JOURNAL OF BACTERIOLOGY, June 1974, p. 1158-1166
Vol. 118, No. 3

Electron Microscopy of the Cell Wall of *Rickettsia prowazeki*

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Received for publication 11 March 1974

Purified *Rickettsia prowazeki* were found to undergo morphological changes resembling plasmolysis when stained with uranyl acetate, resulting in rod-like forms. Sequential electron micrographs of disintegrating organisms provide evidence for the cell wall origin of these rod-like forms. The substructure of the cell wall was discerned by using negative-contrast electron microscopy. The wall was found to be composed of repetitive subunits with a periodicity of 13 nm and was surrounded by a thin membrane.

Evidence accumulated in recent years suggests that rickettsiae are highly fastidious bacteria that share the common property of intracellular parasitism (14, 21). Chemically and morphologically, rickettsiae appear to be similar to gram-negative bacteria, and like these bacteria, their cell walls contain several sugars, a variety of amino acids, muramic acid, and diaminopimelic acid (13, 15, 23). Moreover, they do not contain teichoic acid (24), which is characteristically present in gram-positive bacteria. In general, electron micrographs of thin sections of rickettsiae have revealed the cell wall of the organisms to be a trilaminar structure composed of two dense layers separated by a pale layer (3, 12). In more definitive studies with high-resolution electron microscopy, Anacker et al. (2) recently described a five-layered architecture in the cell walls of *Rickettsia prowazeki* similar to that seen with *Escherichia coli*. Purified cell wall preparations of rickettsiae examined by chromium shadow-casting and various negative stains also show a morphology similar to that of gram-negative bacterial cell walls (23). However, no studies have yet revealed any structural detail of isolated rickettsial cell walls. In the present paper, we report investigations on the structure of purified *R. prowazeki* as shown by high-resolution electron microscopy with negative staining techniques. The presence of a repetitive substructure of the cell wall as well as unusual forms of the organism are shown.

MATERIALS AND METHODS

Growth of rickettsiae and purification. *R. prowazeki*, strain Breinl, was propagated in 6-day-old embryonated hen's eggs from a flock maintained on antibiotic-free feed. The eggs were inoculated via the yolk sac with a dose of rickettsiae adjusted to kill approximately one-third of the eggs between 9 and 10 days after inoculation. Smears prepared from representative eggs were stained by the Gimenez method (10). When the yolk sac smears indicated extensive rickettsial growth, the remaining yolk sacs were harvested and blended to make a 20% (wt/vol) suspension in 0.01 M sodium phosphate-buffered 0.15 M NaCl (PBS), pH 7.0, containing 0.1% Formalin. This suspension was incubated overnight at 4 C to inactivate the rickettsiae. The suspension was then mixed with an equal volume of 50% (wt/vol) sucrose in PBS and centrifuged for 1 h at 30,000 rpm in a no. 30 Spinco rotor. The pelleted rickettsiae were suspended in PBS to a volume equivalent to the original 20% suspension and mixed with Celite (25). The Celite was removed by low-speed centrifugation, and the supernatant containing rickettsiae was mixed with an equal volume of 50% sucrose and centrifuged as before. The sediment from this centrifugation was suspended in PBS and sedimented through a cushion of 30% sucrose (wt/wt) in PBS by centrifugation in an SW25.1 Spinco rotor at 15,000 rpm for 1 h. The resulting sediment was suspended in 18 ml of PBS and layered onto 9 ml of 30 to 50% glycerol-potassium tartrate viscosity density gradients (J. F. Obijeski, A. T. Marchenko, D. H. L. Bishop, B. W. Cann, and F. A. Murphy, J. Gen. Virol., in press). Gradients were centrifuged for 3 h at 40,000 rpm in an SW41 Spinco rotor. A visible band resulted at a density of about 1.3 g/cm^3. It was collected and recentrifuged in the same type of gradient. The final band was collected by side puncture of the tube with a needle and was washed free of gradient material by centrifugation and washing in distilled water. Other rickettsiae used in this study were purified by the same procedure as described above.

Complement fixation. Complement fixation tests were performed by the Laboratory Branch complement fixation method (7). Reference reagents for the test were obtained from the Biological Reagents Section, Center for Disease Control, Atlanta.

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Electron microscopy. Specimens were prepared for electron microscopy by the pseudoreplica technique (19). For staining, Formvar containing entrapped organisms was floated on either 0.5% uranyl acetate (UA) at pH 4.0 or 2.0% potassium phosphotungstate (PTA) at pH 7.0. Double staining was accomplished by mixing equal volumes of 0.5% UA with purified rickettsiae and then counterstaining with PTA by the pseudoreplica technique.

RESULTS

Figure 1 shows a photograph of a glycerol-potassium tartrate density gradient after the final centrifugation of *R. prowazeki* to equilibrium. The organisms form a well-defined, milky-white band at a density of about 1.3 g/cm^3. Electron microscope examination of the banded material showed that it contained intact rickettsiae and no extraneous cell debris. The banded rickettsiae also reacted in complement fixation tests with homologous antisera and failed to react with hyperimmune animal sera to normal egg material.

Figure 2A shows an electron micrograph of purified *R. prowazeki* stained with PTA. The organisms vary in size from 0.3 to 0.5 μm in width and 1 μm or more in length. The convoluted surface morphology typical of gram-negative bacteria is clearly evident, and some cells are sufficiently penetrated by the stain to reveal a layered wall (arrows). Figure 2B shows the same preparation stained for about 20 s with UA. The particles now appear as electron-dense areas surrounded by an electron-translucent structure. The insert shows a single cell from the same UA-stained preparation viewed at a higher magnification. No details of surface structure are evident except that the electron-dense area appears to be a membrane-bound region that has contracted and pulled away from the cell wall (arrow). These same staining characteristics were also seen with purified preparations of *R. mooseri*, *R. rickettsi*, *R. canada*, and *R. akari*, but not with other gram-negative organisms such as *Proteus* OX-19 or OX-2.

When purified *R. prowazeki* and UA were allowed to stand for longer periods of time, particles such as shown in Fig. 2C through 2I were obtained. The preparations from which the electron micrographs were made in this study were allowed to float on UA stain for 1 to 24 h. They had been stored at 4 C for less than 24 h in distilled water. Some preparations were doubly stained with PTA. The particle shown in Fig. 2C has a striated structure and is elongating at what appears to be a tear in the particle (arrow). It has also completely lost its electron-dense center. Figure 2D shows a form which, except for a bleb at one end, is completely elongated. This form has clearly defined striations with a periodicity of approximately 13 nm. The length of the rod-like forms varies and measures up to 4 μm. Their width also varies considerably but generally falls between 60 and 160 nm. Figure 2E shows a form that has folded and is collapsed on the Formvar surface so that it appears devoid of internal components. The figure also shows an elongated form with a double membrane layer (arrows). The electron micrograph in Fig. 2F shows a form that has folded, and the arrow points to areas where the membranes surrounding it are connected at 13-nm periodic intervals.

Figures 2G through 2I show other forms of *R. prowazeki* seen in UA-stained preparations. The form in Fig. 2G shows surface details of the periodic striations. They appear to be composed of regularly occurring subunits. Figure 2H shows a rounded form with clearly evident surface striations. The last figure, Fig. 2I, shows what appears to be an intact rickettsial enve-

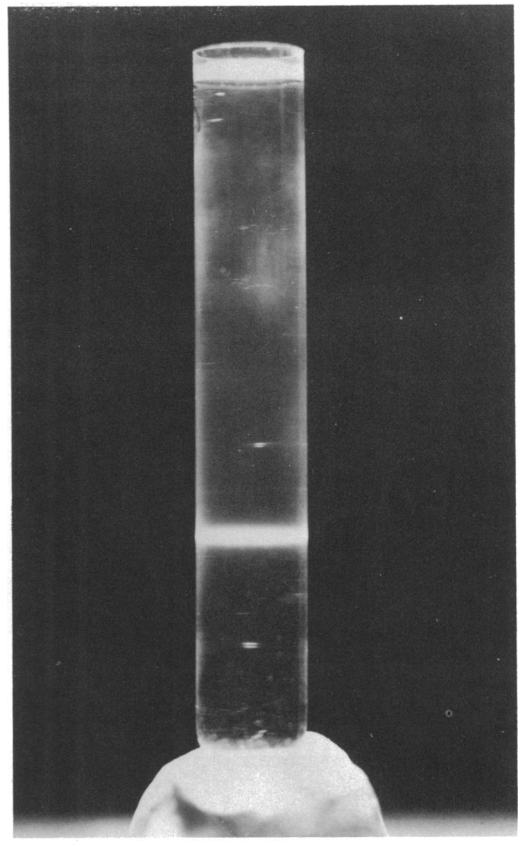

FIG. 1. *Photograph of glycerol-potassium tartrate density gradient after centrifugation of R. prowazeki to equilibrium. The organisms band as a milky-white area at a density of about 1.3 g/cm^3.*

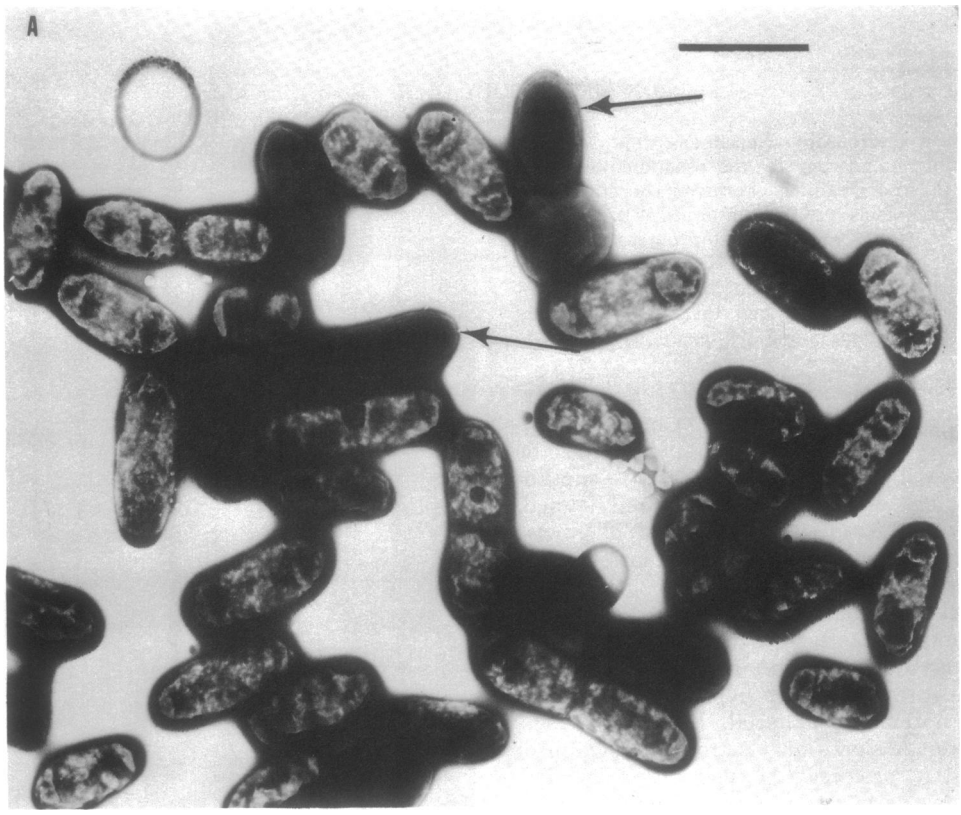

FIG. 2A. *Electron micrograph of purified R. prowazeki stained with PTA. Arrows point to a layered wall surrounding the bacterium. The scale bar represents 0.5 μm.*

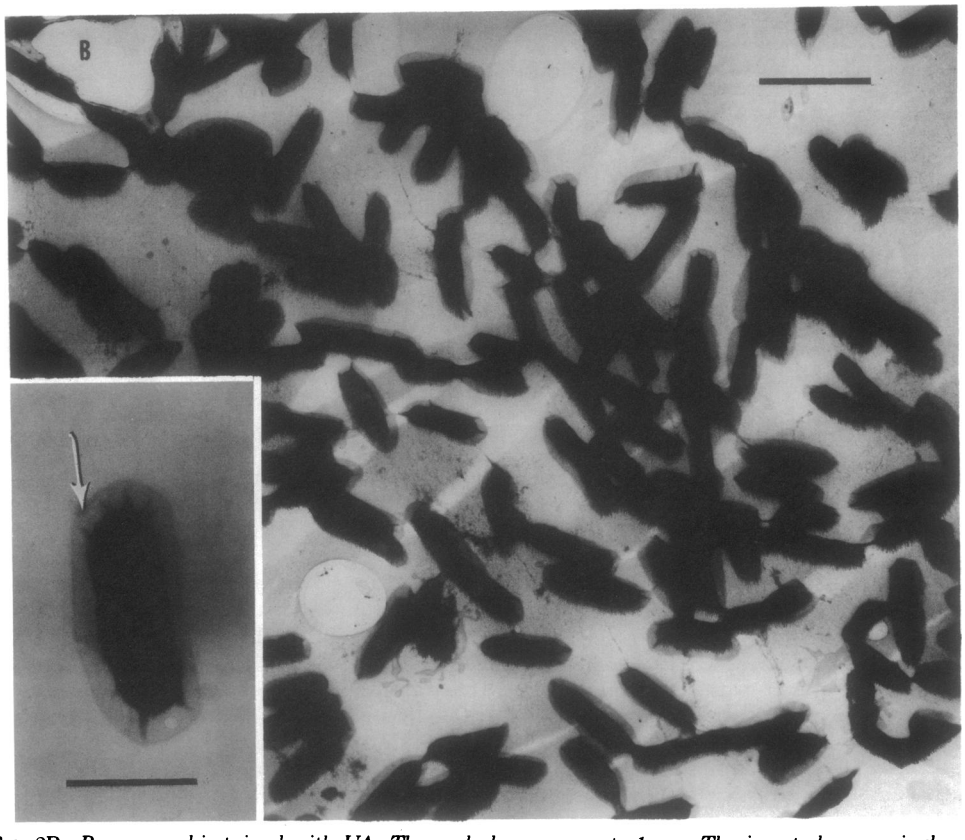

FIG. 2B. *R. prowazeki stained with UA. The scale bar represents 1 μm. The insert shows a single particle with an electron-dense center surrounded by an electron-translucent structure (arrow). The bar represents 0.5 μm.*

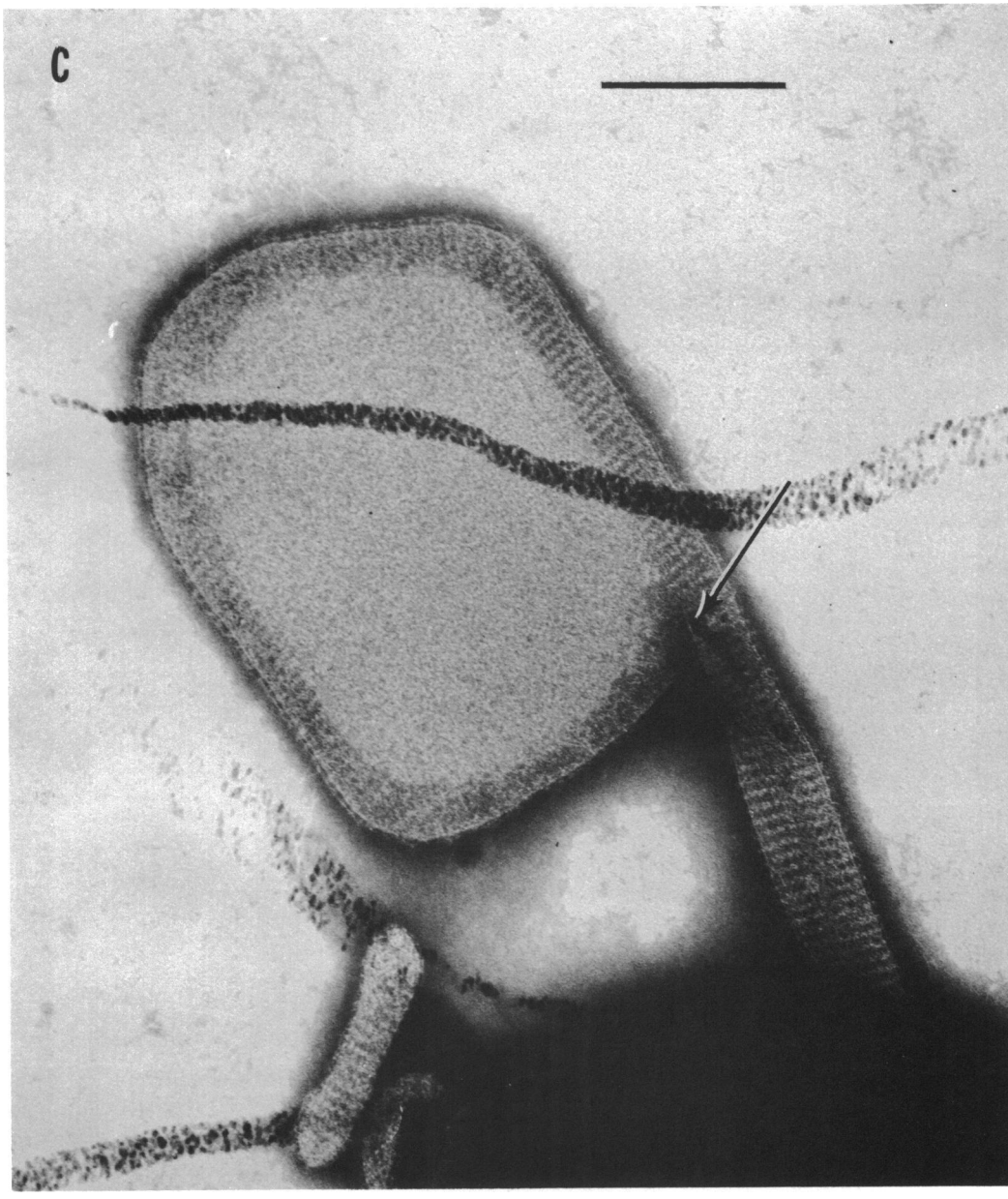

FIG. 2C. *R. prowazeki stained with UA. The particle is elongating at a point of a tear (arrow), and striations are evident. The scale bar represents 0.2 μm.*

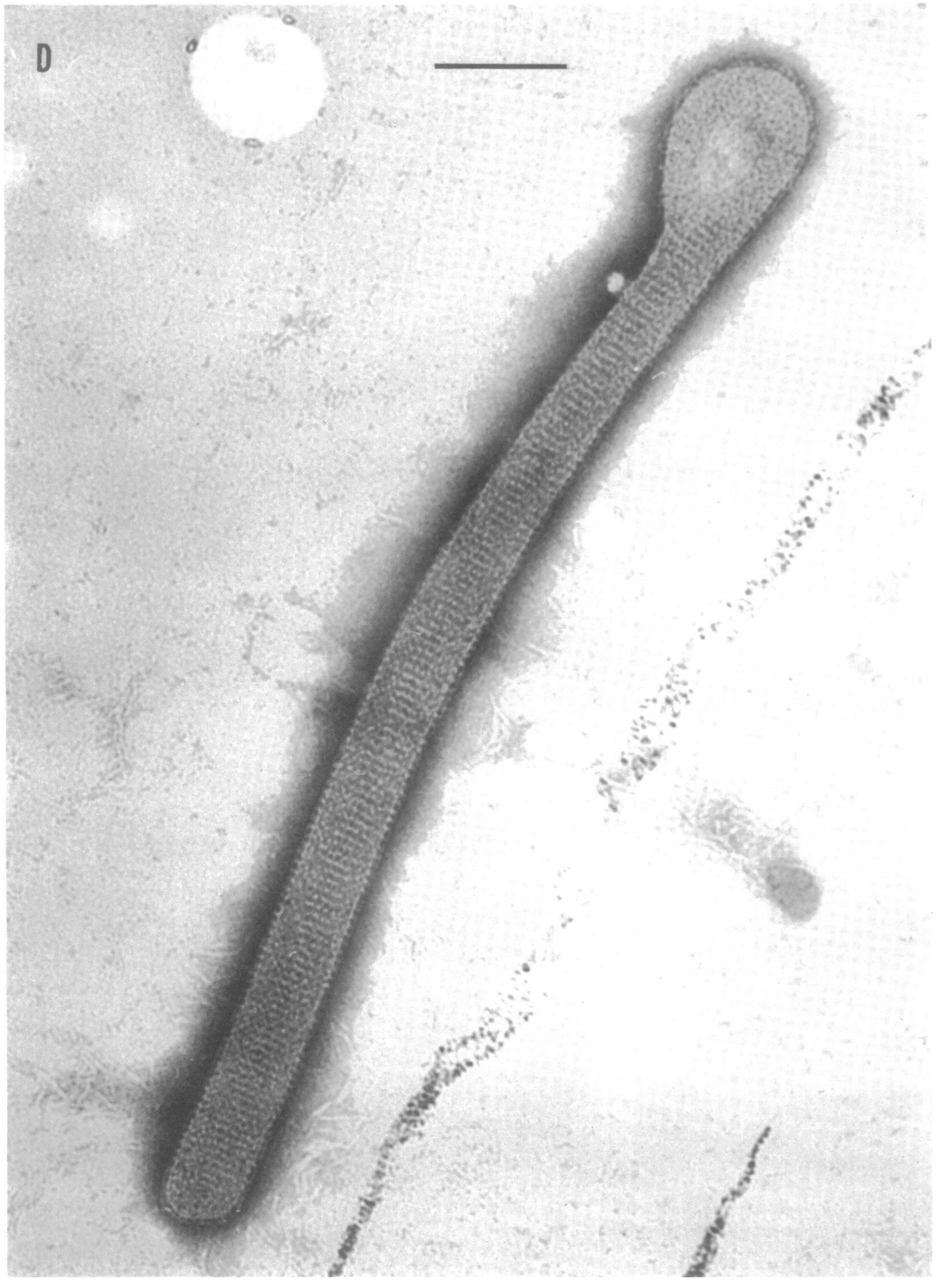

FIG. 2D. *Elongated form of R. prowazeki stained with UA and then counterstained with PTA. Periodic striations are spaced 13 nm apart. The scale bar represents 0.2 μm.*

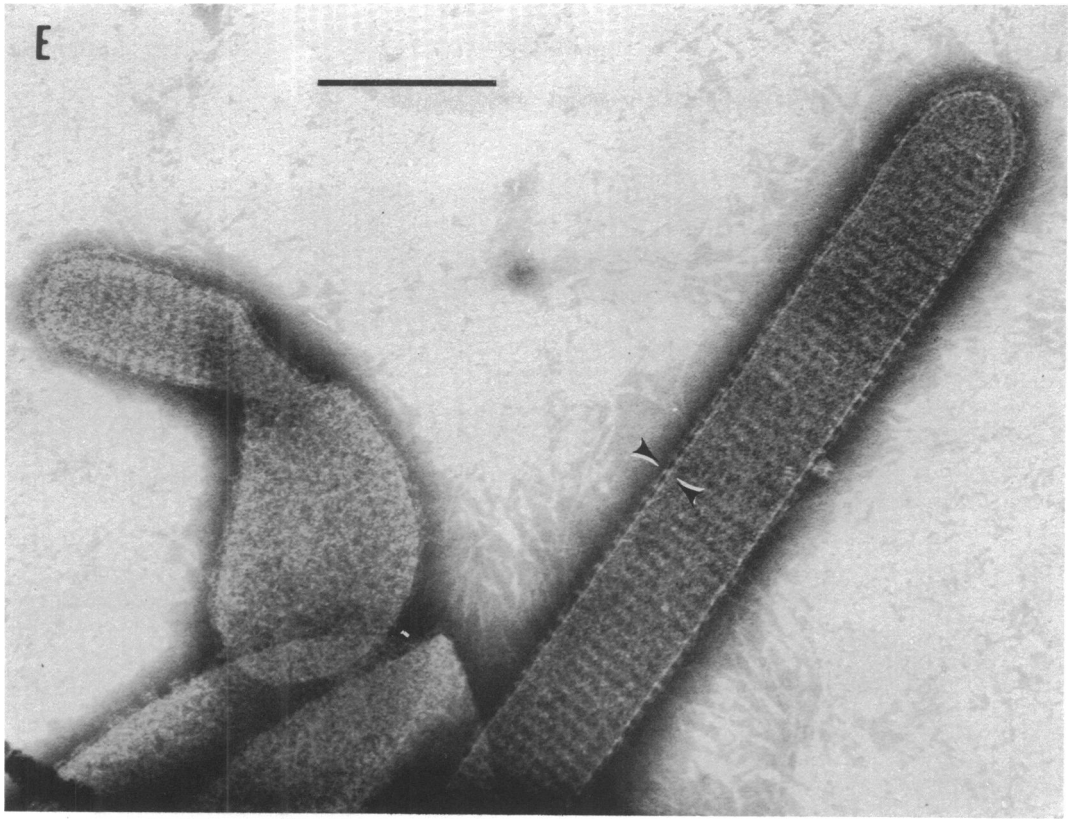

FIG. 2E. *Two forms of R. prowazeki stained with UA and PTA. The smaller form is folded upward, revealing that it is flat. The rod-like form shows an elongated particle with a double membrane (arrows). The scale bar represents 0.2 μm.*

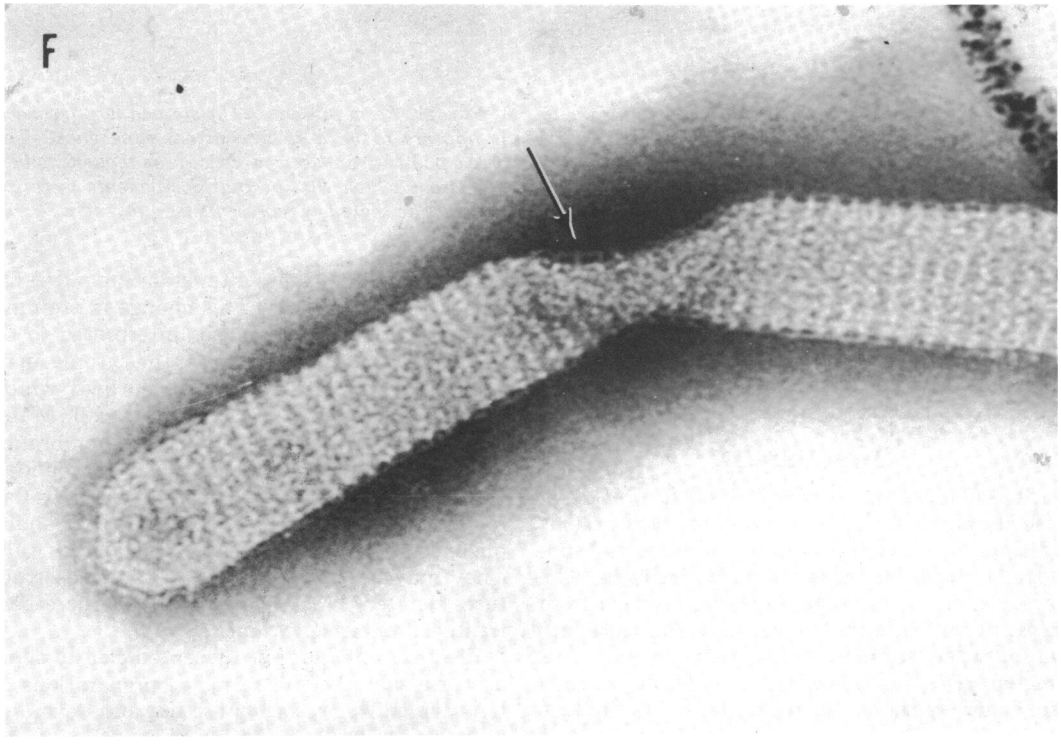

FIG. 2F. *Elongated form of R. prowazeki stained with UA and PTA. The arrow points to a fold revealing that the periodic striations are joined by a thin membrane. The scale bar represents 0.1 μm.*

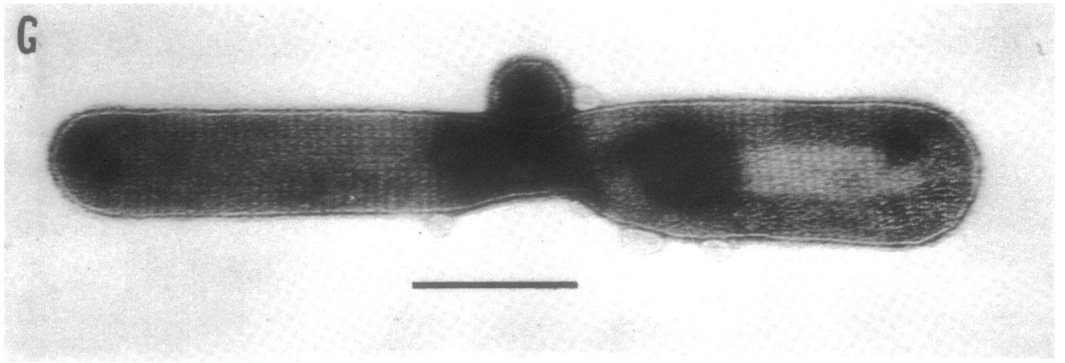

FIG. 2G. *Flat form of R. prowazeki stained with UA. Repetitive subunits of the periodic striations are evident. The scale bar represents 0.2 μm.*

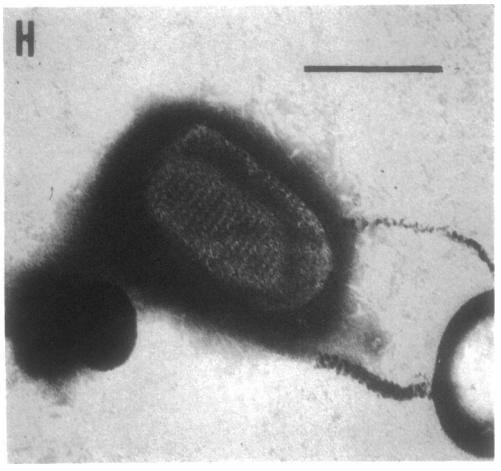

FIG. 2H. *Round form of R. prowazeki stained with UA and PTA. Striations on the particle surface are identical to those seen in elongated forms. The scale bar represents 0.2 μm.*

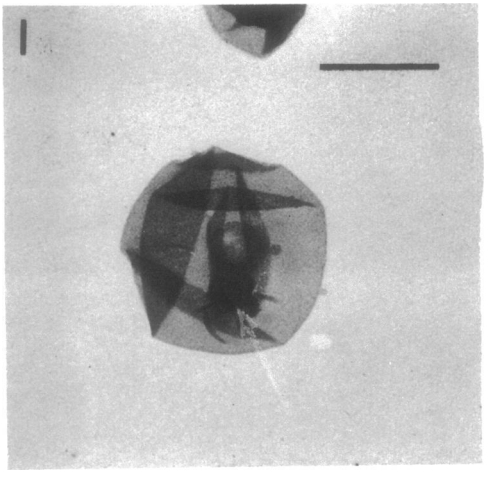

FIG. 2I. *Form present in UA-stained R. prowazeki. It appears to be a rickettsial envelope ghost. The arrow points to an electron-dense area thought to be a hole from which the internal components were expelled. The scale bar represents 0.5 μm.*

lope ghost. The arrow points to an electron-dense area that appears to be a hole from which the internal components of the organism have been expelled. Although the rod-like wall forms are predominant in UA-stained rickettsial preparations, several of these envelope ghost forms that are usually seen in each support grid.

DISCUSSION

It is evident that when purified *R. prowazeki* are stained with UA at pH 4.0 for short periods of time, the organisms rapidly undergo morphological alterations resembling plasmolysis. The cytoplasmic membrane appears to retract from the cell wall, and the cell contents condense into an intensely stained electron-dense region. This region, which is characteristic of cells stained for short periods of time, loses its intense staining characteristics after standing in UA for longer periods of time. This change in staining properties of the organism is interpreted to be the result of a steady loss from the cytoplasm of cellular components such as nucleic acid, which stain intensely with UA at low pH (22). Many cells appear to completely lose their membranes and internal components. This leaves a rounded form that then begins to elongate, forming a flat rod structure. These structures are no doubt pleomorphic forms of the rickettsial cell wall. They may be similar to the rod-shaped structures derived from the cell walls of the ether-treated *Francisella tulanensis* (18).

Electron micrographs presented clearly demonstrate that the rod forms in preparations of UA-stained *R. prowazeki* are flat and devoid of

internal constituents. It is not possible to state whether or not the cytoplasmic membrane is still present in these forms, but no such structures could be demonstrated by the procedures described. The plasmolysis-like process that leads to the formation of the rods suggests that the membrane has been eliminated along with internal components such as ribosomes that would stain darkly with UA.

Many of the empty rod-shaped forms appeared to be bounded by a membrane similar to that seen surrounding the intact organism after it is stained with PTA. When these rods are collapsed flat on the Formvar surface, the substructure of their surface becomes evident. High-resolution electron microscopy shows the surface to have periodic striations composed of repetitive subunits. These striations traverse the width of the wall and appear to be attached to a thin membrane that surrounds the wall. The striations have a periodicity of 13 nm, which is similar to that reported for several other bacteria (6, 9, 20). The length of the rods varies and measures up to 4 μm.

To the best of our knowledge, this is the first description of rickettsial cell walls that demonstrates periodicity of their surface structure and the presence of repetitive subunits. The mechanisms whereby UA brings about such a change in the rickettsial cell wall are not known, but the ability of UA to stabilize bacterial wall subunits has been reported (10). It is possible that the action of UA is a reflection of damage to the organisms incurred after the process of purification and suspension of the cells in distilled water. The highly unstable nature of these organisms when placed in aqueous suspension has been reported: in aqueous suspension they have been shown to readily lose nucleic acids and other cellular components (5, 8). Nevertheless, when stained with UA for short periods of time, all rickettsiae examined appear as electron-dense centers surrounded by an electron-translucent structure. In contrast, we could not demonstrate this staining characteristic with *Proteus* OX-19 or OX-2. Nor did these latter organisms form rod-like cell walls upon extended treatment with UA.

Among the other forms of *R. prowazeki* that we observed were many empty envelope ghosts. We were able to achieve excellent preservation of their morphology by staining these forms with UA. Similar results have been obtained by staining erythrocytes with this stain (17).

It is pertinent to add that other investigators have observed tubular structures with periodic striations in cultures of *Leptospira* after immune disruption (3) and in living and inactivated *Leptospira* material (1). However, these structures had a periodicity of 4 nm. Although their origin was not known, they appeared to be derived from the leptospiral sheath and were sometimes attached to the cell wall. Similar tubular structures have also been observed in cultures of the human pathogen, the Marburg agent (1, 16), but their periodicity differed from that of the tubular form of *Leptospira* and from those reported here for rickettsiae. The significance of these periodically striated forms of *Leptospira* and the Marburg agent is not known, but possibly they reflect an action of negative stain similar to that we have observed with *R. prowazeki* stained with UA.

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