Brief Notes

Cell Wall and Cytoplasmic Membrane of *Escherichia coli*.*

By Edouard Kellenberger and Antoinette Ryter. (*From the Laboratoire de Biophysique, University of Geneva, Geneva, Switzerland.*)‡

The existence of two envelopes in bacteria has been postulated to account for numerous observations related to the presence of an osmotic barrier with differential permeability (1, 2), the occurrence of free, metabolizing protoplasts (3), and the possibility of producing plasmolysis (1, 4). All of these observations are easily explained by assuming both a cytoplasmic membrane as the selective osmotic barrier, and a rigid cell wall conferring mechanical strength and defined form to the bacterial cell. In ultrathin sections of bacteria, systems of layered envelopes have actually been observed (5–10). These have been interpreted either as a double-structured cell wall, or as wall and cytoplasmic membrane. It has not been possible to clarify further these differences in interpretation either by ultrathin sectioning of free protoplasts (11) or by the study of isolated cytoplasmic membranes, which, in contrast to the cell walls, are very difficult to isolate and purify (12). Since the existence of a visible cytoplasmic membrane was not evident, it could be postulated that the functionally defined cytoplasmic membrane was constituted of a so called pseudomembrane. Such a membrane might be formed at the interface of cytoplasm with surrounding medium, by the polarized arrangement of a common constituent of the cytoplasm, in a way comparable to that of detergents at the liquid gas interface.

During investigations on the fixation of the bacterial nucleus and on the intracellular development of bacterial viruses (13, 14), we had the opportunity to make some morphological observations which may contribute to the understanding of integument organization in the gram-negative bacterium *Escherichia coli*.

Material and Methods

Several strains of *E. coli* have been used: B for investigations of development of phages of the T-series; K12S, a non-lysogenic variant of K12; C, obtained as No. 122 of the National Collection of Type Cultures, London.

The methods for fixation and embedding of bacteria are described at length in another publication (13). Here only the main points are given: fixation in a 1 per cent solution of OsO_4 in veronal buffer, pH 6, with calcium added; embedding in soft agar (so that blocks may be cut) with subsequent washing in veronal buffer; further treatment with $La(NO_3)_3$ or uranyl acetate in some cases. After dehydration in acetone, the agar blocks were embedded in a polyester, vinox K3 (15). Sections were cut with a microtome of our own design (16). Observations were made with an RCA-EMU-2D electron microscope fitted with objective aperture.

RESULTS

In ultrathin sections of exponentially growing cells of *E. coli*, a double-structured outer envelope is frequently observed. The space between the two dense constituents is found invariably to be between 20 and 30 A in width. This spacing remains unchanged, even if by artifact this integument is detached from the cytoplasmic body. Similar observations can be made in the micrographs published by Birch-Andersen *et al.* (5). The constant spacing indicates a unity of the double outer envelope. The detachment of this envelope from the cytoplasmic body suggests that it represents the cell wall (17, Fig. 1), and stimulates one to look closely at the surface of the cytoplasmic body for evidence of a membrane or inner envelope. For normal cells of *E. coli*, however, this inner envelope had not been detected with certainty. Fortunately, in a culture of *E. coli* C, we found emptied cells where the outer double envelope is visible together with an inner single structure (Fig. 1). Confirmation of this occasional observation was found in sections of *E. coli* B infected with phage T2 (Figs. 2 to 4): in a lysis-inhibited system the cells become crowded with viruses; the cytoplasm becomes very rarefied, and is partially replaced by a plasma of filamentous phage DNA. In such cells, the outer

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Schematic representation of integument organization.

double envelope is clearly visible; it is frequently detached from the cell body and wrinkled, probably an artifact resulting from shrinkage of the protoplast. Under these conditions, we commonly observe also an inner envelope. This structure is continuous and limits plasmas formed of DNA as well as of cytoplasm. It is always single and of an approximate width of 60 to 80 A. The dimensions of the different envelopes observed are summarized in Text-fig. 1.

DISCUSSION

The observations reported here may justify the interpretation of the multiple-layered outer envelope of *E. coli* as cell wall, and the inner single structure as cytoplasmic membrane. In the case of artifact, these two integuments can separate from each other, but the spacing of the multiple-layered wall remains constant. Our micrographs show that the cytoplasmic membrane persists in cells emptied of cytoplasm, and that it is able to limit other plasmas than the normal cytoplasm. Thus the cytoplasmic membrane seems to be permanently organized, and therefore the hypothesis of a pseudomembrane becomes untenable.

Comparison with gram-positive cells must be made with caution. We have to admit that the gram-positive *Spirillum serpens* (8) may well have a constitution which is different from that of *Bacillus* (7, 9). The observations of Houwink (18) and Salton (12) on free cellular envelopes demonstrate that the integuments of *Spirillum* species show a layer of regular geometric pattern not seen in *Bacillus* species. Sections of *Spirillum* species (8, 9) show that the outer envelope of the integument system separates frequently from the inner, which adheres to the protoplast; both envelopes seem to be single-structured. In the micrographs of *Bacillus cereus* (9), a thick, single-structured outer integument separates in a similar way from a very thin membrane. Observations on *B. megaterium* (7), however, give clear evidence for a multiple-layered outer integument separating as a unit from the protoplast; a very thin membrane limiting this protoplast is barely visible. We ourselves, not having obtained better information with *Bacillus* species, believe that the observations of gram-positive cell walls must be accomplished with still higher resolution, on normal as well as "pathologic" cells, in order to judge the extent of the differences between integuments of gram-positive cells and those of *E. coli* described here.

Chemically, the walls of gram-positive bacteria are known to consist of polysaccharide, some 9 to 10 amino acids and 1 to 4 per cent lipide (12, 19); whereas the walls of gram-negative cells consist of layers of both lipopolysaccharide, associated with 9 to 12 amino acids, and lipoprotein (20), the total lipide content being in the order of 20 per cent (12, 19). The layered structure of the *E. coli* wall may easily be explained on this chemical basis: in polyester-embedding, concentrated polysaccharides such as cellulose and starch are less electron-scattering than the embedding material (21); proteins, at least when impregnated with OsO_4, look darker. Hence the structure of the wall could be the image of three layers, constituted of polysaccharide which is coated inside and outside either by proteins, lipidic groups, or both. This hypothesis has to be tested on free cell walls. It has been shown that the lipoprotein can easily be removed either by phenol treatment (20), or by extraction with duponol (22). After such treatment, lipopolysaccharide remains, in association with some 9 amino acids. The latter can be removed by the action of T2-phage enzyme (20). Such selective agents are now being used in conjunction with electron microscopy to further analyze the envelope of bacterial cells.

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Note added in proof: Now we use for embedding the more constant polyester vestopal W, purchased from M. Jaeger, Geneva, Switzerland.

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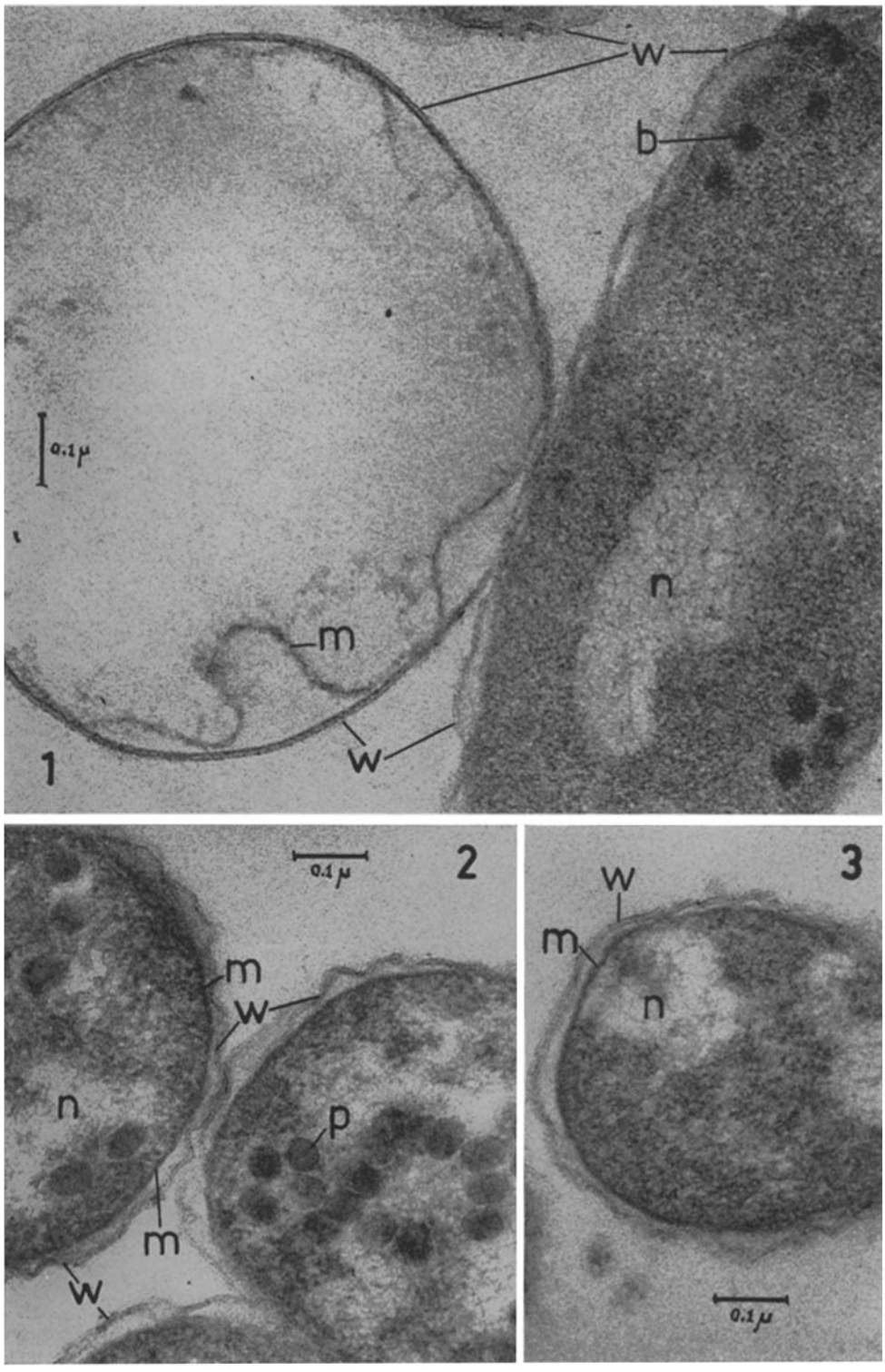
Explanation of Plates

Plate 184

Fig. 1. Normal and emptied cells of *E. coli* C. In the emptied cell the double-structured cell wall (*w*) can be seen. The cytoplasmic membrane (*m*) seems to be single. Some cytoplasm still adheres to the membrane. In the normal cell, the wall is somewhat detached from the protoplast. Its double structure is clearly visible at some places. The protoplast contains the granular cytoplasm, the fibrillar nucleoplasm of the nucleoid (*n*), and inclusion bodies of yet unknown nature (*b*). The cytoplasmic membrane is not visible. × 100,000.

Fig. 2 (detail of Fig. 4). *E. coli* B infected with T2 phage; 30 minutes after infection. Double-structured walls (*w*) and probably single-structured membrane (*m*) are easily distinguishable. Fibrillar plasma constituted by the DNA of vegetative phage (*n*) alternates with diluted granular cytoplasm. Intracellular phage (*p*) sectioned at different angles can be seen. Since DNA contained in phage particles is very concentrated (less than 20 per cent water), it is much more electron-scattering than its diluted form in vegetative phage plasma. The protein membrane of some of the phages can be seen. × 100,000.

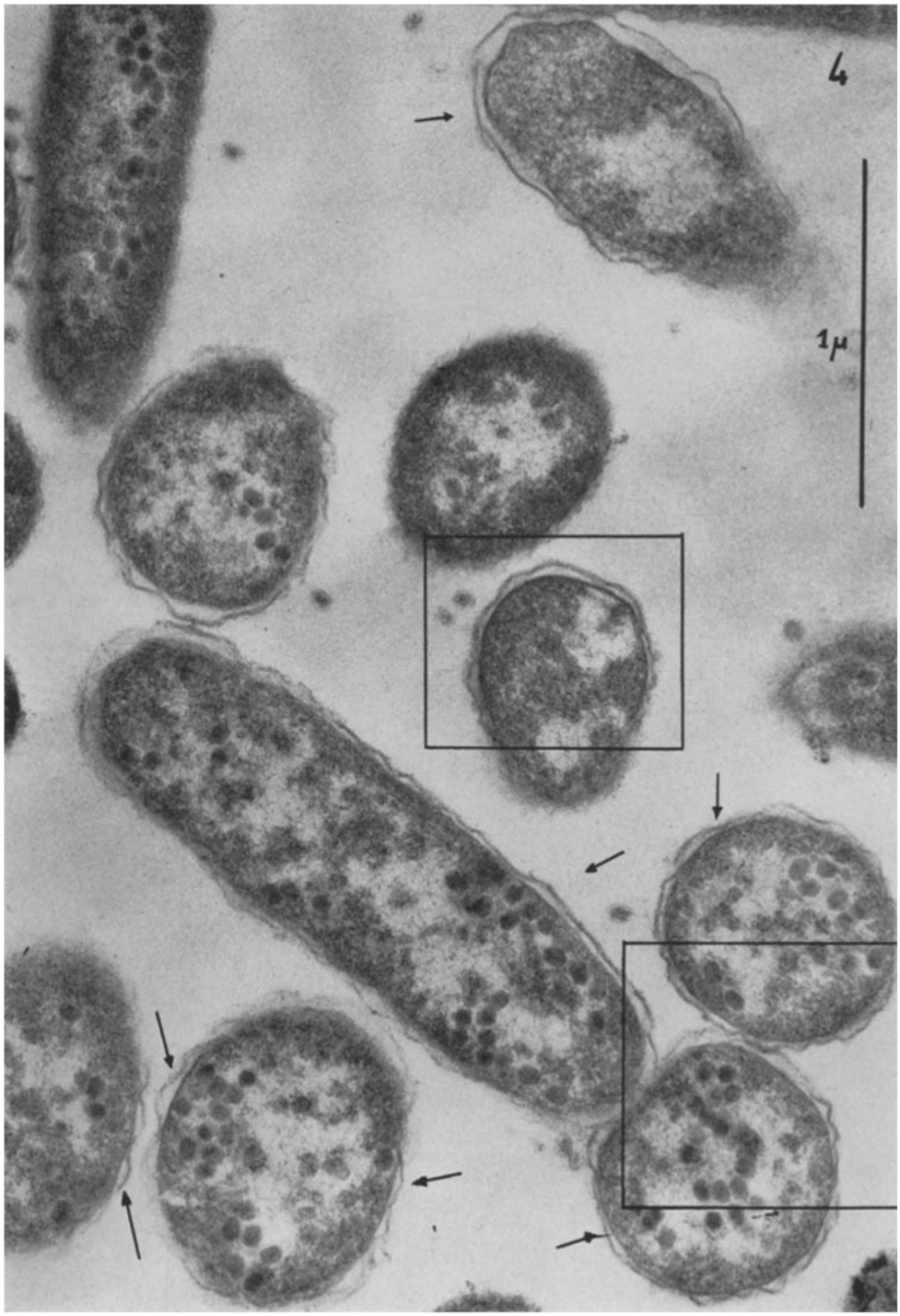
Fig. 3 (detail of Fig. 4). *E. coli* B infected with T2 phage; 30 minutes after infection. No intracellular phage can be seen on this section, but the fibrillar plasma constituted of vegetative phage DNA (*n*) is again visible. Here the membrane is seen to limit another plasma than cytoplasm. × 100,000.



(Kellenberger and Ryter: Cell wall and cytoplasmic membrane)

PLATE 185

FIG. 4. *E. coli* B infected with T2 phage; 30 minutes after infection. Over-all picture, showing the frequency of cases in which bacterial membrane and wall can be clearly distinguished. The surrounded regions are reproduced at a higher magnification in Figs. 2 and 3. Other regions showing clearly the cytoplasmic membrane are indicated by arrows. Note also the polyhedral appearance of the phage and the fibrillar structure of the plasma of vegetative phage, which is distributed all over the cells with islets of granular cytoplasm.



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