLADEN, H. A., and WATERS, J. F., *J. Bact.*, 1963, **86,** 1339). Their micrographs are interpreted to show a "space" between the cell-wall outer membrane and the plasma membrane which has a greater density than the embedding medium. We believe that this "space" structurally corresponds to the cell-wall intermediate layer of *A. suboxydans* and possibly to the "mucocomplex" portion of gram-negative cell walls.

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