ELECTRON MICROSCOPY OF ULTRA-THIN SECTIONS OF BACTERIA

I. Cellular Division in Bacillus cereus

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In the course of cytological studies of a few selected species of bacteria by the techniques of ultra-thin sectioning and electron microscopy, some new and significant information has been obtained concerning cellular division in *Bacillus cereus*.

The cytology of bacterial cellular division involves a number of structures and events which are beyond the resolution limit of the light microscope. The complexity of these structures and the possible variations which differences in species, age of culture, and growth conditions may introduce have made it impossible to make reliable interpretations by indirect methods. This has led to considerable confusion as is apparent from any review of the literature. It is hoped that some of the observations made in the present investigation will remove a little of that confusion and point the way to much greater contributions of the method in the future.

MATERIALS AND METHODS

A rough variant of *B. cereus*, obtained from Dr. F. H. Johnson, was used in this study. The bacteria were grown in brain-heart infusion medium (Difco) in 75 ml broth tubes aerated by means of a Thiberg aerator. Seven hour cultures were transferred to centrifuge tubes and the organisms sedimented by a moderate centrifugation for three minutes. Most of the supernatant broth was decanted, and the sedimented bacteria were resuspended in the remainder. To the 1 ml (approximately) suspension of bacteria which resulted, 5 ml of a 2 per cent OsO_4 solution with 0.8 per cent NaCl were added. The tube was stoppered and put aside for 16 hours. The fixed bacteria were washed several times with distilled water and dehydrated through a graded ethyl alcohol series. They were passed into a mixture of equal parts of absolute ethyl alcohol and normal-butyl methacrylate monomer; passed through several changes of a mixture of three parts normal-butyl methacrylate monomer, one part ethyl methacrylate monomer, and 2 per cent "luperco CDB". Finally they were dispensed into no. 1 gelatin capsules which were stored in an oven at 47 C for several days prior to being used in order to dry them completely. The preparations were maintained at 47 C until polymerization was complete, which was usually within 24 hours. Before sectioning, the gelatin capsules were soaked off in water. Sections of less than 0.1 μ were cut by means of an experimental microtome which will be described elsewhere. For this particular study glass knives prepared in the laboratory from selected glass were used. For collection the sections were floated off the knife edge on to a surface of 30 per cent acetone in distilled water. They were picked up on a cleaned 200 mesh copper screen on which a thin collodion membrane previously had been mounted and dried. The specimens were examined in an RCA, type EMU, electron microscope which had been fitted with a wide field objective and a 50 μ aperture. The electron micrographs were all taken at an original electronic magnification of 9,000 $\times$ and enlarged to 36,000 $\times$ unless otherwise stated.

RESULTS

In our early work on this problem considerable difficulty was experienced with explosions of the cells during the polymerization. The use of long fixation times and of salt in the osmium tetroxide solution seemed to overcome this difficulty except for the occasional lifting of the cell wall from the fixed cytoplasm. The only other immediately obvious possibility of an artifact is the near emptiness of some of the peripheral bodies which suggests a possible partial dissolution of their original contents by the treatment.

Figure 1 is a section of what was probably a slightly curved group of cells. It is a longitudinal

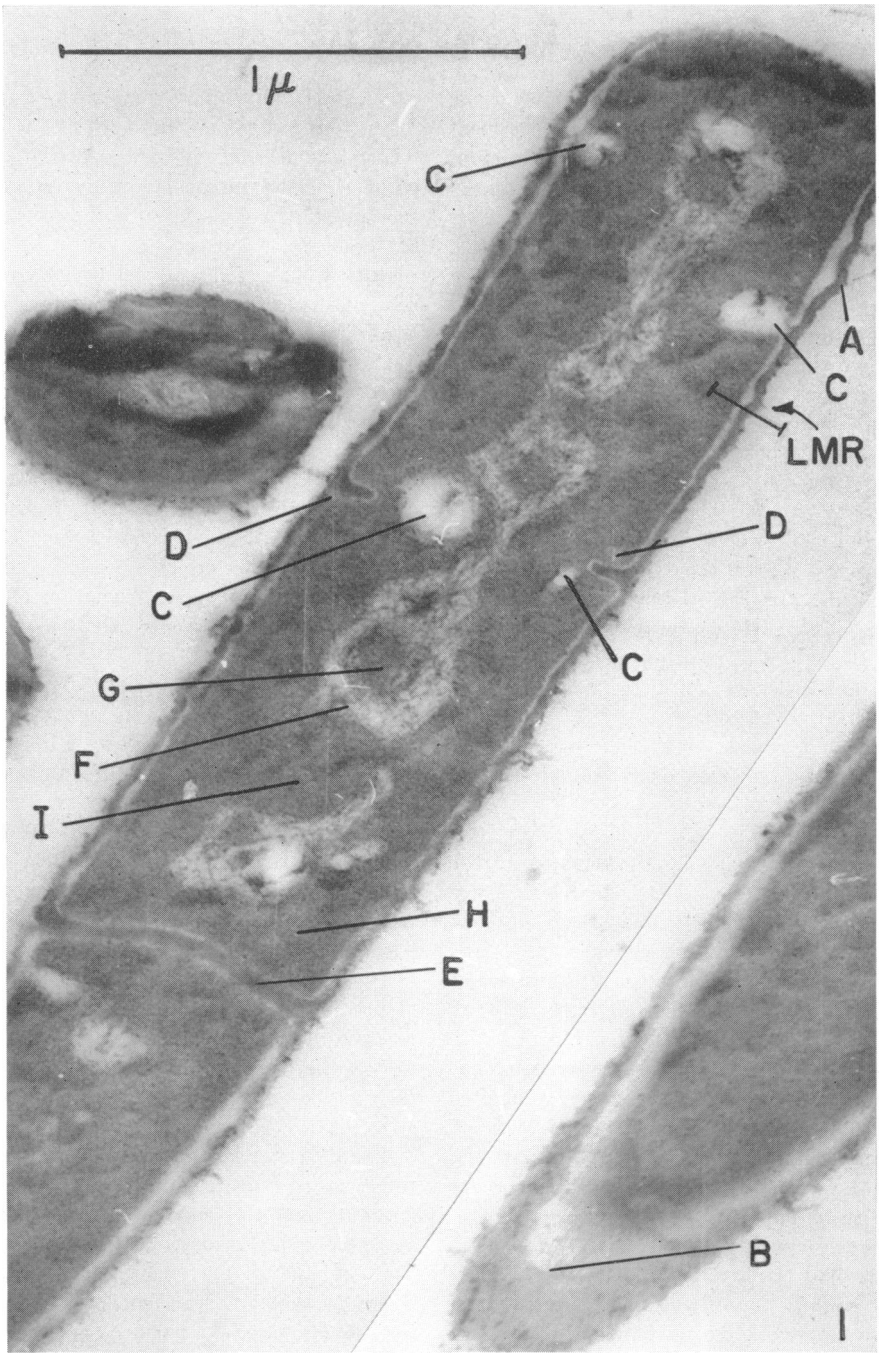


Figure 1. Electron micrograph of an ultra-thin longitudinal section of *Bacillus cereus*. 60,000 ×. The end of the section of the lower cell is shown in the insert.

A—Cell wall showing evidence of the shrinking of the cytoplasm.
B—Very oblique section of the cell wall showing dense particles and the dense inner layer.
C—Indicating four peripheral bodies cut at different levels.
D—Beginning of the centripetally growing transverse cell wall.
E—Completed transverse cell wall before thickening.
F—Low density fibrous component of nuclear apparatus.
G—Dense body in nuclear apparatus which may be inclusion of cytoplasmic material.
H—Small dense particles which appear to be main constituent of cytoplasm.
I—Unidentified cytoplasmic inclusions.
LMR—Scale indicating the *limit* of resolution of a light microscope using visible light.

section which runs close to a median plane of one cell and then passes obliquely through the next cell. The result is a micrograph which summarizes all of the results presented in this paper.

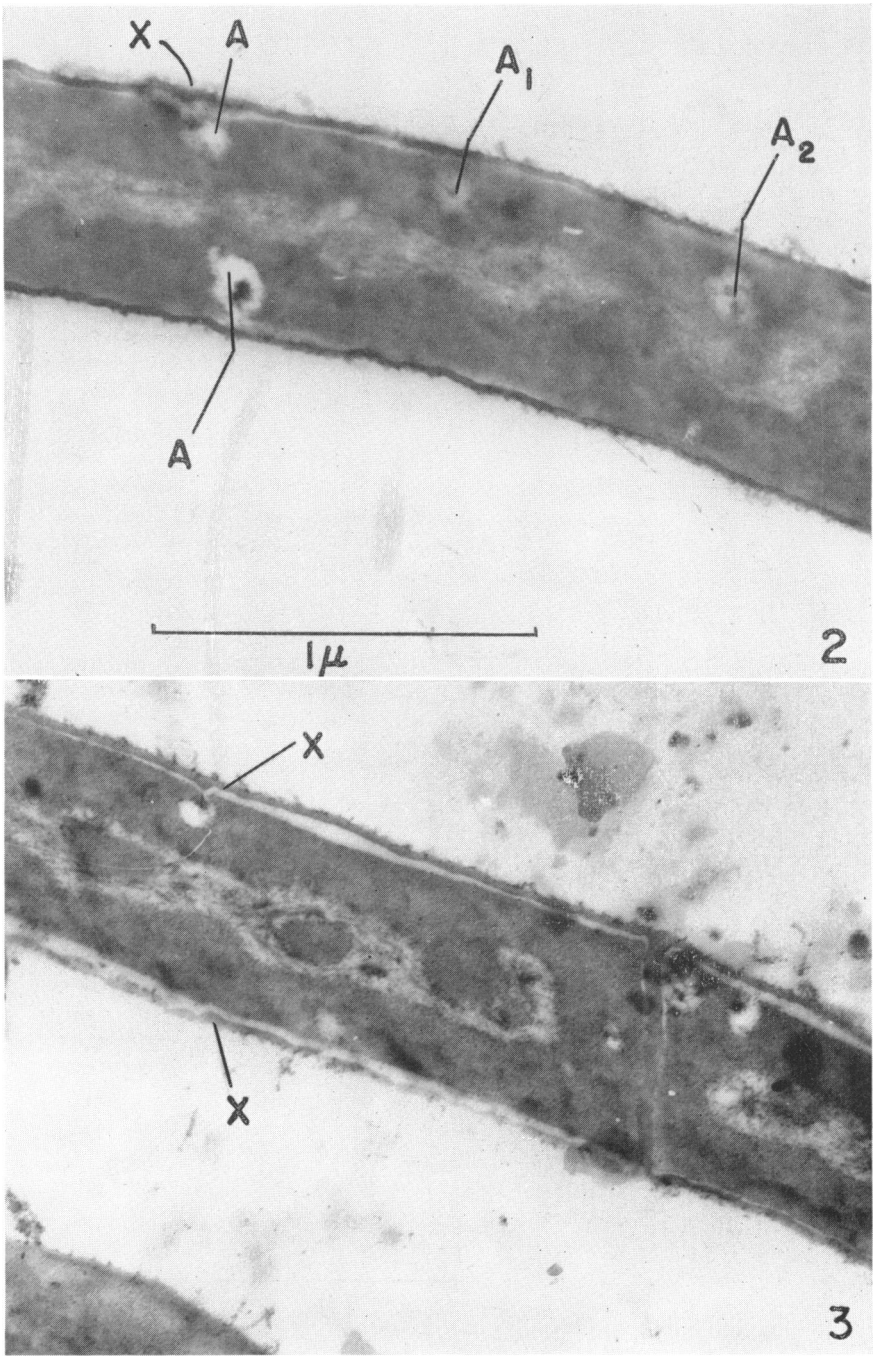
As might have been anticipated from previous electron microscopy of intact bacterial cells, the cytoplasm and some elements in the nuclear sites have densities much greater than is found in ultra-thin sections of mammalian tissue. Light microscopic observation and measurement of the electron micrographs indicate that there has been some shrinkage of the cells during treatment. It is possible that this explains the tendency of the cell wall to be somewhat wrinkled and often to enclose the cell only loosely. The cell wall *A* has a total thickness of approximately 200 A. In cross section the only structures visible in the cell wall are scattered dense particles near the outer surface. However, in the oblique sections of the cell wall it often appears that the inner surface is denser and takes on some semblance of being a discrete layer *B*.

In none of the pictures is there any discrete entity that could be identified as the cytoplasmic membrane.

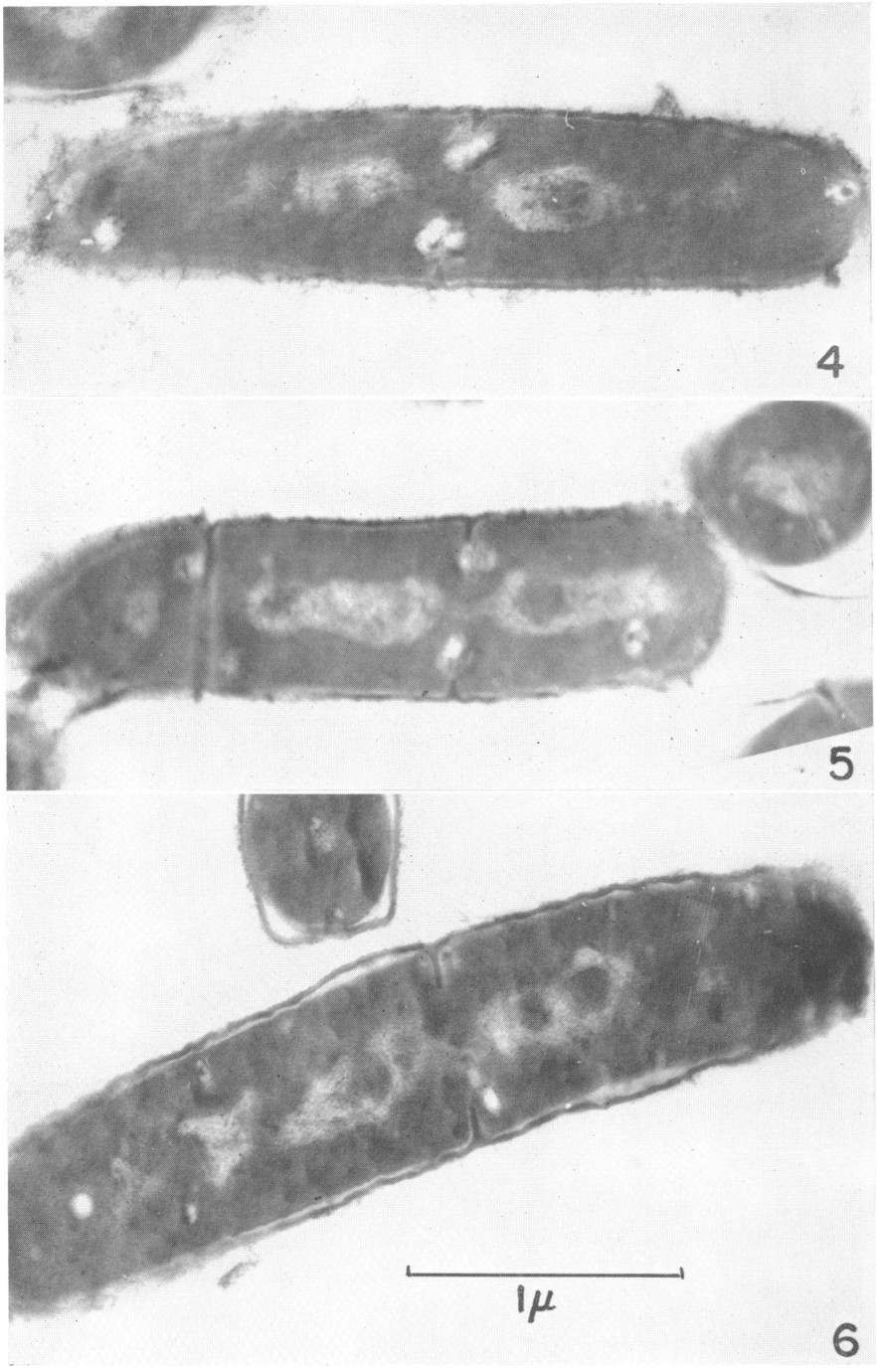
Among the cytoplasmic inclusions are "peripheral bodies" or vacuoles *C* which are of the order of 0.2 μ diameter. These characteristically appear empty except for small, very dense, irregularly shaped granules which seem to be present in each. The beginning of a transverse cell wall is seen at *D*. The transverse cell wall appears to start as an annular disc integral with the lateral cell wall. *E* is a later stage of the transverse cell wall where it has been completed but has not thickened appreciably or divided. The other structures of interest in the bacterial cell are those found at the nuclear sites. The low density observed in previous electron micrographs is also apparent here, *F*. However, as has been suggested (Hillier *et al.*, 1949), the nuclear sites also contain dense bodies, *G*. Unfortunately, in the present work we have no means of distinguishing the material of these bodies from that of the cytoplasm although the ordered location of these bodies in many pictures does suggest that they are of different nature. The less dense material in the nuclear sites has a different texture from that of the cytoplasm, being almost entirely fibrous in character. The cytoplasm is seen to have a fine stippled appearance and contains large numbers of small dense particles, *H*. These are not more than 40 A in diameter. There are also shaded areas in the cytoplasm which, at the moment, are considered to be sectioning artifacts caused by the caseous texture of the fixed cytoplasm. In the high resolution pictures other cytoplasmic inclusions are occasionally observed, *I*. However, nothing is known regarding the significance of these.

Figures 2 to 9 have been arranged to illustrate successive stages in the formation of the transverse cell wall. The criteria for this arrangement are of course the light microscopic observations decribed in the literature. Figure 2 is an elongated cell in which the formation of transverse cell walls apparently has been delayed. It appears possible that the peripheral bodies *A*, A_1, A_2 indicate the positions of formation of the next cross walls. It is to be noted that there is a slight thickening of the cell wall at *X* and that the peripheral bodies *A* straddle a division point of the nuclear apparatus. At A_1 and A_2 are perhaps still earlier stages where the peripheral bodies are not as close to the surface and are not as empty. Figure 3 again shows two very early stages in the formation of the transverse cell wall. In the case marked *X* a very definite ridge is beginning to form on the inner surface of the cell wall. Again there is a peripheral body present. Also note that the dense structure within the body is located adjacent to the cell wall. There seems to be a conclusive correlation between the location of the peripheral bodies and the formation of the transverse cell wall. The dense structure within also appears to tend to be located adjacent to the growing transverse cell wall.

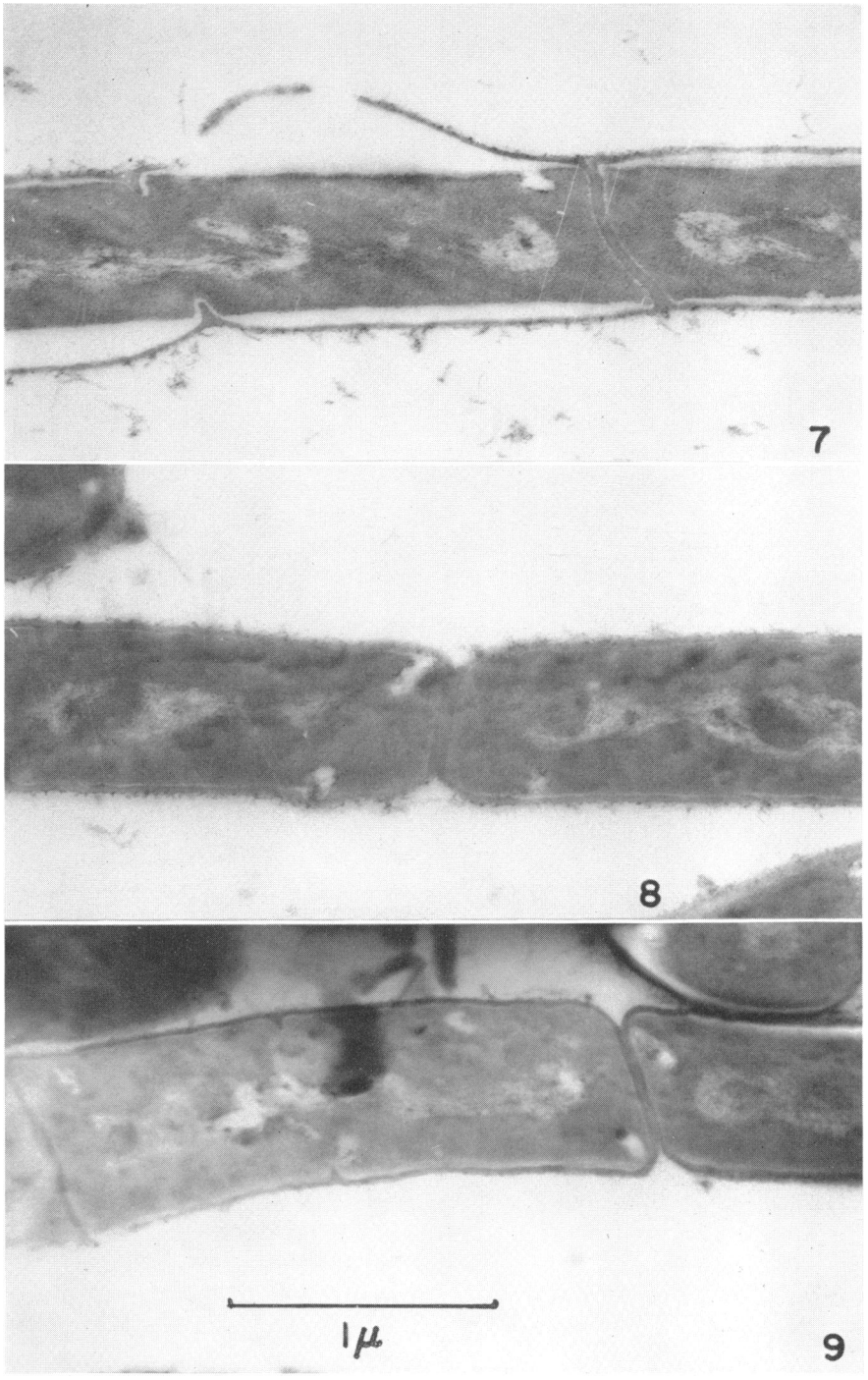
The next step in the sequence of formation of the transverse cell wall has been illustrated in figure 1. Figure 4 shows a still somewhat later stage and gives a particularly clear illustration of the centripetal movement of the peripheral bodies as the cross wall develops. Note also that the nuclear material appears completely separated and drawn into the daughter cells. Figure 5 is only a slightly later stage as far as the formation of the transverse cell wall is concerned. In this case, however, the nuclear material has not quite completed dividing, for the daughter "nuclei" are still connected by a thin, fibrous structure. However, the difference in the appearance of the nuclear material in figures 4 and 5 can be explained



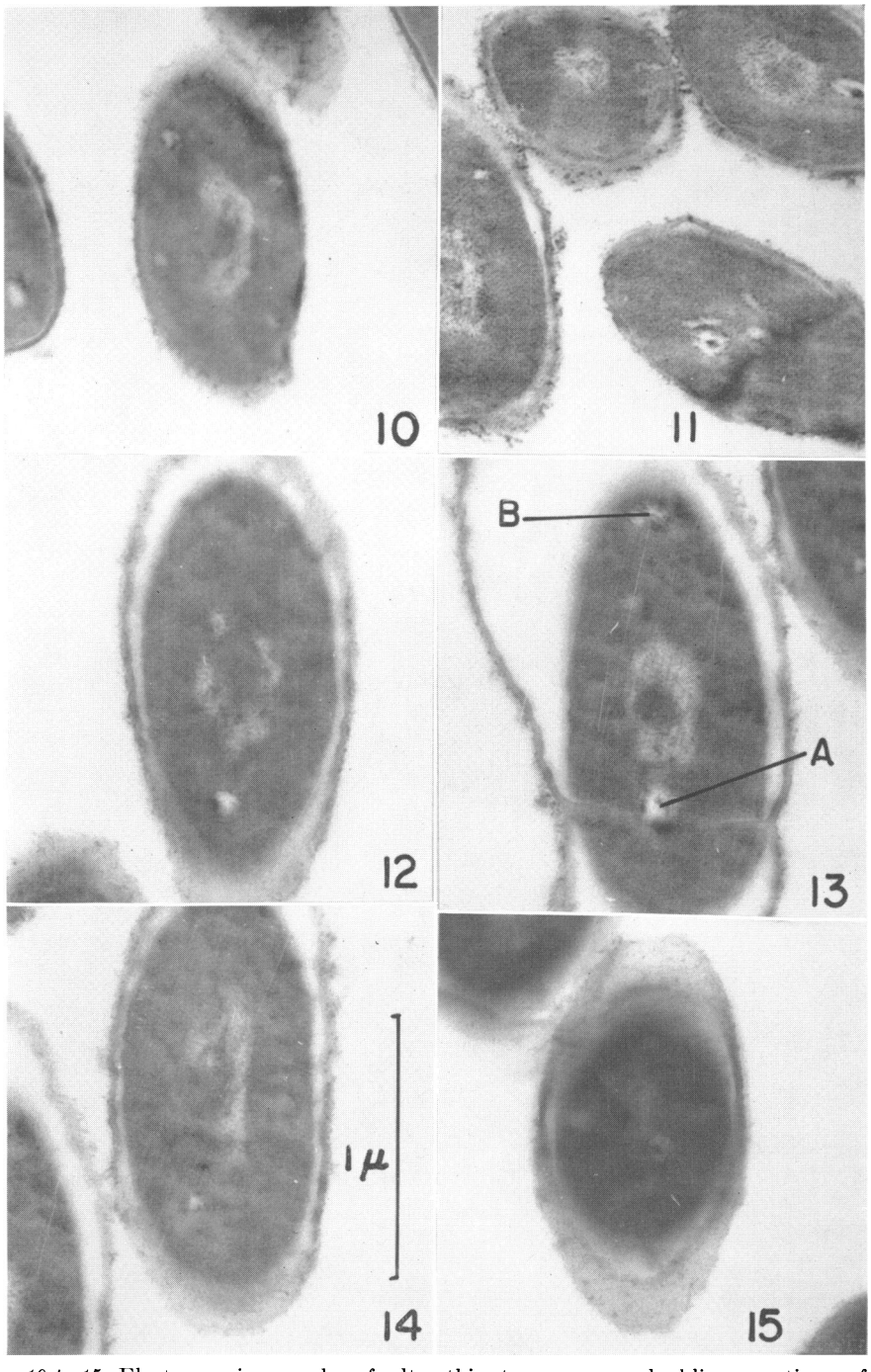
Figures 2 and 3. Electron micrographs of ultra-thin longitudinal sections of *Bacillus cereus*. 50,000 ×. The figures illustrate distribution of the peripheral bodies A, A_1, A_2 and the initial stages of formation of the transverse cell walls X.



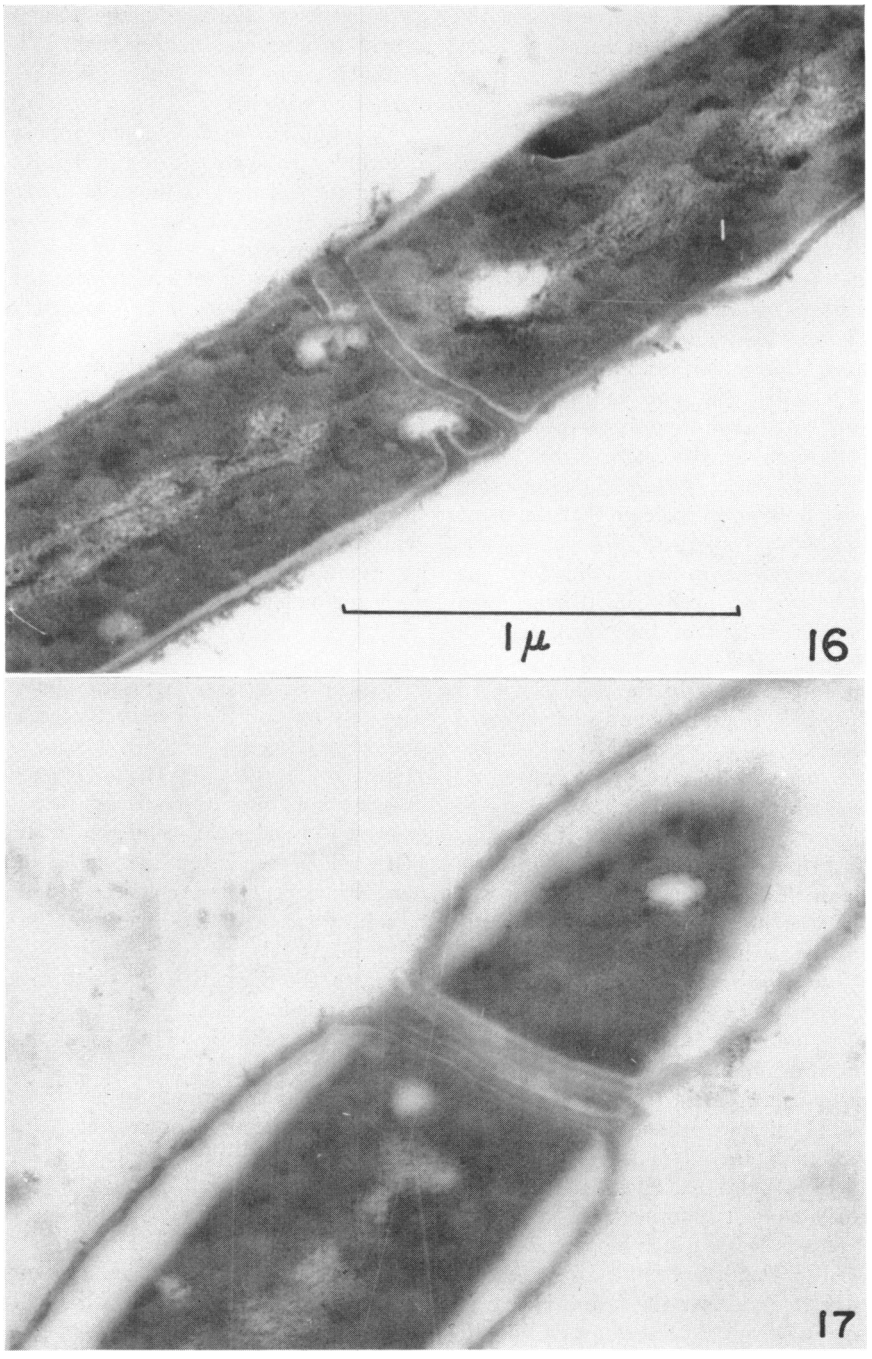
Figures 4, 5, and 6. Electron micrographs of ultra-thin longitudinal sections of *Bacillus cereus*. 36,000 ×. The figures show cells which have been fixed at successively later stages in the formation of transverse cell walls. Figures 4 and 5 show especially well the relationship of the peripheral bodies to the forming transverse cell walls.



Figures 7, 8, and 9. Electron micrographs of ultra-thin longitudinal sections of *Bacillus cereus*. 36,000 ×. Later stages in the formation of transverse cell walls. Figure 7 shows a completed transverse cell wall; figure 8, a completed and thickened transverse cell wall; and figure 9, a still later stage in which each of the adjacent cells has its own end wall.



Figures 10 to 15. Electron micrographs of ultra-thin transverse and oblique sections of *Bacillus cereus*. 36,000 ×. Figures 10, 11, 12, and 13 show the distribution of the peripheral bodies; figures 10, 12, and 14, the abaxial distribution of the nuclear material; and figure 15, the density distribution in the cell wall.



Figures 16 and 17. Electron micrographs of ultra-thin longitudinal and slightly oblique sections of *Bacillus cereus*. 50,000 ×. The figures illustrate two stages in the formation of "supernumerary" transverse cell walls.

most easily as being the result of sectioning the cells at slightly different levels. In all of these pictures it is to be noted particularly that there is absolutely no evidence of any structure preceding the growth of the transverse cell wall except the aforementioned peripheral granules. Figure 6 is a still later stage. In this picture the peripheral bodies are not clearly visible. This is probably simply an accident of cutting.

Unfortunately, no good pictures were obtained of the final stages of the closing of the transverse cell wall. Figure 7 shows a completed transverse cell wall which is beginning to thicken. The stage here is approximately the same as in figure 1 at *E*. It is to be noted that there is no evidence of any special structure at the center of the transverse cell wall in either picture. On the other hand, we have nothing to indicate that the point at which the central aperture disappeared was included in the sections. In figure 7 there is some evidence that there is a layer of slightly increased density on each surface of the transverse cell wall.

Figures 8 and 9 illustrate the final stages of division of the cells. Figure 8 shows a late stage of thickening and an indication of a less dense layer appearing in the middle of the transverse cell wall. There is some indentation at the surface of the cell. However, in this particular picture this is emphasized by the fact that the section is not axial. In figure 9 a more nearly axial cut was obtained. In this it is of interest to note the presence and location of the peripheral bodies.

Figures 10 to 15 are examples of cross sections which demonstrate the peripheral location of the empty granules. As may be expected, no micrographs were obtained of sections made in the same plane as a forming transverse cell wall. The probability of making such a cut is very small. However, figure 13 is an oblique cross section which includes a transverse cell wall which is nearly completely formed. *A* is a peripheral body which is located in the opening of the transverse cell wall and, as has been seen before, has the dense granule near the forming edge of the transverse cell wall. In the other end of the section of the same cell is seen what is considered to be an earlier stage of the peripheral body *B*, which exists just before the beginning of the formation of the transverse cell wall. Figures 11 to 14 also present several near cross sections of the nuclear structures. It should be noted how in some cases the less dense structures seem nearly to surround material which is indistinguishable from the cytoplasm and seems to be connected with it.

Figure 15 is also an oblique cross section and is included to demonstrate the density distribution in the cell wall. It shows rather clearly that there is a dense layer on the inside of the cell wall. However, it is very thin, less than 40 A, and seems to be an integral part of the cell wall. The granular nature of the outer layer of the cell wall is clearly evident.

Figures 16 and 17 illustrate a phenomenon which was observed fairly frequently, namely, the formation of a transverse cell wall in an abnormal position. The formation of these "supernumerary" transverse cell walls seemed to occur most frequently at a short, fixed distance from newly completed transverse cell walls. In figure 17 the supernumerary wall has been completed, thus enclosing a small amount of cytoplasm.

DISCUSSION

Cellular division. The most extensive recent work on cellular division in bacteria has been done by Knaysi, Robinow, and Bisset. Both Knaysi (1941, 1949, 1951) and Robinow (1945) describe the inward annular growth of a ring from the cytoplasmic membrane to form a cell plate which separates the cytoplasm of the daughter cells. They conclude that the cell plate is split then by the centripetal growth of the cell wall. At this point they differ in that Knaysi describes the formation of the transverse cell wall as an independent function of each daughter cell. On the other hand, Robinow, for want of more conclusive evidence, withholds judgment as to whether the growth of the transverse cell wall proceeds doubly or singly.

These earlier results are in agreement with the present work *within the limits imposed by the resolution obtainable with the light microscope.* However, in reality there are several points of disagreement, some of which are quite unexpected. The most important of these is that it is the growth of the cell wall which produces the initial partitioning of the cell. The cytoplasmic membrane, if such exists, remains at all times in contact with the growing cell wall and does not partition the cell independently of the cell wall. The process seems to be one in which cell wall material is secreted as a ring on the inside

surface of the cell wall. In this way an annular disc is formed which closes in the manner of an iris diaphragm until the cell is completely partitioned. The structures designated as "peripheral bodies" undoubtedly are involved in the formation of the transverse cell wall, possibly for the production and secretion of the cell wall material. After the transverse cell wall is completed, it appears to thicken and to divide into two layers. The mechanism of this last event is not elucidated by any of the micrographs obtained so far.

A striking feature of the results obtained in this work is the absence of any structure which can be identified readily as a cytoplasmic membrane. This is rather surprising since earlier electron microscopy has provided some of the best, although not entirely unequivocal, evidence for the existence of a cytoplasmic membrane. Careful examination of the high resolution pictures in this work gives an impression of a slightly increased density at the edge of the cytoplasm. If this is a real membrane, it cannot be more than 40 A in thickness. However, when one examines very oblique sections of this surface, the increased density is no longer visible. This suggests that there is no real membrane and that the observed effect is simply the normal change in distribution one expects to find at any interface.

It appears therefore that the present work tends to support the contention of Henrici (1934) that the cytoplasmic membrane is a surface phenomenon, an optical effect, or both. The plasmolysis of bacteria observed in both the light and the electron microscope with the inferred osmotic response of the bacterial cytoplasm is one of the strongest bits of evidence in favor of the existence of a cytoplasmic membrane. Unfortunately, this gives no suggestion as to its morphology. Light microscopic evidence for the existence of a cytoplasmic membrane would seem very unreliable in view of the very limited resolution. The scale on figure 1 marked *LMR* is 2,000 A long and therefore is a good measure of the *limit* of resolution of the visible light microscope. Obviously, such a coarse means of observation can lead to many mistakes.

It may be argued that the present micrographs include many artifacts. While this may be partially true, the long fixation with osmium tetroxide while the cell is in its natural environment generally is considered to produce less artifacts than the treatments used in light microscopy. The preparations examined here are certainly better than in the early electron microscope work in that they are well fixed and, while they have been subjected to a dehydration series and have been polymerized in plastic, they have *not* been subjected to the violent forces of surface tension on drying or of osmotic effects which arise during the washing of unfixed cells.

It now appears that it was really the limit of resolution of the light microscope which led the earlier workers to decide that the cytoplasmic membrane preceded the cell wall in the partitioning of the cell. It can be seen from the frequency with which peripheral bodies appear in the longitudinal and transverse sections that the number actually associated with a given transverse cell wall formation is probably more than four and possibly as high as six to eight. Since these granules are just at the limit of resolution of the light microscope, are separated by distances of the same order, and are usually arranged approximately in the plane of the developing transverse cell wall, it is obvious that the resulting appearance in the light microscope will be that of an indistinct septum. Thus, our work clearly indicates that the initial partitioning of the cell observed by the early workers was only apparent and that, furthermore, the cytoplasmic membrane, if such exists, is not involved. Our suggestion that what was being observed by the light microscopists was the early planar arrangement of the peripheral bodies is given some support by the staining experiments made by Bisset (1948) and Cassel (1951). It must be noted, of course, that the nature of the process of cellular division undoubtedly varies greatly with species and conditions of growth (Hewitt, 1951).

An interesting phenomenon which, to our knowledge, has never been described in the literature is the occurrence of what we propose to call "supernumerary" transverse cell walls. These abnormal transverse cell walls could be recognized only because they occurred unusually close to normal ones which had just been completed. The extremely short abortive cell which is formed usually seems to contain nothing but cytoplasm. It is also interesting that in the cases which have been observed the separation between normal and abnormal transverse cell wall has been uniformly 1,000 A.

Nuclear apparatus. This report describes some

of the mechanics of cell separation as observed by the ultra-thin sectioning technique and electron microscopy. However, the main intent of the investigation of which this is part is the study of the nuclear apparatus. While the latter is by no means complete, the quality of the micrographs presented here permits some interesting observations to be made regarding the nuclear apparatus and the different forms it may assume.

There is an excellent correlation as to distribution and density between what we identify as nuclear material in our preparations and what has generally come to be accepted as the nuclear apparatus from many other observations. These include electron micrographs of intact untreated cells (Hillier *et al.*, 1949), light micrographs of stained cells as in the work of Robinow (1945), of Johnson and Gray (1949) on luminous bacteria, of Mudd and Smith (1950), etc.

There is also a good correlation with the nuclear regions as shown by the phase contrast studies by Clifton and Ehrhard (1952) of an avirulent anthrax bacillus, Stempen (1950) of *Escherichia coli* and *Proteus vulgaris*, and by Hewitt (1951) of various bacteria. It must be pointed out again, however, that this correlation is valid only within the limits imposed by the resolving power of the light microscope.

The configurations assumed by the nuclear material are strongly suggestive of various stages of division. Of particular significance is the common occurrence of a constriction in the nuclear material in the plane of a forming transverse cell wall (figures 1, 2, 4, 5, and 6). The nuclear material seems to consist of more or less spherical bodies, almost indistinguishable in density and structure from the cytoplasm, surrounded by less dense regions of filamentous material. In a light microscope, properly stained structures of this general arrangement could be interpreted easily as achromatic and chromatic figures. Unfortunately, there is as yet no criterion for the recognition of individual chromosomes in electron micrographs. However, the characteristic arrangement of chromosomes found in mitotic figures should be recognizable. At present no structures have been observed which could be interpreted as being the mitotic figures described by DeLamater (1951), DeLamater and Hunter (1951), DeLamater and Hunter (1952), and DeLamater and Mudd (1951). Superficially the nuclear structures observed bear some resemblance to those described by Delaporte (1950) who was studying much older cultures. However, both these statements must be qualified immediately by pointing out that the longitudinal sections shown represent, on the average, only one-tenth of the total volume of the cells. Up to four serial sections of one bacterium have been photographed, but unfortunately the technical quality of some of the sections and other accidents of preparation have not yet permitted a reasonable reconstruction to be made. For this reason it is not now possible to make any definitive statements regarding the existence of mitotic figures.

It should be noted that no nuclear membrane has been detected in any of the cells. Is it possible that, for these cells which are all undergoing rapid multiplication, an "interphase" nucleus may not occur?

One remaining point is apparent from the pictures, namely that the distribution of the nuclear material is not as axial as it first appears. In some cells it has separated in strands which are still essentially longitudinally arranged but surround a mass of cytoplasm-like material.

No organelles having the characteristic internal structure of the mitochondria of numerous other types of cells (Palade, 1953; Sjöstrand, 1953) have been observed in this work. This observation can be interpreted only to mean that if mitochondria exist in *B. cereus* they do not have the same type of morphology as those in many other types of cells. Moreover, since this work was limited to *B. cereus* (7 hour cultures), the observation has no bearing on the work of Mudd and his co-workers (1951).

SUMMARY

Ultra-thin sections of methacrylate embedded cells of *Bacillus cereus* have been obtained using an experimental microtome. The resulting sections have led to the following description of the division of the cells:

Peripheral bodies arrange themselves annularly at the surface of the cell at a point where the division of the nuclear material is to occur.

A ring of cell wall material is secreted centripetally and the peripheral bodies move inwards with the inner edge of the forming cross wall.

The annular disc of transverse cell wall completely partitions the cell.

The transverse cell wall thickens and splits forming an independent transverse cell wall for each daughter cell.

The adjacent ends of the cells round off and the cells separate.

The sites and appearance of the nuclear apparatus agree with earlier observations, but it was not possible to identify either chromosomes or mitotic figures.

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