ELECTRON MICROSCOPY OF ULTRA-THIN SECTIONS OF BACTERIA

II. Sporulation of *Bacillus megaterium* and *Bacillus cereus*

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Received for publication August 1, 1955

The lack of clarity of the cytological representations of non-nuclear structures appearing in figures which accompany various papers describing the sporulation process has always left the author somewhat dissatisfied. Nuclear elements typically are distinctly stained while the cytoplasm and cell walls usually are either indistinguishable or very faintly discernible. The techniques which have been developed for these studies have admittedly been designed to demonstrate the nucleus. That this aim has been achieved, with some degree of success, can be seen by observing the figures provided by Knaysi (1955), Fitz-James (1954), Robinow (1953*a*), and DeLamater and Hunter (1952). Since it is frequently necessary in cytology to sacrifice one structure in order to demonstrate another, the lack of appearance of non-nuclear structures in the above preparations in no way detracts from the contributions made.

A desire to determine what structures could be observed in the cytoplasm of sporulating cells led the author to apply the techniques of ultra-thin sectioning to the problem. This procedure was described in the preceding paper in this series (Chapman and Hillier, 1953).

Following the publication of this article, there have appeared several papers which have presented the results obtained when ultra-thin sectioning techniques were used. All of the papers owe much of their merit to the added resolution provided by the electron microscope, used in conjunction with ultra-thin sections. Thus, ultra-thin sections have provided striking information concerning cellular division (Chapman and Hillier, 1953), nuclear division (Bradfield, 1954), effect of culture age on cell appearance (Birch-Andersen *et al.*, 1953), spore structure (Robinow, 1953*b*), phage-host interaction (Maaløe *et al.*, 1954), and the cytology of sporulation (this paper). The only attempt to add to the techniques in this new area of bacterial study was made by Bradfield, who developed a modification of the Feulgen stain. Although the results presented in all of the papers have been highly informative and often dramatically clarifying and have helped to establish the basis upon which further analytical work may proceed, it is only by the development of auxiliary techniques that we may expect to obtain a substantial part of the information which ultra-thin sectioning promises to reveal.

MATERIALS AND METHODS

Spores of *Bacillus megaterium*, obtained by growing the bacteria for 72 hr on casein hydrolyzate agar—10 ml of 10 per cent casein hydrolyzate (Nutritional Biochemicals Corp., Cleveland, Ohio) to each liter of 1.5 per cent nutrient agar (Difco)—were supplied to the author in distilled water suspension by Dr. Katherine P. Zworykin. Immediate fixation and routine processing of some of these spores provided the blocks from which the sections of mature spores were obtained. In order to obtain cells in various stages of sporulation, several drops of the above spore suspension were inoculated onto the surface of casein hydrolyzate agar in a petri plate and incubated at 37 C for 12 hr. (It had been determined by light microscopy that nearly all of the spores germinated within 5 to 6 hr underwent several vegetative cell divisions, and then started to sporulate.) The cells were harvested in 0.8 per cent solution of NaCl and processed routinely.

Cells of *Bacillus cereus* in different stages of sporulation were obtained by taking samples after various periods of incubation at 30 C on nutrient agar (Difco) slants to which 0.8 per cent NaCl had been added. The American Type Culture Collection strain no. 7064 was used.

Electron microscopy was performed with two different RCA, type EMU, electron microscopes, each of which had been fitted with a wide field objective lens and a 50 μ aperture. The electron micrographs of *B. megaterium* were taken at an original magnification of 9,000 $\times$ and were en-

larged to 36,000 ×; those of *B. cereus* were taken at 9,650 × and were enlarged to 29,000 × or 38,600 ×.

RESULTS AND DISCUSSION

The explosions of cells and tissues which frequently occur in making ultra-thin sections and which it was thought had been eliminated by long fixation have reappeared from time to time. In a given field, some cells may appear quite satisfactorily prepared while others are exploded to various extents. When this problem arises, one may either reject the entire sample or select those cells which are intact to illustrate his purposes. In the present work, there was no explosion problem with *B. megaterium*, but there was with *B. cereus*. Accordingly, selection of intact cells of *B. cereus* was resorted to. This alternative seemed justified since, in remnants of exploded cells, one could always find those structures which are pointed out in the included micrographs of intact, or nearly intact, cells. The explosion problem then, while it is discouraging and exasperating, does not render useless all samples from a population of cells.

In spite of this somewhat encouraging fact, explosion still remains the principal producer of artifacts in bacterial preparations. It is hoped that someone will soon uncover the cause and cure for this troublesome phenomenon.

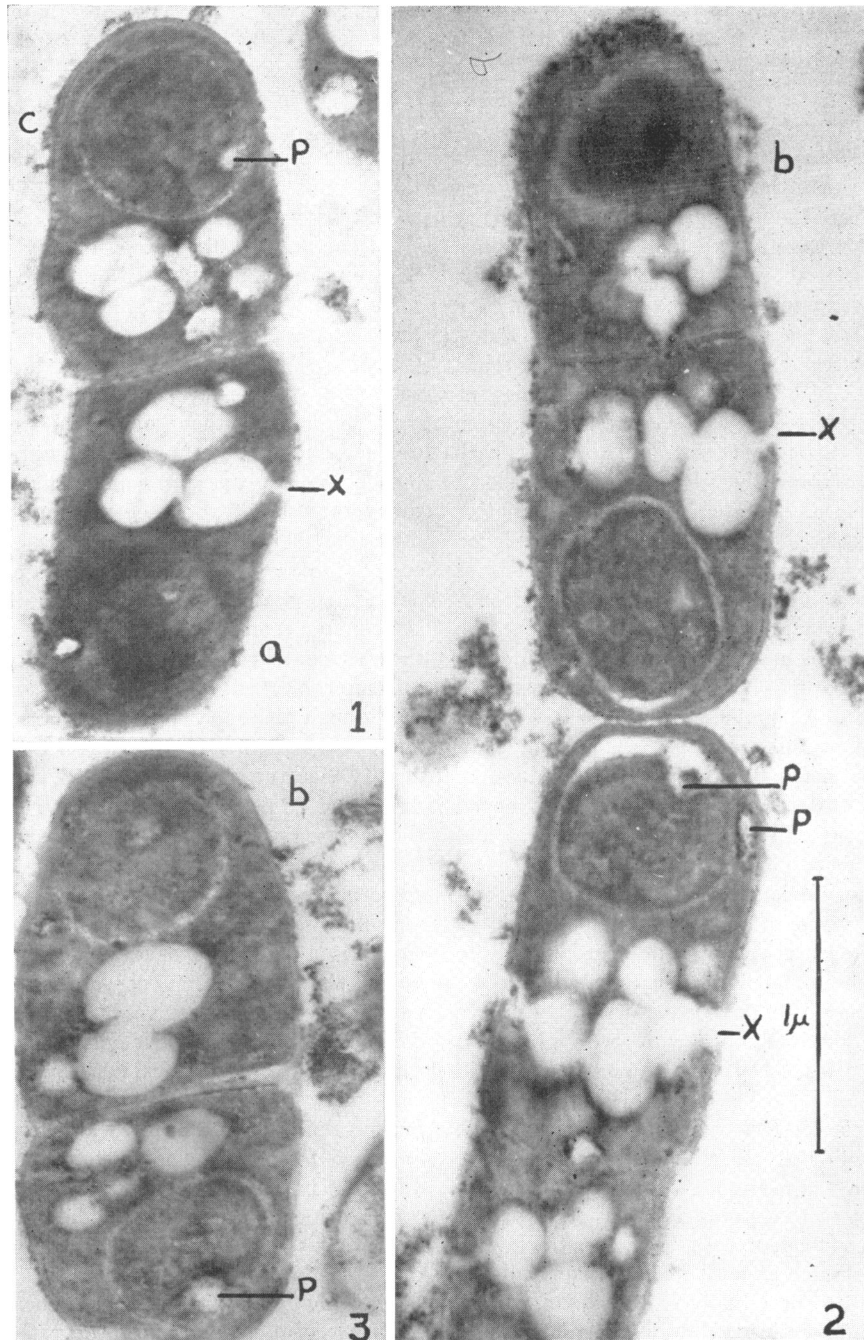
One other obvious artifact appears in some of the micrographs, that is, the opening through the cell wall from the vacuole-like inclusion body to the surrounding medium (figures 1 and 2 at ×). These inclusions appear to be of a fatty nature since they stain readily with Sudan black B (Burdon, 1946). They are also clearly visible in unstained, living cells observed with the television microscope (Zworykin and Chapman, 1955, unpublished data) wherein they have been noted to persist in the medium for some time after disintegration of the sporangium. Whether or not the apparent tendency of these bodies to coalesce is an observation at variance with that made by Burdon (1946) or actually represents an artifact cannot be determined. However, the latter may be more likely since, in the television studies mentioned above, no tendency of the inclusions to coalesce was observed in the living cells.

Figures 1 to 9 are of *B. megaterium*. They have been arranged in an order which places the earliest detectable stages of spore formation first, the mature endospores later, and the free spores last.

In figure 1, cell a and in figure 2, cell b represent stages in sporulation which are so early as to be very nearly undetectable. No suggestion of a spore coat exists, and one must be careful if the margins of the endospore are to be seen. It should be noted that no granules of sufficient size to account for the "growth of a granule" or "aggregation of granules" theories, long held by some visible-light microscopists to account for the mechanism of spore formation, are observed. (See Knaysi's 1948 review.) At various loci in the sporangia, granules of about 200 A in diameter may be seen, but these, of course, are fully an order of magnitude below the resolution limit of the light microscope and could not account for the reported observations. Furthermore, it seems rather unlikely that granules of a size great enough to be visible in the light microscope could lie undifferentiated in the cytoplasm of these cells. It therefore seems reasonable to reject the two theories mentioned earlier, at least as far as these cells are concerned.

Although the spore primordium in cell b of figure 2 appears to have a slightly greater electron density than the general cytoplasm, such density differences do not occur consistently. In general, there appears to be no greater density to electrons of the cytoplasm of the most mature endospore in comparison to the density of the cytoplasm of its sporangium than occurs in the same comparison at earlier stages. These figures, then, fail to show an electron density increase comparable to the progressive increase in optical density on the part of developing endospores in comparison with the optical density of sporangia which Bayne-Jones and Petrilli (1933) showed for unstained, living cells and which Lewis (1934) indicated for stained and unstained cells. This difference in observations is not surprising, however, when one realizes that the determination of optical density is a quite different matter from the determination of electron density.

The only cytoplasmic change (associated with maturing of the endospore) suggested by the included figures is that of a progressive refinement of the endospore cytoplasm as the endospore matures. Visibly, this change appears as an increasing textural smoothness. Whether this alteration represents a visible indication of a reduction in molecule size or of a rearrangement



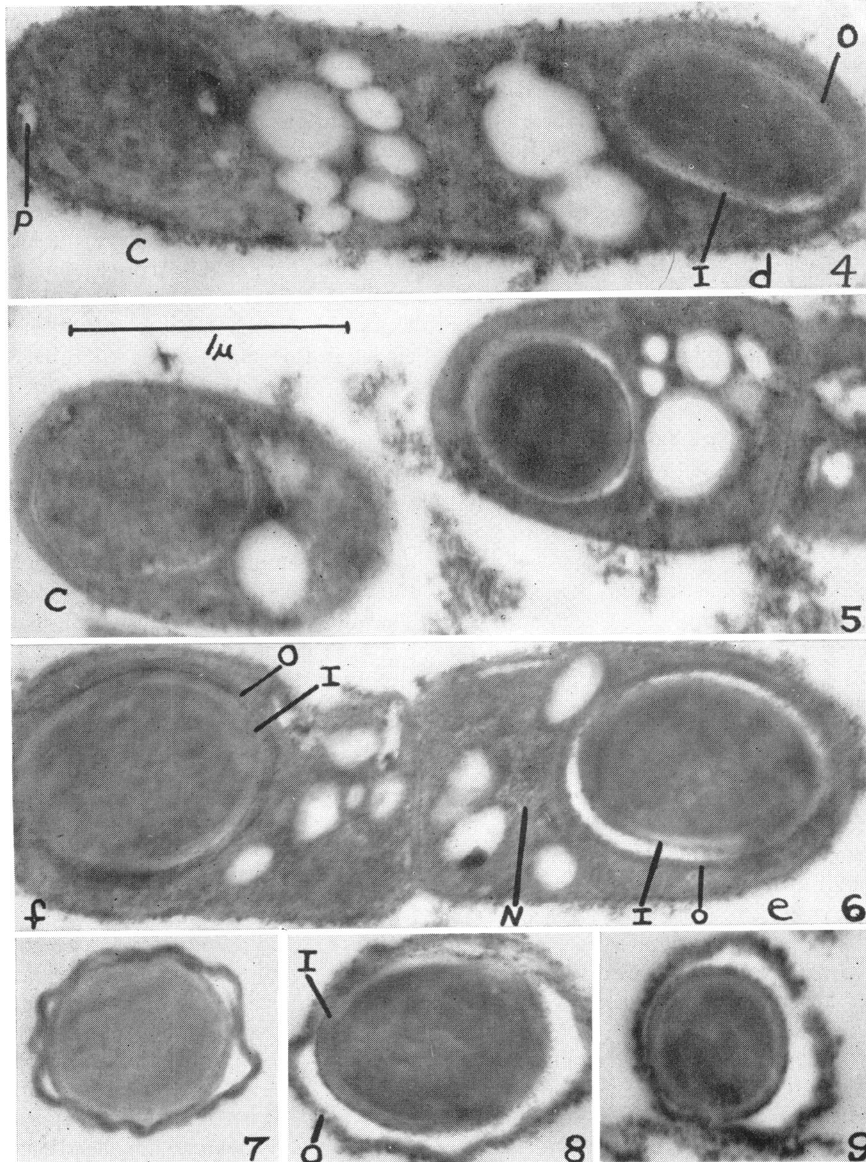
Figures 1, 2, and 3. Electron micrographs of ultra-thin sections of *Bacillus megaterium*. 36,000 ×. The figures show cells in early and intermediate stages of sporulation. X, opening to outside of inclusion body; P, peripheral body.

of molecules or of some other phenomenon cannot be determined. It may correspond to the change in staining properties undergone by the forespore nucleoplasm, as reported by Knaysi (1955), and considered by him to consist "in a loss of metachromasy and a rise in isoelectric point to the neighborhood of that of common proteins."

The author is led to conclude from the above

observations that the process by which the spore cytoplasm is delineated from the sporangial cytoplasm is best described as a differentiation *in situ*.

While the endospore cytoplasm is becoming distinct (apparently both micromorphologically and chemically, as well as physiologically) the spore coats are formed about it. The very fine line of increased electron density which appears clearly in cells c of figures 1, 4, and 5 has not yet appeared in cells a and b of figures 1, 2, and 3. Cell d of figure 4 depicts an early "two-coat" stage. The inner coat I can be seen at several regions, if one looks along the periphery of the endospore proper. At O, the outer coat is marked. Cell e of figure 6 represents a slightly more mature stage. The inner and outer coats are now readily discernible. Cell f of figure 6 represents the most advanced stage observed, exclusive of free spores.



Figures 4 to 9. Electron micrographs of ultra-thin sections of *Bacillus megaterium*. 36,000 ×. Late stages of sporulation and mature spores are shown. I, inner spore coat; O, outer spore coat; N, nuclear element; P, peripheral body.

Study of the figures leads one to the conclusion that the inner coat is formed in a somewhat immature condition (thinner than a mature inner coat) before the outer coat appears, and that the inner coat matures as the outer coat appears and matures. The process by which a coat develops seems best described as a condensation of the cytoplasm—of the sporangial cytoplasm in the case of the outer coat and of the endospore cytoplasm in the case of the inner coat. No evidence of an iris-like proximal extension of any spore coat from the end of the sporangium, as reported by Lewis (1934), was observed.

Figures 7, 8, and 9 are electron micrographs of sections of free spores. The two spore coats are clearly visible. These sections appear quite different, however, from the sections of Robinow (1953*b*). While both have two spore coats, the inner coat of these spores appears to be much more intimately bound to the spore proper than does the inner coat of Robinow's spores, which is connected to the spore proper by only an isthmus of spore proper material. As Robinow has shown, and as this paper substantiates, considerable difference between species exists. It is not, then, too surprising that strain differences also occur. (Actually, Robinow's *B. megaterium* resembles his strain of *B. cereus* and that which will be described shortly more closely than it resembles the *B. megaterium* strain herein described.)

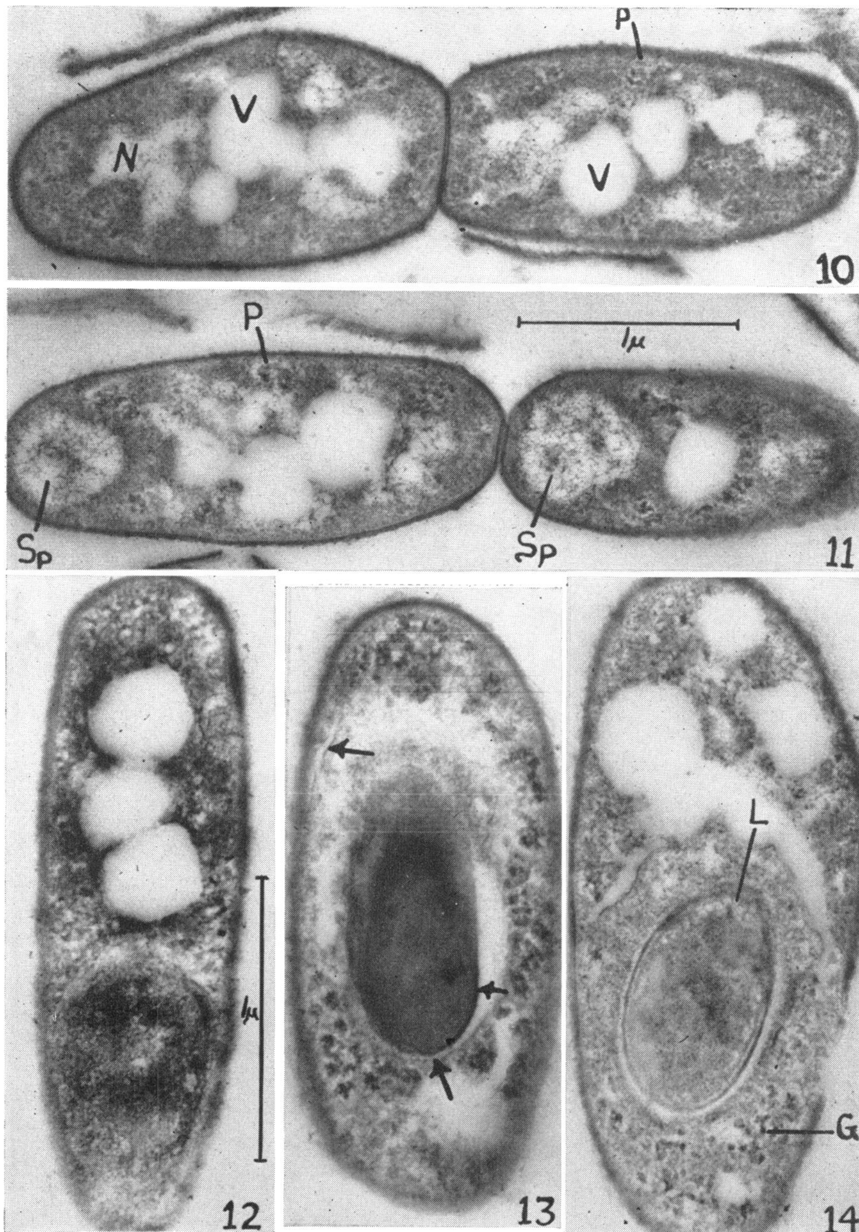
Other differences which appear between these two strains of *B. megaterium* are the absence of a low density cortex and of low density peripheral areas of the core (nuclear elements) from the present strain and their presence in Robinow's strain. The first difference might be explained simply as a strain difference, but the failure to observe the nuclear elements is more difficult to account for. However, the fact that Robinow found these elements often not detectable and never so well defined as they were in *B. cereus* leads one to suspect that the nuclear elements of *B. megaterium* may be more sensitive to the preparative treatments than are those of *B. cereus*. Perhaps the additional spore coat of *B. cereus* decreases this sensitivity.

Figures 10 to 19 are of *B. cereus*. They have been arranged in the same order as the figures of *B. megaterium*.

The cells of figure 10 are from an actively dividing culture. No evidences of spore primordia are visible. The vacuole-like inclusions (V) are readily apparent. Other cell structures have not been well preserved. The cells of figure 11 show early stages of formation of spore primorida (Sp). These primordia show a lower electron density than is seen in most primordia and forespores. This decreased density may be representative of a sporulation stage so early that it can be characterized only by an aggregation of the low electron density nuclear material in the prospective area of formation of the spore primordium. It has been shown by Robinow (1953*b*) and by Chapman and Hillier (1953) that the nuclear material in these preparations typically appears of lower density than the cytoplasm. In figure 12, the inclusions have been displaced to one end of the cell, and a spore primordium of density approximately equal to that of the cytoplasm is faintly visible. The cell of figure 13 shows, albeit indistinctly, the earliest stages of formation of the three spore coats. Arrows point to the sites at which this may be seen most clearly. Figure 14, which is the highest resolution figure included in this paper, shows the three coats quite clearly. Figure 15 shows the coats in a very nearly mature condition. Figure 16 is a cross section of a cell at about the same stage of sporulation as figure 15.

Figures 17, 18 and 19 are electron micrographs of sections of mature spores. These figures bear a marked resemblance to the figures of *B. cereus* spores which appear in Robinow's (1953*b*) paper. The exosporium (E), outer spore coat (O), and nuclear elements (N) correspond to the similar structures shown by Robinow's figures, except that here the "outer spore coat" corresponds to his "spore coat." This difference in nomenclature must be used since an additional spore coat, an inner spore coat (I), appears in the included figures. This inner coat may have remained indistinguishable in Robinow's work due to the slightly greater thickness of his sections or to the fact that he used much shorter fixation times, or both. (Actually, there is a suggestion of the existence of an inner spore coat in figures 12 and 17 of Robinow's paper.) The various structures listed above, as well as the remnant of the sporangium cell wall (W), all of which show clearly in the figures, are indicated on figure 19.

It should be noted that there appears to be a difference in the manner in which the two species form spore coats. *B. megaterium* seems to form

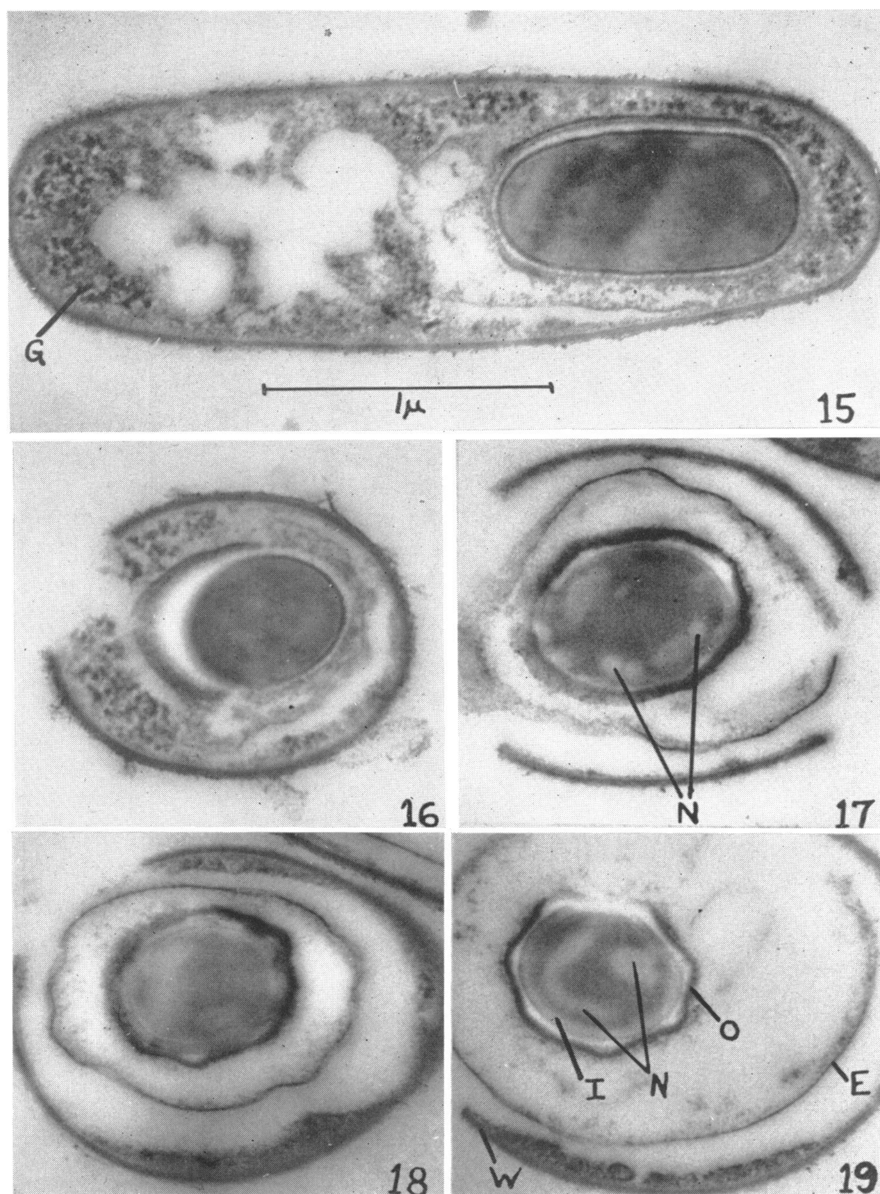


Figures 10 to 14. Electron micrographs of ultra-thin sections of *Bacillus cereus.* 29,000 × and 38,600 ×. The figures are arranged to show cells in successively later stages of sporulation. Arrows point to areas in which the three spore coats may be detected; N, nuclear element; P, peripheral body; G, dense granule; Sp, spore primordium; V, inclusions; L, low density particles.

one coat and then the other, while *B. cereus* seems to form all three coats simultaneously.

Another observation which should be noted is that the spores of these two species attain complete morphological maturity while still encased in the sporangium. No additional spore coats were observed to be formed after the release of the spore from the sporangium.

With the possible exception of figure 6, at N, no evidence for the existence of nuclear material in any of the figures of *B. megaterium* could be found. That even a vegetative supportive nu-



Figures 15 to 19. Electron micrographs of ultra-thin sections of *Bacillus cereus*. 38,600 ×. The figures show cells in late stages of sporulation and just prior to liberation from the sporangium wall. E, exosporium; O, outer coat; I, inner coat; N, nuclear element; W, sporangium wall; G, dense granule.

cleus, as observed by DeLamater and Hunter (1952), was not seen is especially puzzling. However, this failure to demonstrate nuclear material in these cells may be accounted for by the possibility that either all of the cells are of an age at which the vegetative supportive nucleus has degenerated, or that the nuclear material which is incorporated into the spore is simply not differentiable from the general cytoplasm by the preparative techniques used. It is also possible that the sections selected missed the nuclear material. In spite of these disturbing features associated with the study of *B. megaterium*, one cannot but be encouraged when it is seen that nuclear material may be identified in the vegetative cells of *B. cereus*, figure 10, in the earliest

stages of sporulation, figure 11, and in the mature spores, figures 17 and 19.

Several general comments concerning the micrographs seem appropriate. Careful observation of the figures will reveal what are considered to be peripheral bodies (P) at several sites in the sporangial cytoplasm (figures 2, 4, 10 and 11) and in the endospores (figures 1, 2 and 3). The possible role of these bodies in cellular division has been described elsewhere (Chapman and Hillier, 1953).

At various sites in the sporangial cytoplasm, one may observe extremely dense granules (G in figures 14 and 15) which are about 250 A in diameter. These granules may appear singly or in clumps and resemble similar granules in the figures of Maaløe *et al.* (1954). The developing endospore of figure 14 also reveals a clump of low density particles (L) of about the same diameter as the high density granules mentioned above. The significance of these inclusions is unknown.

It is hoped that this paper, the second of a series, will serve as an indication that fairly high resolution electron micrographs of ultra-thin sections of bacteria may be obtained routinely and that thereby more investigators will be tempted to apply the method to their own problems. There is obviously much to be learned.

ACKNOWLEDGMENTS

The author wishes to thank Dr. Frank H. Johnson for making available the various facilities of his microbiological laboratories.

SUMMARY

A series of electron micrographs of ultra-thin sections of methacrylate-embedded cells of *Bacillus megaterium* and *Bacillus cereus* have been obtained which illustrate various stages in the sporulation of these organisms.

Sporulation seems best described as a process in which the spore proper is formed by a differentiation *in situ* of the sporangial cytoplasm, following incorporation of nuclear elements. The spore coats appear to be formed by a condensation of sporangial and spore cytoplasm. *B. megaterium* has two spore coats and *B. cereus* has three spore coats. *B. megaterium* spores apparently lack the exosporium, or outermost spore coat, of *B. cereus*.

Nuclear elements, closely resembling those described by Robinow (1953*b*) have been seen in the spores of *B. cereus* but not in the spores of *B. megaterium*.

Lipid inclusions, low density particles, high density granules, and peripheral bodies have been identified in the cells.

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