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Further Observations on the Spodogram of *Bacillus cereus* Endospore

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ABSTRACT

KNAYSI, GEORGES (Cornell University, Ithaca, N.Y.). Further observations on the spodogram of *Bacillus cereus* endospore. J. Bacteriol. **90**:453-455. 1965.—Endospore spodograms of *Bacillus cereus* C_3 were examined in dark contrast by use of the phase microscope with oil-immersion objectives. When the spodograms were mounted in air, their central part, or core, appeared bright; when mounted in water or oil, the central part appeared dark. The central part of the normal, unincinerated spore appeared bright in air and water, but dark in oil. This indicated that whether a body appears bright or dark when observed with the phase microscope does not depend only on its refringence relative to that of its surround, but also on its thickness and on the magnitude of the difference between its refractive index and that of its surround. Present observations showed that, contrary to a previous conclusion, mineral matter is more highly concentrated in the core than at the periphery of the endospore.

Several years ago, I reported (Knaysi, 1961) on bacterial spodograms observed with the phase microscope and pointed out the potential value of such a study for bacterial cytology. Removal of the organic material by incineration simplifies the picture of a cell or a cellular structure. Its chief limitation has been the low resolving power of dry objectives heretofore exclusively used for observation. For instance, in the work referred to above, observation was made with a Zeiss Neofluar 40/0.75 which has a theoretical resolving power of 0.33 μ. In subsequent work, however, I used a Neofluar 63/0.90 and found it far superior to the 40/0.75. It was much later that I considered the use of oil-immersion objectives. To my surprise, these objectives gave images of excellent qualities. The spodograms had sharp outlines and filaments 0.2 μ thick, were clearly resolved, and stood out in excellent contrast (Fig. 4; also compare Fig. 3 with 1 and 2), probably because of a certain continuity between the individual spodograms and the cover glass so that no air phase intervened between the spodograms and the objective.

It was the purpose of this investigation to make use of the greater resolution and magnification afforded by the oil-immersion objectives for a further study of the properties of the endospore spodogram.

MATERIALS AND METHODS

The organism used in this study was strain C_3 of *Bacillus cereus*, which I have used often in previous cytological studies. It was grown in microcultures on collodion films supported by 2% agar (Difco) in water, as described by Hillier, Knaysi, and Baker (1948). Sporulation was complete in 2 days. A spore microculture to be studied was floated on water, picked up with a cover glass, and air-dried or dried from the frozen state by use of a new technique to be described in a subsequent report. Microcultures to be incinerated were picked up with Pyrex cover glasses. Incineration was carried out at 525 C for 30 min.

For observation, the cover glass carrying the microculture was inverted over a glass slide to which it was attached by four minute droplets of vaspar (a mixture of equal masses of vaseline and paraffine). Preparations mounted in thin films of water were often sealed with vaspar to prevent evaporation.

RESULTS AND DISCUSSION

Figures 1 to 3 confirm my previous observation (Knaysi, 1961) that the endospore spodogram of *B. cereus* consists of a bright center surrounded by a dark area when observed in dark contrast with the phase microscope. Since in dark contrast the phase microscope usually shows objects that are more refringent than their surrounds dark, and objects less refringent than their surrounds bright, it was natural to conclude that "the spore mineral matter is concentrated in a peripheral layer surrounding a minerally poor core" (Knaysi, 1961). Furthermore, the mineral residue of the vegetative cell and of the periphery of the spore appeared dark when similarly ob-

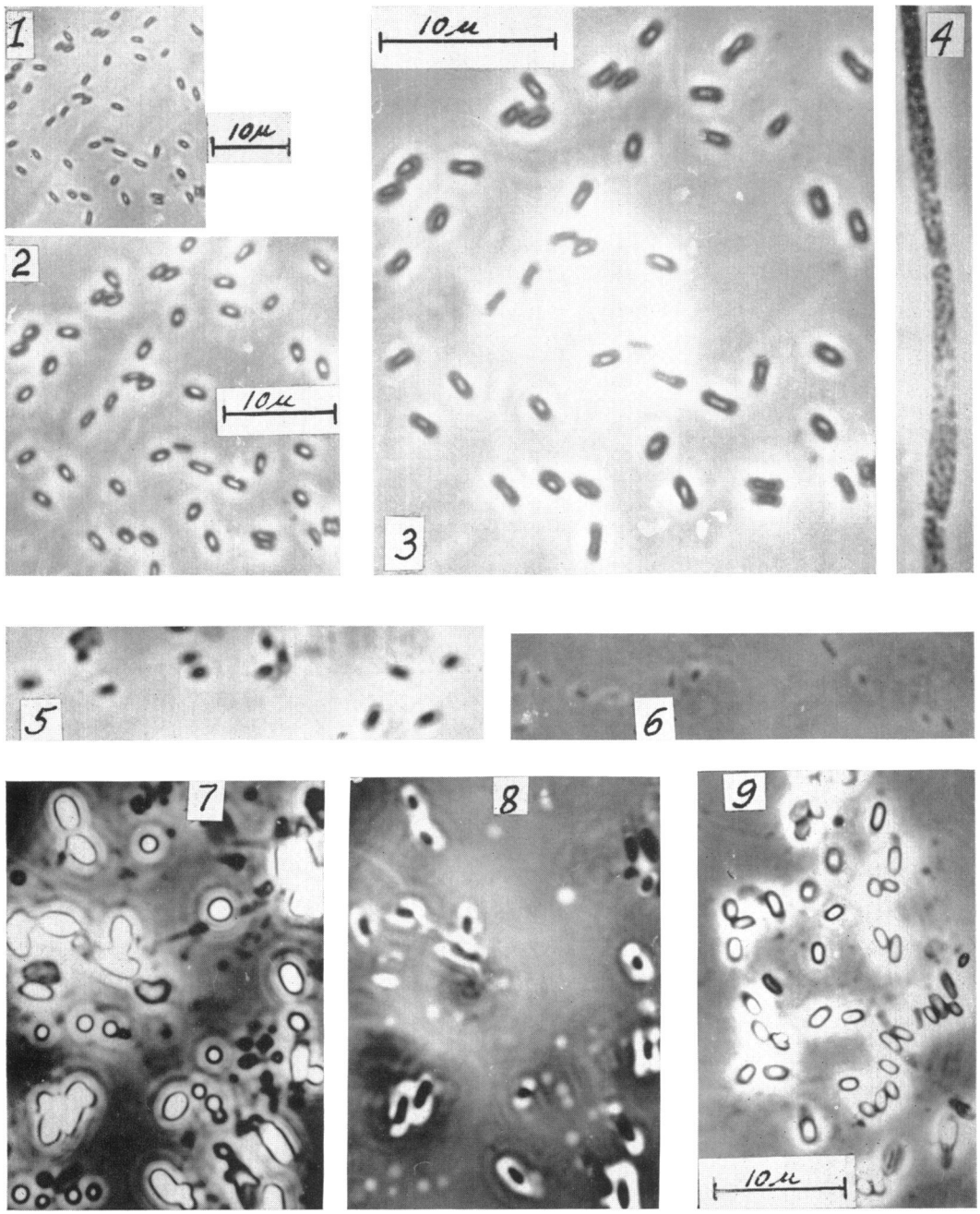

FIG. 1–9. *All figures represent Bacillus cereus C_3. Fig. 3 to 8 have the same scale of magnification.*

FIG. 1–3. *Spodogram of a 2-day-old microculture of spores developed on a collodion film at 30 C and incinerated at 525 C for 30 min. All three figures represent the same part of the microcolony mounted in air, and photographed in Fig. 1 with a Zeiss Neofluar 40/0.75 dry objective; Fig. 2, Neofluar 63/0.90 dry objective; Fig. 3, Neofluar 100/1.30 oil-immersion objective. All three negatives were printed at the same enlargement.*

FIG. 4. *Spodogram of vegetative cells in an actively growing microculture, mounted in air and observed with an oil-immersion objective.*

FIG. 5 and 6. *Spore spodograms. Figure 5, mounted in water; Fig. 6, mounted in mineral immersion-oil.*

FIG. 7–9. *Spores developed in microcultures on collodion films. Figure 7, mounted in air; Fig. 8. mounted in mineral immersion-oil; Fig. 9, mounted in water.*

served, and it seemed unlikely that the residue of the central part of the spore would behave differently and would continue to exhibit the peculiar optical property of the intact spore, namely, that in spite of its high refringence it would appear bright when observed in dark contrast. The fact that the core remained bright in charring experiments was attributed to a low organic as well as mineral content. The low resolution and magnification available to me at the time did not allow further experimentation with the optical properties of the spore spodogram.

In subsequent experiments aimed at identification of some of the mineral components of the spore spodogram, I noted that the spodogram was not readily soluble in water, and that, when mounted in water, the central part, or core, appeared dark (Fig. 5). This indicated, by the same token as above, that it had a higher refractive index than water; indeed, slightly higher than oil (Fig. 6). Accordingly, the bright central part, or core, of the spore spodogram could not represent an empty space or a region poor in minerals, but instead, a region of the spore rich in minerals. This conclusion contradicts the one drawn from the early work referred to above, a contradiction obviously due to misinterpretation of the microscopic picture in terms of refringence. I originally assumed that a body more refractive than its surround would always appear similarly, usually dark, when observed in dark contrast with the phase microscope, and, in contrast, that a body less refractive than its surround would always appear similarly, usually bright, when similarly viewed. The present case and other observations made on spores and vegetative cells show that the appearance of a body more refractive than its surround, observed with the phase microscope, depends not only on whether its refractive index is higher or lower than that of its surround, but also on its thickness and on the magnitude of the difference between its refractive index and that of the surround. The greater that difference is, the greater the tendency of the body is to appear bright; the smaller the difference is, the greater the tendency of the body is to appear dark when viewed in dark contrast. Thus, the core of the spore spodogram appears bright when mounted in air and dark in water or in oil. The spore itself appears bright when mounted in air and in water, but dark in oil (Fig. 7 to 9). The mechanism of this phenomenon is not clear. It may be due to an effect of the mounting medium on the optical path of light or on the proportion of light that undergoes diffraction and reflection.

In recent years the electron microscope has been adapted to spodography, which should greatly enhance the value of spodography and of electron microscopy to cytology, as shown by the work of Thomas (1964). With respect to distribution of mineral matter in the spore, Thomas noted that, in shadow preparations, the core of the spore spodogram cast a longer shadow than the peripheral zone, showing that mineral matter is more concentrated in the core than at the periphery. This is in agreement with my emended conclusion stated above.

Until it becomes possible to identify at least some of the mineral elements in the spodograms, it will not be possible to determine the distribution of calcium dipicolinate in the spores. However, observation of spore spodograms at different stages of germination indicates that the core becomes gradually smaller while the surrounding zone becomes darker, suggesting that the bulk of the dipicolinate is in the core. This suggestion is also supported by the relatively considerable amount of mineral matter in the core, which alone could account for the high calcium content of the spore (Tinelli, 1955). It is not unlikely, however, that calcium is present in both the core and the peripheral zone.

I do not share the pessimism of some workers about the prospects of identifying at least certain minerals in the spodograms with either the light or the electron microscope (*see* Scott, 1943).

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