

ELECTRON MICROSCOPY OF ULTRATHIN SECTIONS OF BACTERIA

III. CELL WALL, CYTOPLASMIC MEMBRANE, AND NUCLEAR MATERIAL

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Ten years have passed since Baker and Pease (1949) published the first electron micrograph of an ultrathin section of a bacterial cell. During this time, more than thirty papers have appeared which have been devoted to the elucidation of the fine structure of the bacterial cell. Due to technical difficulties, to individual differences among species, and to the fact that some authors have limited their discussions to specific aspects of bacterial cell structure, e. g., the nucleus or the spore, relatively little information has been provided concerning some features of bacterial anatomy. Particularly deficient is the information pertaining to the cytoplasmic membrane. Indeed, only four or five authors have presented electron micrographs in which there was even a hint of the existence of such a membrane.

Although cell walls have been more regularly seen, they, too, have been neglected. This is surprising for the bacterial cell wall is one of the most rigid and resistant structures possessed by the bacterial cell. The cell wall is also one of the cell's most conspicuous components. It was perhaps prophetic that the 1949 paper revealed no cell wall and it is disturbing that the recent paper by Glauert and Glauert (1958), although describing the advantages of Araldite as an embedding medium, reveals only indistinctly the cell walls of bovine tubercle bacilli.

The nuclear apparatus, although it has been quite thoroughly and most competently investigated by Kellenberger and Ryter (1955) and by Kellenberger *et al.* (1958) and has been given at least some attention by other workers, remains rather enigmatic.

It is the purpose of this report to present some recent observations of the structure of the bacterial cell wall, cytoplasmic membrane, and nuclear apparatus.

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MATERIALS AND METHODS

In investigations during the last eight years of the fine structure of bacteria, the author has employed a wide variety of fixatives, dehydrating agents, and fixing and embedding schedules. The fixative which has proved most effective in preserving bacterial structure consists of 2.5 ml of a buffer (9.714 g sodium acetate·3H₂O and 14.714 g sodium Veronal, made up to 500 ml of solution), 1 ml of 8.5 per cent NaCl, 3 drops of 0.11 M CaCl₂, 2.75 ml of distilled water, and 6.25 ml of 2 per cent OsO₄. The pH of this fluid was about 9.4. Cells fixed in this fluid at room temperature for 4 hr differed only slightly in appearance from those fixed in the same fluid at room temperature for 4 hr followed by 9 hr at 10 C. (The difference is a more pronounced granularity in the latter case. However this difference might be attributable to the fact that the cells which were fixed longer were dehydrated for a shorter time.) In each case, the bacteria were centrifuged from a broth culture and resuspended in 2 to 5 ml of the fixative. Following fixation, the cells were washed in a fluid consisting of the fixing solution minus the OsO₄, dehydrated by passage through a graded ethyl alcohol series, and were finally embedded in no. 1 gelatin capsules in a mixture of 3 parts normal butyl methacrylate to 2 parts ethyl methacrylate polymerized at 70 C in the presence of 1.5 per cent luperco CDB. Ultrathin sections were cut with a Servall Porter-Blum ultramicrotome or with the experimental, thermal expansion advance ultramicrotome described by Hillier and Chapman (1954). Glass knives, prepared in the laboratory essentially as described by Latta and Hartmann (1950), were used for the sectioning. The sections were floated off the knife edge onto the surface of the 40 per cent acetone in a collecting trough mounted behind the knife edge. The sections were removed from this surface on 200 mesh copper screens on which a thin collodion film had been mounted and were examined in an

RCA EMU-2D electron microscope which had been fitted with a 0.015 in externally centerable (Canalco) condenser aperture and a 50 μ aperture in the standard objective pole piece.

RESULTS AND DISCUSSION

Cell wall. Some of the most striking and provocative electron micrographs of bacterial cell walls have resulted from shadowed preparations, as exemplified in the work of Houwink (1953). Houwink's demonstration, by the use of shadow-casting techniques, of spherical macromolecules, of from 120 to 140 A in diameter, in the cell wall of a *Spirillum* species is a most impressive achievement. Houwink's figures become rather disturbing, however, when it is realized that 10 years of ultrathin sectioning studies have revealed no such spherical macromolecules in the cell wall. Indeed, only rarely, as for example in figures 10 and 11 of the paper by Chapman and Zworykin (1957), does one observe in ultrathin sections any structure in bacterial cell walls. Cell walls usually appear quite homogeneous. The explanation for this dilemma may lie in the observation by Knaysi and Hillier (1949) that the beaded threads, held together by a cementing material, which constitute the spore coat, are invisible in the intact coat, but that they become visible and are finally liberated by the dissolution of the cementing material. In the light of this observation, it is perhaps more understandable that one rarely observes fine structure in the intact bacterial cell wall. (It seems reasonable to apply this observation of the bacterial spore coat to the bacterial wall for it has been shown by Chapman and Zworykin (1957) that the vegetative cell wall pre-exists as the innermost coat of the mature spore.)

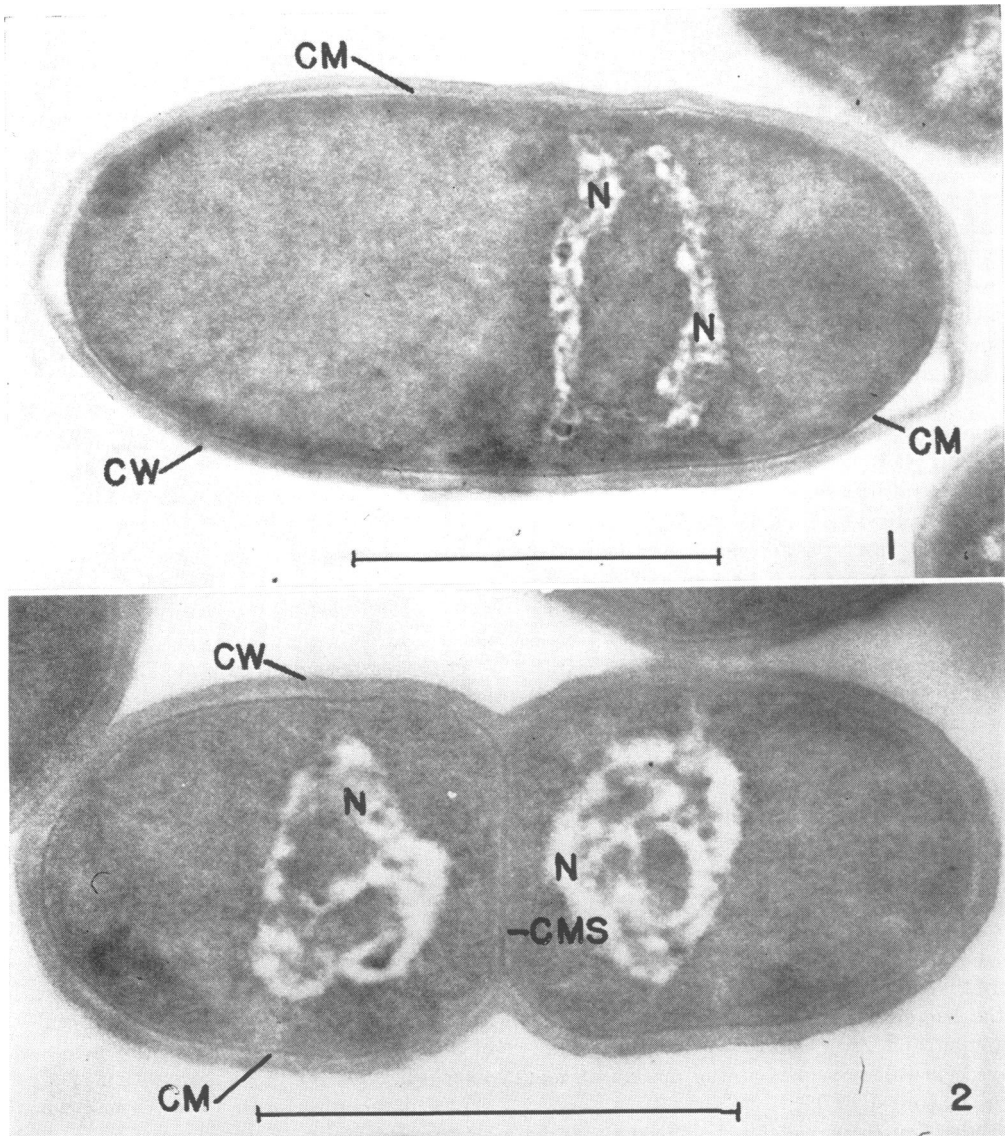
In the figures included with this paper, the cell wall assumes one of two configurations: it is either of uniform density (figures 2 to 6, 8, 9, and 11 to 14) or it appears as two dense lines separated by a less dense line (figure 10). (Figures 1 and 7 reveal only a slight density increase at the surfaces of the wall.) This latter appearance has been described by Piekarski and Giesbrecht (1956) for *Bacillus megaterium* and by Kellenberger and Ryter (1958) for *Escherichia coli*. Figure 10 is a section through cells from an old culture of the gram-positive *Bacillus subtilis*. (The report by Murray (1957a), that the high concentration of lipids and absence of certain amino acids from the walls of gram-negative as

compared to gram-positive bacteria are reflected in a dense-light-dense layering in the former, whereas the latter have a dense and almost homogeneous wall, is not supported by the above observations.) The fact that the spacing of the two dense layers of the cell wall remains the same in these old cells which have lost their protoplasts further supports the belief of Kellenberger and Ryter (1958) that the multiple-layered structure represents a cell wall. Each of the two dense layers in the *B. subtilis* wall measures about 40 A thick. The low density space between the two dense layers measures from 60 to 80 A wide. These measurements are about twice those found by Kellenberger and Ryter (1958) for the corresponding regions in *E. coli* and about one-half those found by Piekarski and Giesbrecht (1956) in *B. megaterium*.

Kellenberger and Ryter (1958) have suggested a plausible explanation for the layered wall appearance based on the possibility that the low density wall region is a polysaccharide (low electron scattering ability) and the high density layers are proteins or lipoproteins (high electron scattering ability). They point out that the hypothesis has to be tested on free cell walls. It seems equally possible that the layered appearance could reflect merely an accumulation of dense material (reduced osmium?) at the two surfaces of the wall. Or, the dense layers could represent a greater concentration of the molecules of wall material or a different and more compact arrangement of those molecules.

A comparison of figures 7 and 9 indicates to some extent the degree to which bacterial cells of different species can vary in the thickness of their cell walls. Figure 7 is a section of an unidentified bacterium with a 400 A thick cell wall. This wall exhibits only a very slight density increase at its two surfaces. Figure 9 is a section through *Bacillus acidilactici* which has a homogeneous cell wall 100 A thick. (The author has observed other bacteria with cell walls up to 600 A thick.)

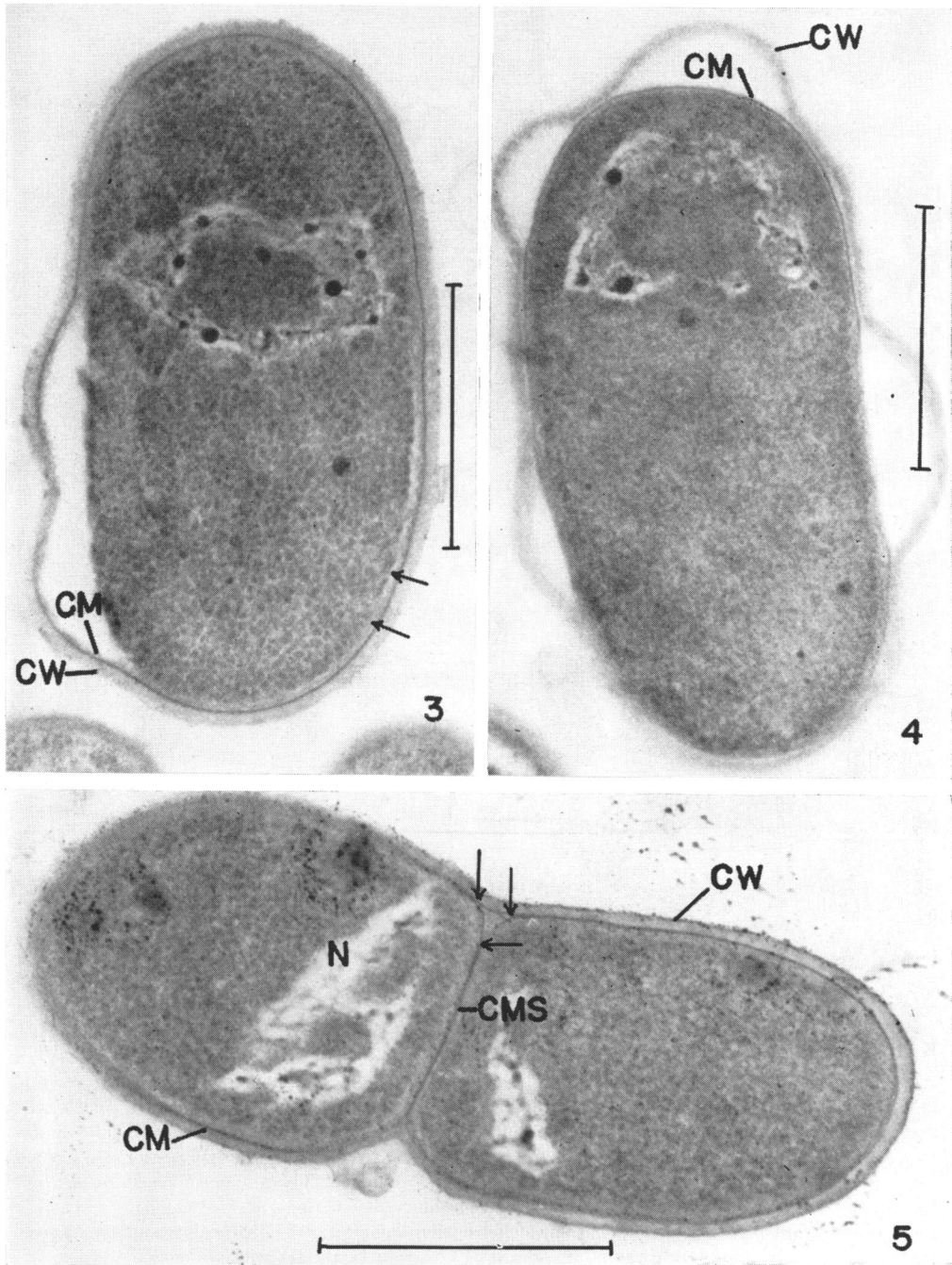
Cytoplasmic membrane. In spite of the fact that indirect evidence, such as is provided by plasmolysis, differential permeability, and metabolizing protoplasts, has indicated the probable existence of a bacterial cytoplasmic membrane, ultrathin sections have rarely revealed the presence of a clearly defined membrane structure internal to the cell wall. (Tomlin and May, 1955) reported that 1 cell in about 100 cells observed



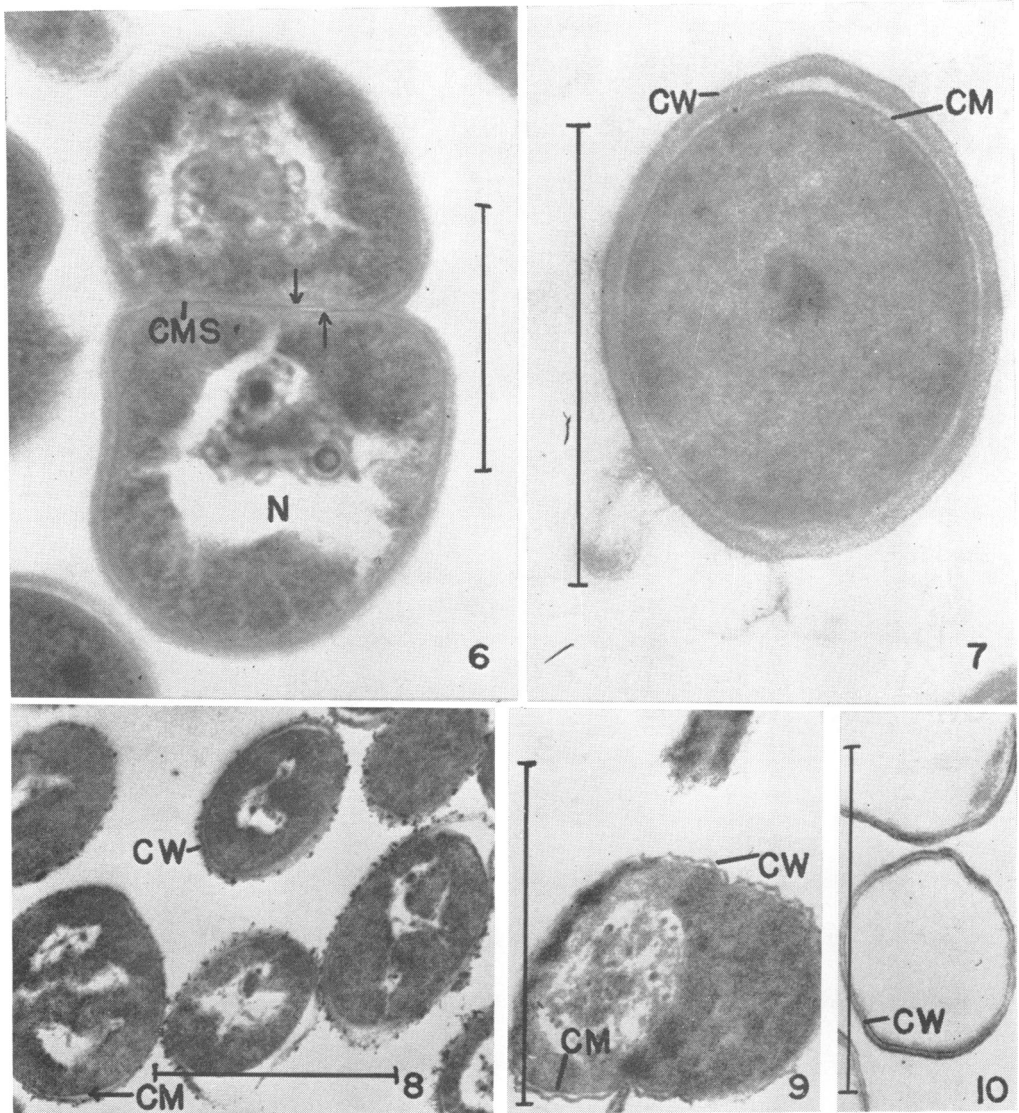
Figures 1 and 2. Electron micrographs of ultrathin sections of an unidentified bacterium. In each figure the magnification mark equals 1μ . Note the fine granularity of the cytoplasm of these cells fixed for 4 hr and kept overnight in 85 per cent alcohol. *CM* = cytoplasmic membrane; *CMS* = cytoplasmic membrane septum; *CW* = cell wall; and *N* = nuclear material.

revealed a plasma membrane.) Yet, although the authors do not mention them, membranes do appear, admittedly rather indistinctly, in the electron micrographs illustrating the papers by Birch-Andersen *et al.* (1953) and by Piekarski and Giesbrecht (1956). (In the former paper, the cell wall has erroneously been called the "cell membrane.") Somewhat more convincing repre-

sentations of a cytoplasmic membrane appear, and are mentioned, in papers by Chapman and Kroll (1957) and Murray (1957*b*). The most impressive demonstration of a cytoplasmic membrane appears in a paper by Kellenberger and Ryter (1958). Here, in cells of *E. coli* which have been infected with bacteriophage or which have been emptied of cytoplasm (by an unex-



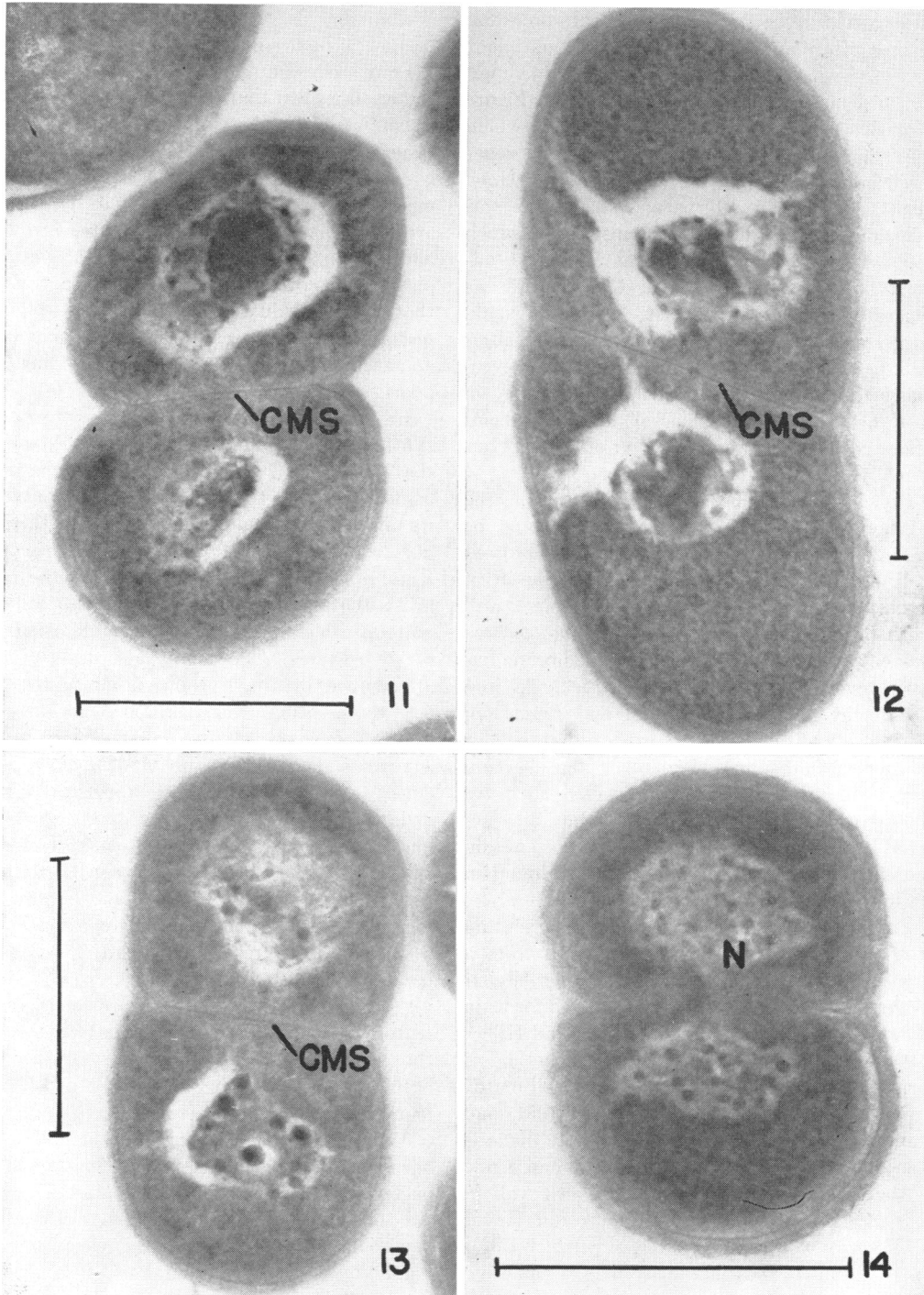
Figures 3 to 5. Electron micrographs of ultrathin sections of an unidentified bacterium. In each figure the magnification mark equals $1\ \mu$. Note the coarse granularity of the cytoplasm of these cells fixed overnight. In figure 3, the cytoplasmic membrane (CM) has lifted away from the cytoplasm with the cell wall (CW). In figure 4, the cytoplasmic membrane (CM) has remained adherent to the cytoplasm although the cell wall (CW) has lifted away from the protoplast. Figure 5 illustrates the delamination (arrows) of the cytoplasmic membrane. Note the complete cytoplasmic membrane septum (CMS).



Figures 6 to 10. Electron micrographs of ultrathin sections of unidentified bacteria (figures 6 and 7) and *Corynebacterium pseudodiphtheriticum* (figure 8), *Bacillus acidilactici* (figure 9), and *Bacillus subtilis* (figure 10). In each figure the magnification mark equals 1μ . In figure 6, the cytoplasmic membrane septum (CMS) reveals a split (arrows). The nuclear material (N) has been poorly preserved. Figure 7 is a transverse section through a cell such as that of figure 1 or figure 2. The nuclear material is not included in the plane of the section. Figures 8 and 9 reveal the homogeneous appearance of the cell wall (CW) generally found in gram-positive bacteria. A slight increase in density at the protoplast surface suggests the presence of a cytoplasmic membrane (CM). Figure 10 illustrates the layered appearance of the cell wall frequently observed in the gram-negative bacteria, but which has been found also in the gram-positive *Bacillus megaterium* (see text) and *B. subtilis* (here).

plained means), one can clearly see a 60 to 80 Å thick homogeneous cytoplasmic membrane. The authors believe that the persistence of the single structure inside the cell wall of cells emptied of

cytoplasm and the separation of the two integuments from each other in cases of artifact justify the interpretation that the inner single structure is a cytoplasmic membrane, and that the layered



Figures 11 to 14. Electron micrographs of ultrathin sections of unidentified bacteria. In each figure the magnification mark equals 1μ . These figures illustrate the appearance of cytoplasmic membrane septa (CMS) (figures 11 to 13) and a stage in the formation of a septum (figure 14). Figure 14 also reveals particularly well the appearance of a constricted "amitotic" nucleus (N). Note the appearance of the granules in the nuclear zone.

outer structure is a cell wall. This interpretation seems quite reasonable. Yet, the question might be raised whether the "membrane" could represent a delaminated layer of the cell wall. Figure 3 of this paper illustrates an instance in which the 80 Å thick cytoplasmic membrane has separated from the cytoplasm with the cell wall, which might suggest an affirmative answer to this question. Figure 4 illustrates an instance in which the cytoplasmic membrane has remained adherent to the cytoplasm while the cell wall has separated from the protoplast. This figure would suggest a negative answer to the question. Figures 2, 5, 6, and 11 to 13, in which a transverse cytoplasmic membrane septum (CMS) appears, seem to support the negative answer, i. e., the "membrane" does not represent a layer of the wall but is really an autonomous structure.

Conclusive evidence of the nature of the relationship between these structures could be provided by concomitant chemical analysis of cell wall and cytoplasmic membrane. The report of the difference in chemical nature of the cell wall from the cytoplasmic membrane of *B. megaterium*, a bacillus in which a cytoplasmic membrane has been revealed by the work of Piekarski and Giesbrecht (1956), by Weibull and Bergström (1958), might lead one to expect that similar findings would appear wherever the electron microscope revealed configurations such as reported here. It should be noted that the bacterial cytoplasmic membrane is of essentially the same thickness as cell membranes found in a wide variety of plant and animal cells.

Figure 14 indicates that the membrane septum (CMS) forms by a centripetal growth from a ring of membrane material, in a manner similar to that in which the transverse cell wall forms in *B. cereus*, as was shown by Chapman and Hillier (1953). It should be noted that this method of formation of a membrane septum or "cell plate" was described by Knaysi (1941, 1949, 1951) and Robinow (1945). It is interesting to note that the "peripheral bodies" which apparently had a role in the formation of the transverse cell wall in *B. cereus* as reported by Chapman and Hillier (1953), do not appear in these cells which differ further from *B. cereus* in possessing a cytoplasmic membrane. (It will be recalled that Chapman and Hillier observed no cytoplasmic membrane in *B. cereus*.) One wonders if perhaps the biochemist might find that the cytochromes reported in the protoplast membrane of *B. megaterium* by

Weibull and Bergström (1958) are present in the peripheral bodies of *B. cereus*.

From an examination of figures 5 and 6, at arrows, it is clear that the cytoplasmic membrane is laminar. It is reasonable to assume that the transverse cell wall results from an extension, by the synthetic activity of the cytoplasmic membrane, of the area delimited by the two arrows in figure 6 and by the three arrows in figure 5. In short, it would seem that the cytoplasmic membrane forms a laminar septum and that the individual layers synthesize cell wall material which passes through the membrane and accumulates between the layers, forcing them apart.

In connection with this discussion of the synthesis of cell wall material, it should be noted that in figure 3 (at arrows) there appears in association with the cytoplasmic membrane, at its inner surface, material which is indistinguishable from the material on the outer surface of the membrane; this appearance suggests the possibility that the membrane synthesizes cell wall material which occasionally accumulates on both of its surfaces. One might also interpret the appearance of the cytoplasm in figures 3 to 5 as representing a mass of 250 Å granules dispersed homogeneously in a lower density ground substance. This ground substance is of the same density as the cell wall. Such appearances make it difficult to resist speculating that the cell wall material might be derived at least in part, from synthetic activity in the cytoplasm. If, for some reason, the cytoplasmic membrane became less permeable to this material, an appearance such as is found in figure 3 would result. The cell wall has been shown by many workers to be of complex chemical composition. There is, therefore, reason to maintain an open mind to the idea that cell wall material may be synthesized by both the cytoplasm and the cytoplasmic membrane.

Nuclear material. Although considerable effort has been expended to clarify the nature of the bacterial nucleus, the nucleus remains an enigma. All that can be said with certainty is that the bacterial nucleus is represented in electron micrographs by a zone of material which is lower in density than the cytoplasm in general. Since the preparation of the first paper in this series, which presented the evidence in support of the interpretation that the low density zones represented nuclear material, an additional investigation

has supplied further corroborative evidence. This is the work of Caro *et al.* (1958), who, employing radioautographic methods, have observed that the radioactivity is found in the low density areas when a thymine-requiring strain of *E. coli* is grown in a medium containing tritium-labeled thymidine.

Examination of the included figures reveals the usual low density areas in the cytoplasm. In figure 1, the low density areas are disposed as two bars, reminiscent of the bars in a Robinow preparation. One can, however, also distinguish a threadlike component in the low density area. Such threads appear in many of the other nuclear zones and resemble the nuclear threads in micrographs of Piekarski and Giesbrecht (1956), Giesbrecht (1958), Giesbrecht and Piekarski (1958), and Kellenberger and Ryter (1955). Piekarski and Giesbrecht consider these threads to represent the chromosomes or chromatids of *B. megaterium*.

It should be noted that no nucleus represented in this paper reveals the fibrillar component—the fibrils being much thinner than the threads—which was described by Chapman and Hillier (1953) and which has recently been accepted by Kellenberger *et al.* (1958) as a real structure and used by them in a profound interpretation of the nature of the bacterial nucleus. It seems, however, that the appearance of the threadlike and fibrillar nuclear structures is highly dependent on the preparative treatments. (The report by Whitfield and Murray (1956) of the dependence of the state of the bacterial chromatin, dispersed or aggregated, on the concentration of electrolytes in the cell environment further illustrates the importance of preparative treatments and environmental conditions.) The author at present reserves final judgment on the significance of the threadlike and also the fibrillar structures but is inclined to believe that they are alternate forms of the chromatin material. In figures 2, 5, 6, and 11 to 13, the membrane septum has compartmented the cytoplasm and a nuclear zone is observed in each daughter cell. In figure 14, an isthmus of nuclear material connects the two larger masses of nuclear material in a cell which is incompletely compartmented by the centripetally growing membrane septum. Such a disposition of the nuclear material is strikingly remindful of the classical figures of amitosis, such as those of Nowikoff (1910), and of the electron micrograph of Bradfield (1954) showing nuclear

division in a staphylococcus. It should be noted that Bradfield described his figure as one representing amitosis. Figure 14 is also interesting because the nuclear material includes many dense round or oval profiles. These would seem to correspond to the granules on the threads described by Piekarski and Giesbrecht (1956). (Such granules appear in the nuclear zones in other figures in this paper and in other papers.) At present it is impossible to determine the significance of the granules. They may, however, represent specialized regions on the nuclear zone threads, as Piekarski and Giesbrecht (1956) believe.

The nuclear material may, then, be considered to be represented by a low density zone within which threads, filaments, and granules may appear. The nuclear zone may be invaded by extensions of cytoplasmic material (figures 3 and 4). These extensions, especially when the connection (figure 4) to the general cytoplasm is not included in the section, might be thought to resemble the achromatic cores of the nuclear bodies as described by Spiegelman *et al.* (1958). However, the observations that (a) digestion of the protoplast does not result in the digestion of the cores and (b) an occasional section does show the continuity between the extension and the general cytoplasm indicate that the cores and extensions are very likely different structures. No counterpart of the cores is then seen in these sections. At cellular division, the nuclear material undergoes a constriction reminiscent of classical amitosis and is ultimately separated into two masses.

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SUMMARY

A study of ultrathin sections of osmium fixed and methacrylate embedded bacterial cells has revealed the presence of a cell wall which may vary in thickness from 100 to 600 Å. This wall may be homogeneous or it may appear as two dense layers separated by a less dense layer.

A cytoplasmic membrane, usually appearing about 80 Å thick, is observed in some bacteria. This membrane is capable of delaminating into layers about 40 Å thick. The centripetal growth of the membrane forms a cell septum which partitions the cytoplasm.

The nuclear material appears as a low density zone often containing threads and granules. Prior to cellular division or membrane septation the nuclear zone is seen to constrict and divide by a process reminiscent of classical amitosis.

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