JOURNAL OF BACTERIOLOGY, Jan. 1971, p. 355-360
Vol. 105, No. 1

Electron Microscopic Observations on the Structure of the Envelopes of Mature Elementary Bodies and Developmental Reticulate Forms of *Chlamydia psittaci*

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Received for publication 23 September 1970

Purified suspensions of *Chlamydia psittaci* were prepared from L cells. Thin sections of intact elementary bodies and intact developmental reticulate bodies and of their purified envelopes were observed by electron microscopy. In both intact organisms and partially purified envelopes, two membranous structures, each appearing in electron micrographs as two darkly stained layers, were observed. In the elementary body sections, the outer membrane was round, apparently rigid, and was not soluble in 0.5% sodium dodecyl sulfate. The inner layer was irregular in shape and was completely removed by detergent treatment. We interpret these results to indicate that the outer rigid layer of the envelope is the cell wall and the inner layer is the cytoplasmic membrane. When the fragile reticulate body envelopes were similarly studied, the outer cell wall was clearly visible, and some evidence of an inner membrane was seen. After treatment with nucleases and detergent, all evidence of inner or cytoplasmic membrane was removed, but the outer cell wall remained. Thus, it appears that the cell wall of this organism is continuous throughout the growth cycle and that the fragility and lack of rigidity of the reticulate body cell is due to changes in chemical composition or structure of the cell wall.

In the multiplication cycle of *Chlamydia* in susceptible cells, two different cell types are involved (1). One is the small, dense-centered infectious elementary body (EB) about 0.3 μm in diameter, and the other is the large, noninfectious reticulate body (RB) about 0.5 to 1.0 μm in diameter, this being the reproductive form. For a clear understanding of the pathway of multiplication of these microorganisms from dense cell through the reticulate cell and again to dense cell, it appeared necessary to study each cell separately and to compare their characteristics.

In a series of our studies on the meningopneumonitis (MP) organisms, the methods of purification for EB, RB, and their envelopes were established (4, 10, 11, 13). From these studies, it has been demonstrated that there are distinct differences in physical and chemical characteristics of EB and RB cells, especially in their envelopes. In this communication, electron microscopic studies of the thin sections of purified envelopes of EB and RB of MP organisms will be described. This study was designed to determine whether the cell wall of MP is continuous throughout the developmental cycle.

MATERIALS AND METHODS

The Cal 10 strain of the MP organism was used. The methods for propagation were essentially the same as those described previously (10). Purified suspensions of EB and RB of the MP organism were prepared essentially as described previously (10, 13). Preparation of envelopes from both forms followed the methods described previously (4, 11).

To prepare thin sections for electron microscopy, the suspensions of dense or reticulate cells, their envelopes, or L cells infected with MP organisms were centrifuged at appropriate speeds to make pellets. After centrifugation, the supernatant fraction was removed carefully, and the sediment was overlaid with 5 ml of ice-cold 0.75% glutaraldehyde-0.1 M phosphate buffer (*p*H 7.4) without disruption of the pellet. After prefixation for 30 min in an ice bath, the liquid layer was removed, and the pellet was cut into small pieces with a thin spatula, immersed in 1% OsO_4-Veronal-acetate buffer solution (*p*H 7.4; reference 7), and fixed for 1.5 hr in an ice bath. During fixation with OsO_4, the liquid layers were gently agitated. After postfixation with OsO_4, the blocks were

dehydrated in acetone and then were embedded with Vestopal W (9) or epoxy resin (3). Sections were cut on a LKB-ultrotome and then stained to enhance contrast by a combination of lead hydroxide (6) and uranyl acetate (14). The stained sections were sandwiched with carbon and examined in a JEM 6 C electron microscope at 80 kv, with a 30-μm objective aperture.

RESULTS

Envelopes of EB. Each purified EB particle is surrounded by a distinct envelope (Fig. 1). Electron-dense structures can be observed in the nucleoid, and ribosomes are scattered throughout the cytoplasm.

A suspension (4 ml) of purified EB in 0.033 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.4) was mixed with 4 g of glass beads (no. 18) and homogenized by use of a Mickle apparatus (60 cycles/sec) at 5 C for 5 min or a Tominaga type B1-100B sonic oscillator at 5 C for 2 min. After homogenization, the treated suspension was allowed to stand for a few minutes and the supernatant fluid was separated with a capillary pipette. The glass beads were washed six times with 1 ml of 0.033 M Tris buffer (*p*H 7.4). The supernatant fraction and the washing fluid were pooled, and 5 to 7 ml of this homogenate was layered on 20-ml density gradient columns of 5 to 45% sucrose and centrifuged at 8,000 rev/min for 30 min in the SW 25 rotor of a Spinco centrifuge. The turbid band of envelopes which formed at the middle in the density gradient column was harvested by capillary pipette, diluted three- to fivefold with distilled water, and centrifuged at 10,000 $\times$ *g* for 1 hr. Figure 2 is an electron micrograph of cross sections of these envelopes. Although the materials inside the particles are almost eliminated by this treatment, the envelopes maintain their rigid shape. Beneath the outermost layer, other membrane structures are seen. These are irregular in shape and sometimes very close to the outermost membranes. The finding that the EB forms of the MP organism are surrounded by these two structures led to the conclusion that these envelopes correspond to the cell walls and cytoplasmic membranes which are found in bacteria.

The suspension of envelopes obtained above was suspended in 2 to 10 ml of 0.2 M Tris buffer (*p*H 7.4) containing 0.02 M $MgCl_2$ and then was sonically treated for 1 min to make a homogeneous suspension. This suspension was incubated with 0.1 mg each of ribonuclease and deoxyribonuclease per ml at 37 C for 2 hr. After incubation, 1 mg of trypsin per ml was added, and the suspension was further incubated for 2 hr. The suspension of cell walls was then diluted threefold with 0.2 M Tris buffer (*p*H 7.4) and centrifuged at 800 $\times$ *g* for 1 hr. In the electron microscopic observation of thin sections of the pellet obtained, no differences from Fig. 2 were observed.

After enzyme treatment, the pellet obtained above was suspended in 4 to 10 ml of water, and an equal volume of 1% sodium dodecyl sulfate (SDS) was added dropwise with vigorous shaking. The suspension was incubated at 37 C for 2 hr and was then centrifuged at 10,000 $\times$ *g* for 1 hr. Figure 3 shows the electron micrograph of a thin section of the envelopes obtained by this procedure. SDS treatment dissolved the inner unit membranes observed in Fig. 2 and only the outer cell wall remained. Maintenance of the shape of these walls after treatment with SDS indicates the rigidity of the structure. These cell walls are about 9.5 to 10 nm in thickness, as calculated from electron micrographs.

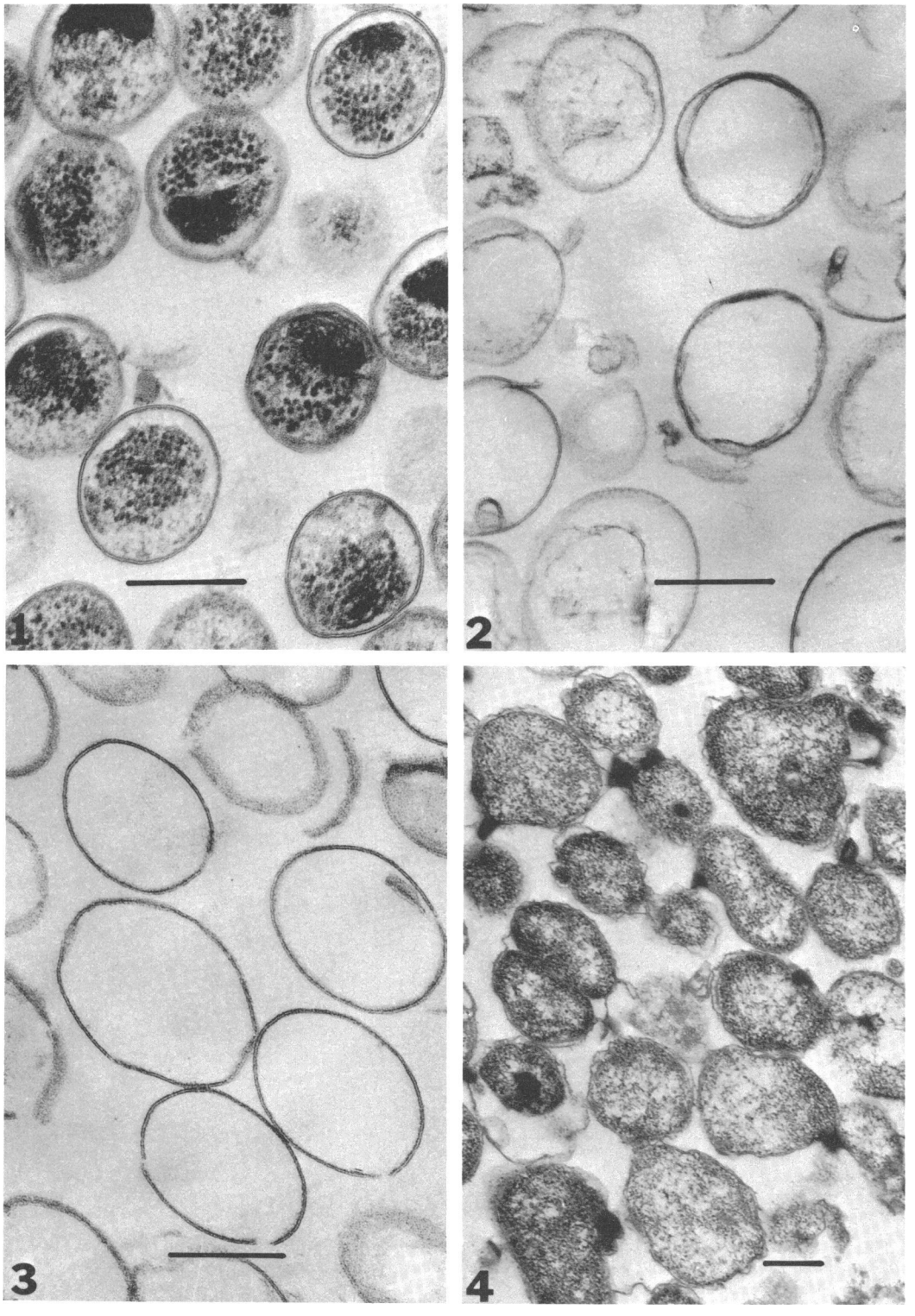
Envelopes of RB. Purified RB were prepared by infecting L cells at a high multiplicity, after which the cells were carefully washed in phosphate-buffered saline (PBS), resuspended in fresh medium, and incubated at 37 C on a rotary shaker for 18 hr. The cells were then collected by centrifugation and homogenized in a Teflon grinder for 10 min in an ice bath. The homogenate was centrifuged at 500 $\times$ *g* for 10 min, and the supernatant fluid was layered over 30% sucrose in 0.033 M Tris buffer (*p*H 7.4) and centrifuged at 5,000 $\times$ *g* for 1 hr. The precipitate was resuspended in 10% potassium tartrate solution containing 0.033 M Tris buffer and layered on top of potassium tartrate density gradient columns (20 to 40%) and centrifuged at 12,000 rev/min for 60 min in a SW 39 Spinco rotor. The RB were harvested from the band in the middle of the

FIG. 1. *Electron micrograph of a thin section of purified meningopneumonitis elementary bodies. A clearly defined double-layered cell wall is clearly seen, but distinct inner membranes are not distinguishable. Electron-dense nucleoids and ribosomes are clearly shown. Marker indicates 250 nm.*

FIG. 2. *Electron micrograph of a thin section of partially purified envelopes of meningopneumonitis elementary bodies. The outer cell wall and an inner cytoplasmic membrane are clearly seen. Marker indicates 250 nm.*

FIG. 3. *Electron micrograph of a thin section of purified envelopes of meningopneumonitis elementary bodies after extraction with sodium dodecyl sulfate. The cytoplasmic membranes seen in Fig. 2 have been completely removed. Marker indicates 250 nm.*

FIG. 4. *Electron micrograph of thin section of purified meningopneumonitis reticulate bodies. Each cell is surrounded by an envelope containing two membrane layers. Electron-dense intercellular bridges are seen. Markers indicate 250 nm.*



FIGS. 1–4.

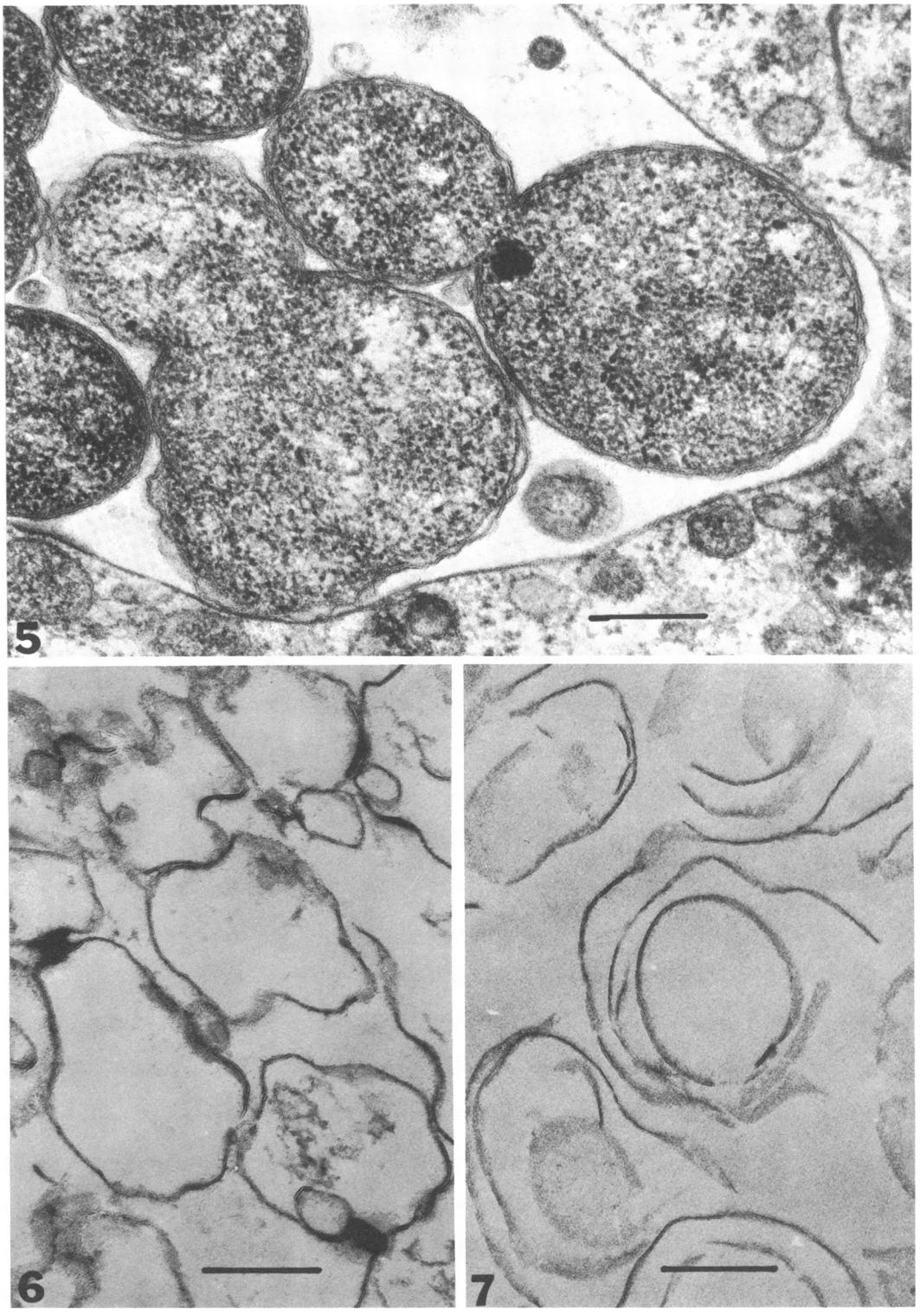


FIG. 5. *Electron micrograph of thin section of meningopneumonitis reticulate bodies in an infected L cell 24 hr after infection. Both the outer cell wall and the cytoplasmic membrane can be clearly seen in various areas of each organism. Markers indicate 250 nm.*

FIG. 6. *Electron micrograph of thin section of partially purified meningopneumonitis reticulate body envelopes before sodium dodecyl sulfate treatment. Electron-dense intercellular bridges and internal membranous structures are seen in several envelopes. Marker indicates 250 nm.*

FIG. 7. *Electron micrograph of thin section of purified meningopneumonitis reticulate body envelopes after sodium dodecyl sulfate extraction. No internal membranous structures are seen, but the outer cell walls remain intact. Marker indicates 250 nm.*

column, washed by repeated centrifugation, and resuspended in PBS.

In thin sections of purified RB, the RB are often irregular in shape, or are round or oval, and are composed of rather homogeneous, amorphous, or reticulate material of moderate density (Fig. 4). Electron-dense connections between two particles are often seen, which may be an organelle acting as a division centrum described previously by one of the authors (1). The purified RB shown in Fig. 4 are surrounded by an envelope, but the inner or cytoplasmic membrane is not clearly evident. However, when thin sections were made of infected L cells at 20 to 26 hr after infection, it was clearly demonstrated that the RB in the vesicles were surrounded by two structures as seen in EB (Fig. 5).

Purified RB envelopes were prepared by resuspending RB in 0.1 M Tris buffer (*p*H 8.5) followed by incubation with trypsin (200 μg/ml) at 37 C for 30 min and then at 2 to 4 C overnight. After centrifugation at 800 × *g* for 10 min, the resulting supernatant fluid was centrifuged at 8,000 × *g* for 30 min, and the thin sections were prepared from the pellet. Figure 6 shows an electron micrograph of a thin section of this material. RB envelopes form uneven outlines. Electron-dense bridges which were observed in Fig. 4 remain after trypsin treatment. Membranous structures can be observed on the inside of some envelopes, but they appear to be partially broken or shrunken. In spite of many efforts, RB envelopes showing two clearly defined layers such as were seen with EB envelopes were not found.

Pellets of envelopes of RB cells after trypsin treatment were suspended in 0.1 M Tris buffer (*p*H 7.4) containing 0.01 M $MgCl_2$, and 0.1 volume of ribonuclease and deoxyribonuclease solution (100 μg/ml each, final concentration) was added to the suspension. After incubation at 37 C for 2 hr, the mixture was again centrifuged at 8,000 × *g* for 30 min, and the pellet was suspended in distilled water. This suspension was mixed with an equal volume of 0.5% SDS and incubated at 37 C for 2 hr. The suspension was then centrifuged at 800 × *g* for 10 min, and the supernatant fluid was further centrifuged at 8,000 × *g* for 30 min. Figure 7 shows an electron micrograph of a thin section of the pellet obtained. All inner membranes and the electron-dense bridges observed in Fig. 6 are completely eliminated by this treatment. The outer layers of the envelopes were not soluble in detergent but appear to become stretched out and to form layers, indicating a lack of rigidity.

DISCUSSION

The existence of cell walls similar in many respects to those of bacteria was suggested by the inhibition of *Chlamydia* by penicillin (5, 12, 15) and was demonstrated directly by the isolation and partial chemical analysis of the envelopes of these organisms (2, 4, 8). This similarity to bacteria suggested that the envelope should consist of a cell wall and cytoplasmic membrane, but such structures have not been previously demonstrated.

We have already reported that envelopes isolated from both EB and RB of MP differed significantly in such characteristics as rigidity, resistance to sonic treatment, lipid content, and amino acid content (4, 11). From these data, we speculated that two possibilities exist: (i) the extracellular EB are surrounded by a cell wall and cytoplasmic membrane, but the developmental RB have only a cytoplasmic membrane and multiply like mycoplasma; or (ii) both EB and RB have both cell wall and cytoplasmic membrane, but the cell wall of the RB is significantly altered as it begins to undergo growth and binary fission.

The results reported in this paper clearly indicate that the second of these alternatives is true. The EB envelopes were found to be composed of a cell wall, not soluble in 0.5% SDS, and a detergent-soluble cytoplasmic membrane. The RB envelope also appeared to be composed of two distinct morphological components, although both were fragile. The outer layer was insoluble in detergent, resembled the cell wall of EB in morphology, and is obviously a continuation of the cell wall found in EB. A probable explanation of the change from a rigid nonpermeable cell wall of EB to a fragile, flexible, permeable wall in the dividing form of the organism is provided by the report of Matsumoto and Manire (Bacteriol. Proc., p. 109, 1969) on the isolation of a formamide-insoluble subunit from the EB cell wall, with these structure being absent in the RB envelopes. A full report on this study will be presented in another paper.

These studies give further evidence for the unique nature of the *Chlamydia*. The EB is essentially an extracellular form of the organism with a cell wall which is resistant to such an environment. After infection and before division, there occurs conversion to an intracellular form which in permeability is especially adapted for parasitism within the protection of the host cell.

ACKNOWLEDGMENTS

We are grateful to Eiichi Fujiwara for technical cooperation on the electron microscopy and also to Mitsue M. Ikemura for exceptionally skilled technical assistance.

This investigation was supported by Public Health Service grant AI-00868 from the National Institute of Allergy and Infectious Diseases and by National Science Foundation grant GB-7587.

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