STUDY OF GERMINATING *BACILLUS CEREUS* SPORES EMPLOYING TELEVISION MICROSCOPY OF LIVING CELLS AND ELECTRON MICROSCOPY OF ULTRATHIN SECTIONS

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Received for publication January 2, 1957

The literature on spore germination fails to provide conclusive evidence concerning the origin and time of appearance of the vegetative cell wall. Reports range from the belief that the inner of two spore coats becomes the germ cell wall (Brefeld, 1881) to the statement that the germ cell wall is not pre-existent in the spore but is formed during germination (Preisz, 1919; Knaysi *et al.*, 1947; Knaysi, 1955).

The technique of ultrathin sectioning has led to the discovery of some interesting facts concerning spore structure and spore formation (Robinow, 1953; Chapman, 1956). It seemed reasonable, therefore, to apply the method in an attempt to determine the origin of the vegetative cell wall.

The application of television microscopy of living cells permitted the establishment of appropriate time intervals for the fixing of samples for embedding and ultrathin sectioning (Zworykin and Chapman, 1956).

MATERIALS AND METHODS

The culture used in this study was the American Type Culture Collection *Bacillus cereus* strain 7064. Following growth for from several days to several weeks on slants of nutrient agar to which 0.8 per cent NaCl had been added, yields of up to 95 per cent spores may be harvested. The spores were washed from the slants in brain heart infusion (Difco) broth. Samples to be embedded and sectioned were made by spreading 3 ml of the broth suspension onto the surface of a 6 inch petri dish of the slant medium. Several such plates were prepared. At intervals indicated appropriate by observation of living germinating spores on the television screen, the growth on a plate was taken up in 0.8 per cent NaCl, centrifuged, and resuspended in 3 ml of 2 per cent OsO_4 to which 0.8 per cent NaCl had been added. Following fixation for 18 to 24 hr, the cells were embedded in methacrylates as described by Chapman and Hillier (1953), except that polymerization was carried out at 72 C instead of at 47 C. Borysko (1955) has reported that polymerization temperatures higher than the 47 C which is generally used result in less damage to a variety of tissues. Since even long fixation does not eliminate explosions in bacterial cells, and since it was observed by Chapman and Kroll (1957), following Borysko's lead, that spirilla are much better preserved at 72 C than at 47 C, the higher temperature was used. Sectioning and electron microscopy were performed as in the earlier paper (Chapman and Hillier, 1953). The electron micrographs were taken at an electronic magnification of 9,000 × and were enlarged to 45,000 ×.

Samples for observation with the television system were prepared by spreading a 2 mm loop of the spore suspension in broth over the surface of a solidified drop of the slant medium on a no. 1 cover slip. A ring of agar around the drop permitted the drop to be suspended in the small chamber formed when the cover slip was sealed with "vaspar," agar down, to the top glass of a constant temperature slide. (This slide has been described by Zworykin and Chapman, 1956.) Distilled water from a constant temperature water bath was passed through the slide at a rate of 1 drop per sec. The temperature of the input water was 37 C.

The slide was placed on the stage of a Bausch and Lomb light microscope equipped with a 90X, 2 mm, N. A. 1.30, apochromatic, oil immersed objective and a 7.5X compensating ocular. Illumination was provided by a Bausch and Lomb microscope illuminator type 31-33-85. The

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light was passed through distilled water in the container provided with the illuminator. No other filters were used.

A Vidicon television camera was mounted over the microscope ocular and was cable-connected to a monitor and control unit. Permanent records of the images produced on the monitor viewing screen were obtained by photographing the screen with a Graphic View camera, equipped with a supermatic (X) lens, operated at f/4.5. "Kodak super-XX" film pack was used. Prints were made on an enlarger, using "kodabromide" no. 4 paper. Final magnification of these figures is approximately 5,000 ×.

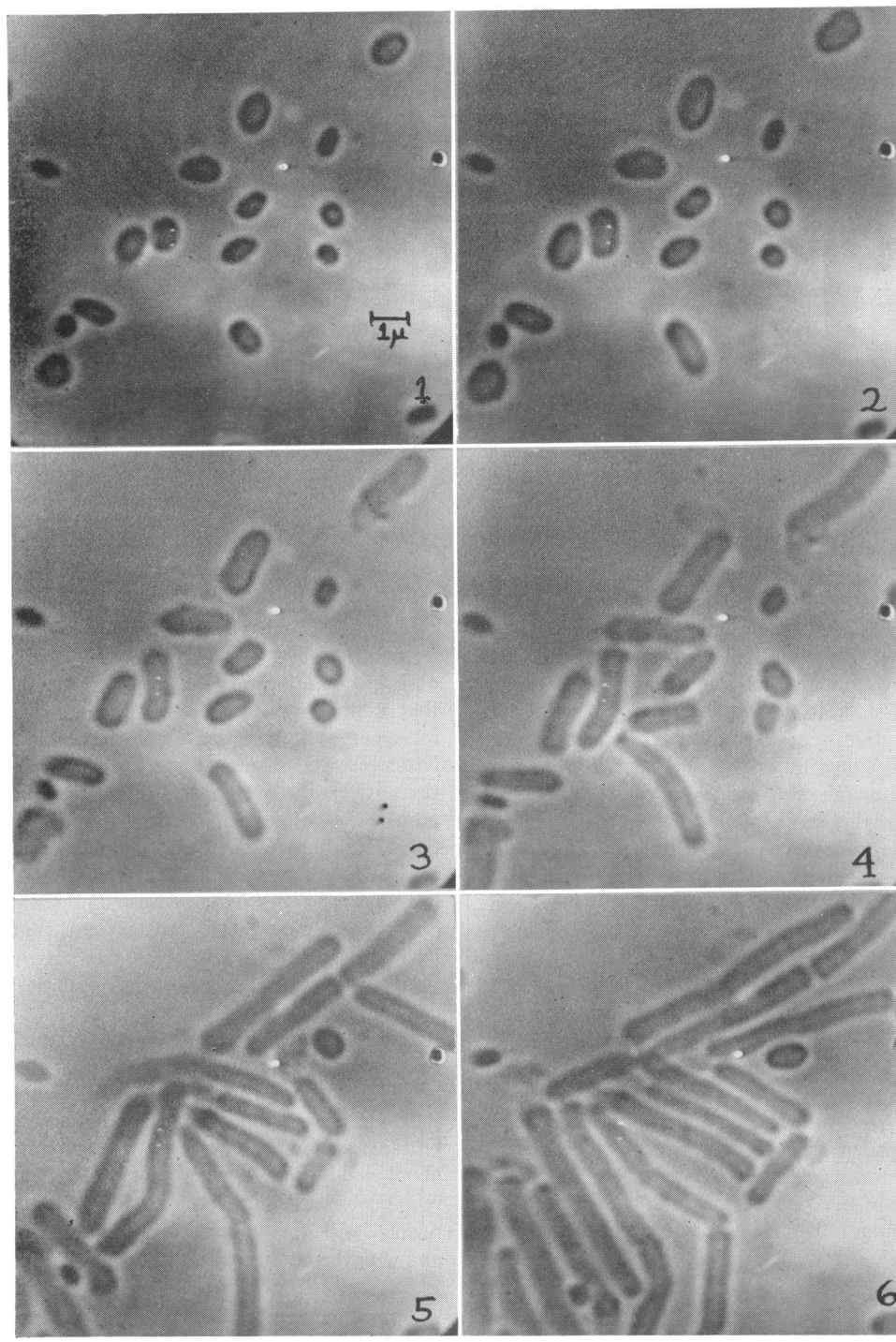
RESULTS AND DISCUSSION

Figures 1 to 6 are of a single microscope field and were obtained from the monitor viewing screen at 30 min intervals over a 2½ hr period. As stated previously, this technique proved useful for establishing time bases because it could readily be seen at what stage of germination the majority of spores in a field had arrived.

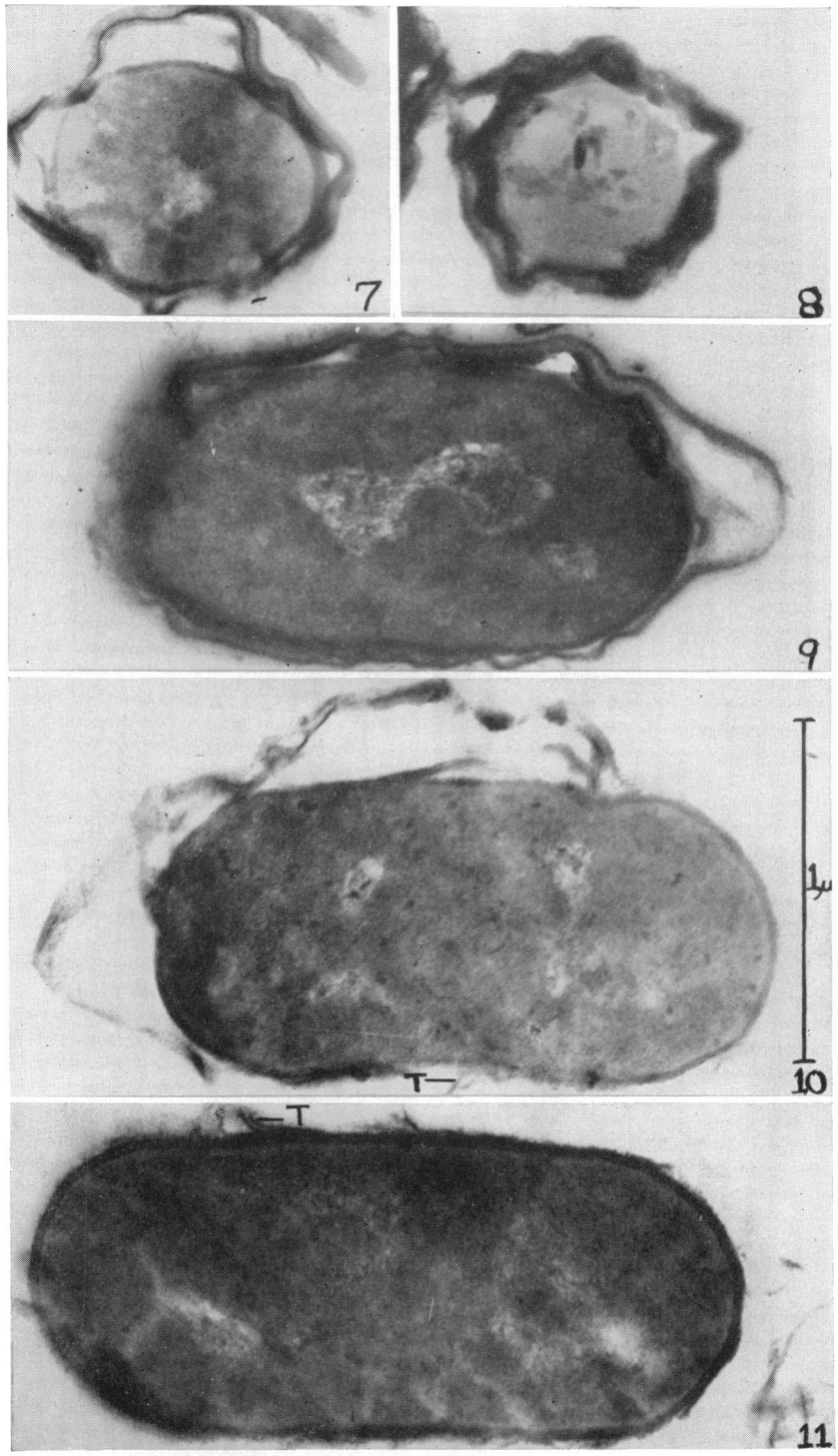
Figures 7 to 13 are electron micrographs of ultrathin sections of spores in various stages of germination up to the 2-cell stage. Although samples for fixation were taken at intervals corresponding to the times of taking the television pictures, and although each sample provided a majority of cells in the developmental stage represented by the majority of cells in the television field, all of the included micrographs were obtained from a single sample taken at 1½ hr after the inoculation of the plate with the spore suspension, because this particular sample provided the best electron micrographs.

Figures 7 and 8 illustrate the appearance of young germinating spores. It would be inaccurate to designate these spores as "resting spores" for several reasons. In the first place, their contact with fresh nutrient medium for longer than 2 hr makes it likely that they differ physiologically from resting spores. Furthermore, the resting spores of *B. cereus* shown by Robinow (1953) and Chapman (1956) clearly show a peripheral orientation of the agranular, low density nuclear areas; the spore in figure 7 has its nuclear material centrally disposed and this nuclear material exhibits a granularity similar to that shown by the vegetative nuclear areas. There is no reason to believe that because these spores have remained essentially unchanged after about 1½ hr on the fresh medium they are nonviable. Several of the spores in the figures in the television microscope series (figures 1–6) remain essentially unchanged until, as in figure 4 (which was taken after the spores had been on the fresh medium for nearly 2 hr), they begin to elongate and subsequently grow. The three spore coats, which are characteristic of *B. cereus*, are quite clearly distinguishable. Observation of the close association between the two outer spore coats in these figures makes it easy to understand why there has been considerable difference of opinion concerning the number of these coats. Indeed, the inclination may still be to view one of these outer coats as a delaminated layer of the other. However, the best micrographs indicate that they actually are independent. It may be noted in these figures that the two outer spore coats are in close contact around much of the spore circumference. Such close contact is in marked contrast to the considerable separation between the two outer coats exhibited by the resting spores of the same *B. cereus* strain shown by Chapman (1956). This difference in appearance could be due to the fact that in the former case, the spores apparently represent young germinating spores while in the latter case they are resting spores. These conditions arise directly from the different methods of preparation. It is also possible that the higher polymerization temperature employed in the present study has resulted in the difference. It follows that the close apposition of these two outer spore coats is the more natural condition, for it has been shown that the higher polymerization temperature results generally in less cell damage.

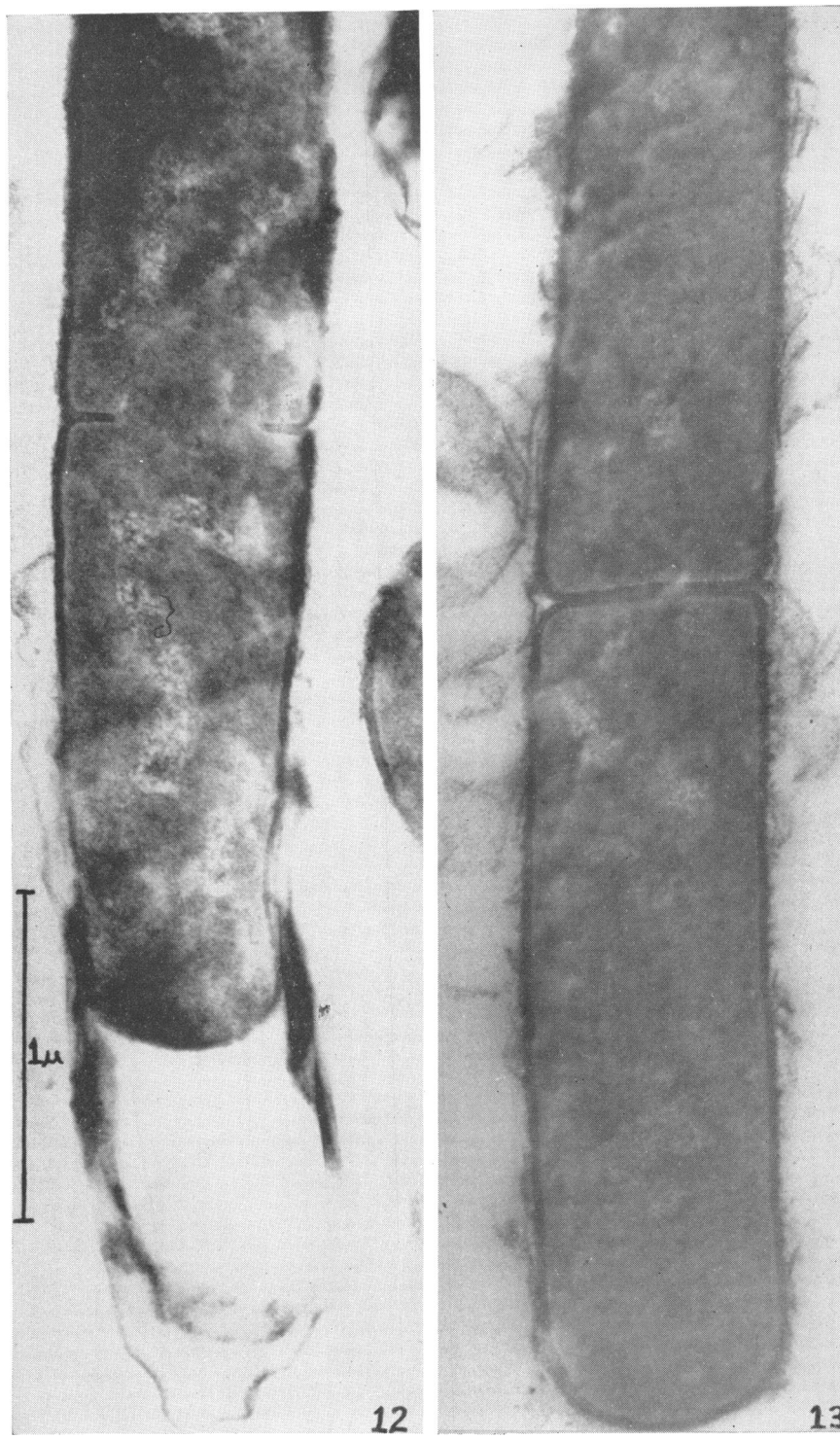
Figure 9 shows a germinating spore. The dumbbell shaped zone of low-density nuclear material is quite evident. The innermost and outermost spore coats are still quite clearly distinguishable, but the middle coat appears to be absent from about one-fifth of the periphery, to be in close relationship with the other two coats in some regions, with the innermost coat in other regions and with the outermost coat in still other regions. It was not possible to ascertain at present whether this variety of relationships is due simply to the forces of shrinkage or whether there is actually some useful association between the middle and innermost coats—with perhaps some



Figures 1–6. Photographs, at 30 min intervals, of a single field of germinating *Bacillus cereus* spores as they appear on television viewing screen. 5,000 ×.



Figures 7–11. Electron micrographs of ultrathin sections of *Bacillus cereus* spores and young vegetative cells. 45,000 ×. The three spore coats can be distinguished in all save the last figure. Apparently, the innermost spore coat becomes the vegetative cell wall. *T*, threadlike component of wall.



Figures 12 and 13. Electron micrographs of ultrathin sections of young vegetative cells of *Bacillus cereus*. 45,000 ×. Appreciable portions of the two outer spore coats still cling to the cell in figure 12; mere remnants persist in figure 13.

absorption of middle coat material by the young germ cell as Lamanna (1940) has indicated to occur in *Bacillus megaterium*. Figure 10 illustrates further diminution of the outer spore coat and again suggests the possible incorporation of some of the middle coat material into the wall of the germ cell. The young vegetative cell of figure 11 is completely free of the outer spore coats while the somewhat older cell of figure 12 (which has already initiated cross wall formation) still is encumbered by the remnants of the two outer spore coats. Figure 13 is a cell which has just completed cross wall formation. Remnants of the outer spore coats are still adherent to the cell wall. A threadlike component of the spore coats, similar to the matrix of beaded threads described for the spore coat of *B. megaterium* by Knaysi and Hillier (1949), is especially noticeable in figures 11 and 13. In figure 11, the thread (T) measures about 75A in diameter. This is in reasonably close agreement with the 90A thread cross section reported by Knaysi and Hillier (1949) for the threads in the spore coat of *B. megaterium*.

As previously noted, in connection with the comments on figures 7 and 9, the nuclear areas of these germinating spores appear as low-density zones. The great variation in the configurations assumed by these zones in the different cells, with the resulting rare occurrence of the dumbbell form, is really not too surprising considering the findings of the light microscopists. Thus, Delaporte (1950) has indicated that a great variety of shapes are assumed by the nuclear material of osmium tetroxide fixed germinating spores of *Bacillus mycoides*. Furthermore, Mason and Powelson (1956) have shown clearly, with their phase contrast pictures of growing *B. cereus*, that chromatin configurations are indeed complicated. Sections, which represent perhaps one-tenth of the cell volume, could hardly be expected to present a more regularly arranged disposition of these nuclear zones. Variation, therefore, remains the rule in these configurations.

ACKNOWLEDGMENT

The authors wish to thank Dr. Arthur K. Parpart for his assistance and interest in this work.

SUMMARY

The method of television microscopy of living germinating spores of *Bacillus cereus* facilitated the establishment of a time basis for fixing specimens for ultrathin sectioning and electron microscopy.

A series of photographs, obtained from the television viewing screen, illustrates the process of spore germination in a single microscope field over a 2½ hour period.

Electron micrographs of ultrathin sections of spores in various stages of germination indicate that the wall of the young vegetative cell was preexistent in the mature spore, as the innermost spore coat. Germinating spores may lose the two outer spore coats at various stages in the germination process. Remnants of the spore coats may remain adherent to the vegetative cell wall for some time. A threadlike component of the spore coats has been described.

Considerable variation in the configurations of the nuclear material of the germinating spores has been noted.

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