DETERMINATION, BY SPODOGRAPHY, OF THE INTRACELLULAR DISTRIBUTION OF MINERAL MATTER THROUGHOUT THE LIFE HISTORY OF *BACILLUS CEREUS*

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ABSTRACT

KNAYSI, GEORGES (Cornell University, Ithaca, N. Y.). Determination, by spodography, of the intracellular distribution of mineral matter throughout the life history of *Bacillus cereus*. J. Bacteriol. **82**:556–563. 1961.—The intracellular distribution of mineral matter throughout the life history of *Bacillus cereus* strain C_3 was investigated by microincineration for which the term spodography is suggested. The organism was grown in microcultures on collodion membranes supported by agar media. At various stages of development, microcultures were floated on distilled water, picked onto cover glasses, air-dried, and incinerated at 500 to 525 C. The mineral residue is deposited in situ and shows the distribution of mineral matter in the cells. Study of the spodograms thus obtained shows that in the spore mineral matter is concentrated in a peripheral layer surrounding a minerally poor core. As soon as the spore begins to germinate, often before one notes any change in its optical properties, the minerally rich layer increases in thickness while the core becomes gradually smaller and finally disappears. The germ cell appears nearly homogeneous, with evidence of mineral aggregation into discrete granules more readily seen in subsequent generations. The nuclei of the vegetative cells are sites of mineral accumulation. In the compound nuclei, minerals seem to be concentrated in a superficial ring around a minerally poor center. In the young forespore, mineral matter is homogeneously distributed. As the spore stage is approached, however, the mineral matter tends to occupy a peripheral position as in the completed spore. The results indicate that the calcium dipicolinate of the spore is principally in the minerally rich, peripheral layer. The relation of this layer to the other peripheral structures of the spore has not been determined. Observation was made with a phase microscope in dark contrast and using a dry objective.

The importance of the roles played by minerals in the vegetative as well as the reproductive phases of bacterial development is now generally recognized. It suffices to mention the role of magnesium in cell division (Webb, 1951), of various mineral cations in capsule (Hoogerheide, 1940) and spore (Grelet, 1952, *a*, *b*) formation, and of calcium in spore resistance (Curran, Brunstetter, and Myers, 1943; Grelet, 1952*b*). Hence, the desirability of studying the intracellular distribution of mineral matter throughout the complete development of an organism has developed.

FIG. 1. *A. (60-H, 11). Microculture on collodion, prepared as in text. Age: 2 days at 30 C + 15 days at 3 C; shows a high degree of sporulation. B. (60-H, 24). Spodogram of A.*

FIG. 2. *A. (60-C, 22). Spodogram of a microculture similar to that of Fig. 1, incinerated at 500 C for 30 min. B. (60-C, 32). Spodogram of the same spores as in A after further incineration at 600 C for 30 min.*

FIG. 3. *A. (60-H, 32). Microculture similar to that of Fig. 1, after incubation for 5 min at 35 C on broth containing 0.3% beef extract, 0.5% glucose, and 0.5% tryptone. Only two spores show darkening. B. (61-A, 6). Spodogram of A. Note the reduction in size, or absence, and darkening of the core even before any noticeable change in the appearance of the spores.*

FIG. 4. *A. (61-B, 10). Microculture similar to that of Fig. 1, after incubation for 1 hr at 35 C on the broth described under Fig. 3A. To reduce shrinkage, the preparation was exposed to water vapors for 10 min before fixation with formol vapor. B. (61-B, 18). Spodogram of A. Note richness of the germ cells in mineral matter and the formation of mineral aggregates.*

Note: The numbers and letters in parentheses after the numbers and letters indicating the figures identify the corresponding negatives in the author's library. Figures 1, 3, and 4 have the same scale of magnification.

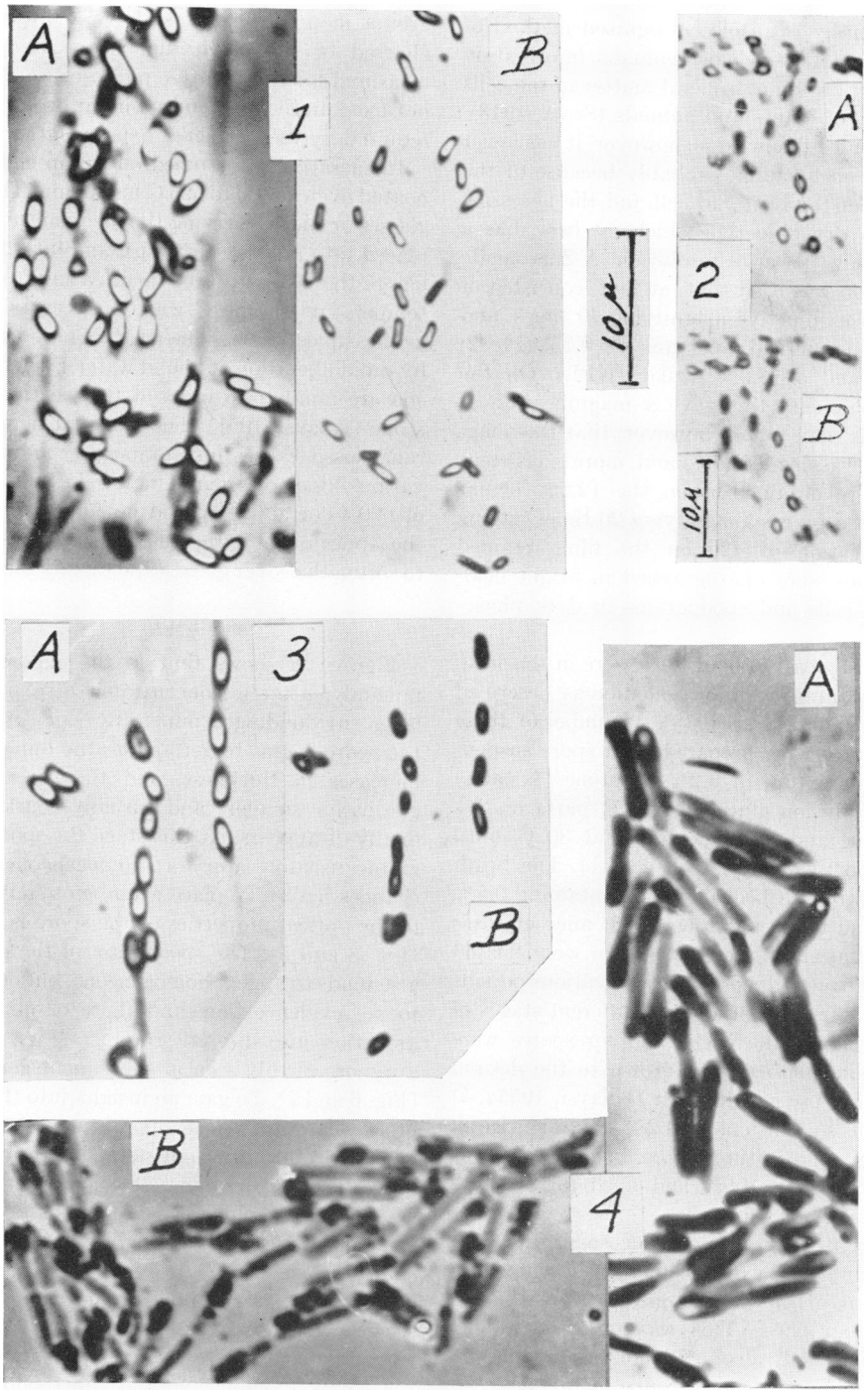

FIG. 1–4

MATERIALS AND METHODS

Strain C_3 of *Bacillus cereus*, which had previously been employed in several cytological studies, was selected to be investigated by a microincineration method. It is suggested that the name *spodography* (from the Greek *spodos*, meaning ashes) be applied to this technique. The term spodogram has been occasionally used by French and German authors.

Spodography is an old, recognized method of cytology, and its use yielded valuable information on the distribution of mineral matter in the cells and tissues of plants and animals (Scott, 1943). To the author's knowledge, however, it has never been used on bacteria, probably because of the small size of the bacterial cell and the necessity of using a dry objective which, at best, has a relatively low power of resolution. A Zeiss-made combination was used that, at first, consisted of a dry, phase-objective magnifying 40 times, and having a numerical aperture of 0.75 (Ph 2; 40/0.75), and a 12.5× ocular (KPL). On the photographic film this gave a magnification of 260. It was soon found, however, that this magnification was inadequate, and more consistent results were obtained when the 12.5× ocular was replaced by one magnifying 20 times, giving a magnification of 420 on the film. Stained preparations were photographed in bright field, unstained cells and spodograms in dark phase-contrast.

All investigated preparations were in the form of microcultures grown on collodion as described by Hillier and Knaysi (1948). A number of these microcultures were prepared from spore suspensions in a mixture of 0.2% tryptone (4 parts) and beef infusion glucose broth (1 part) on 2% Difco agar, and were incubated at 30 C until sporulation was complete (Fig. 1*A*). The broth was half-strength beef infusion containing 0.5% glucose and 0.5% tryptone. Such microcultures were used as spore preparations, or were floated on meat extract, glucose broth for various periods (Knaysi, 1959) and studied at different stages of their development. Nuclei and forespores were observed in microcultures grown to the desired stage on glucose-acetate agar (Knaysi, 1955*a*, *b*) from spore suspensions in 0.2% tryptone. In all cases the microculture to be investigated was mapped (Knaysi, 1957) and fixed for about 2 min with formol vapors.

Microcultures grown on glucose-acetate agar were mounted in a film of methylene blue solution, about pH 5, and cell groups were selected and photographed. This was followed by decolorization with alcohol, incineration, and rephotographing the same groups mounted in the air. Most other microcultures were mounted in water after fixation with formol vapors and selected groups photographed. This was followed by incineration and rephotographing of the same groups mounted in air as above. Cultures to be charred or on which dimensions were to be measured for comparative purposes were usually not fixed and were mounted in air or in water as required by the particular experiment.

Incineration was carried out in an electrically heated oven at 500 to 525 C for 30 min. The cover glasses or slides, carrying the microcultures, were placed into a pyrex petri dish and into the oven before the heat is turned on. Heating from room temperature to 150 C was done very slowly to avoid explosive phenomena that may be caused by rapid liberation of bound water. Such phenomena are familiar to users of the electron microscope (Knaysi, 1951). The maximal temperature range used causes instantaneous combustion of various kinds of organic material. Temperatures of 600 C or above caused no further change in the appearance of the cells (Fig. 2) but tended to soften the cover glasses and distort their shape.

RESULTS

Figure 1*B* shows that in the endospore the mineral matter is concentrated in a peripheral layer surrounding a minerally poor core. When the spore begins to germinate, the mineral layer increases in thickness, and the core becomes gradually smaller and usually darker, and finally disappears. At this stage the spore spodogram usually appears homogeneous. These changes may take place even before any change in the optical properties of the spore is detected (Fig. 3 and 5). The spodogram of the germ cell also tends to appear homogeneous, but one begins to see evidence here and there of mineral aggregation into discrete granules (Fig. 4) which are more readily seen in subsequent generations (Fig. 6 and 7). To gain an insight into the nature of these granules, the organism was grown on glucose-acetate agar and stained with methylene blue. The spodograms of such microcultures show that the mineral aggregations occupy the same positions as the stained granules previously shown (Knaysi, 1955*a*) to be nuclei. In the large granules (compound nuclei), the mineral matter seems to occupy a superficial layer, which corresponds to the position of the primary nuclei in the compound nucleus, and the spodograms of these granules consist of mineral rings surrounding minerally poor circles (Fig. 8). In the young forespore, mineral matter is homogeneously distributed, but as the spore stage is approached,

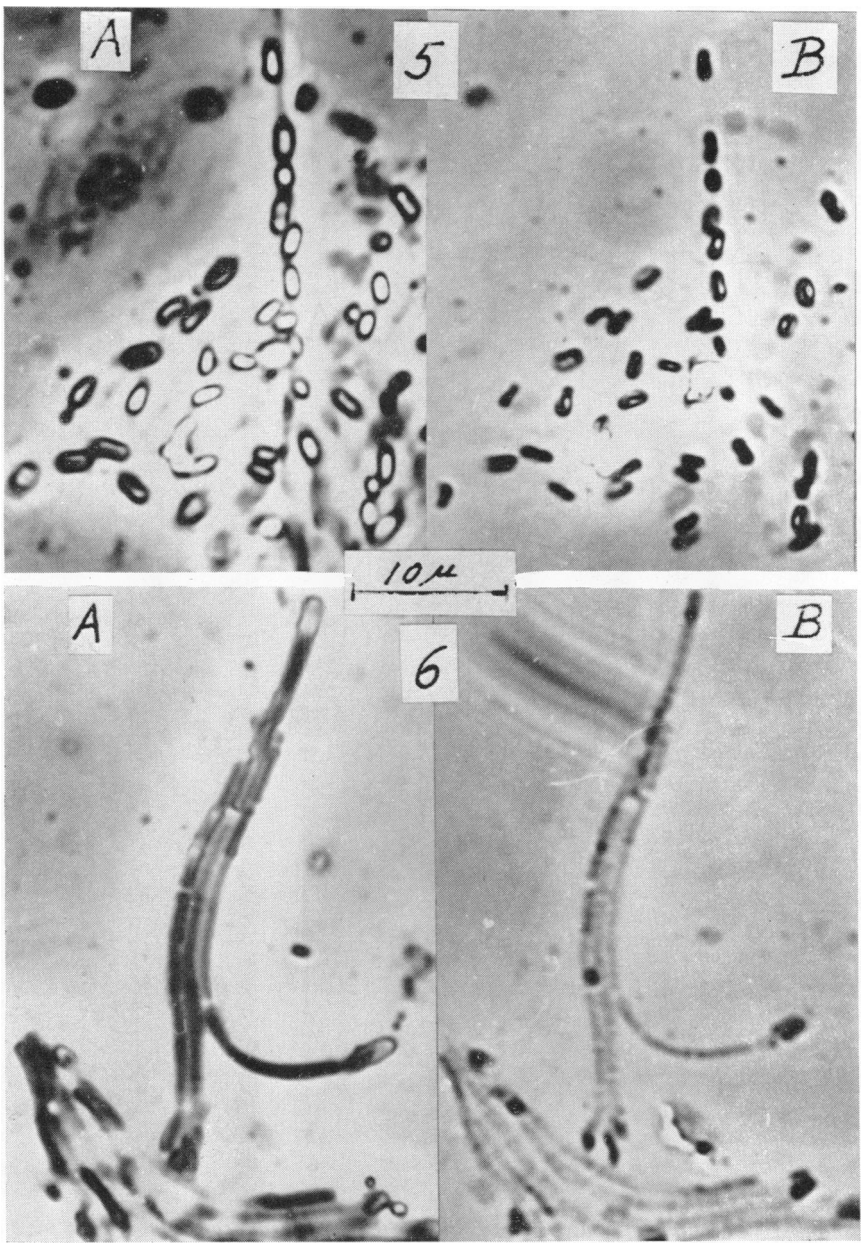

FIG. 5. *A. (61-B, 12). Microculture similar to that of Fig. 1, after incubation for 5 min at 35 C on the broth described under 3A. Fixed 2 min with formol vapors. B (61-B, 21). Spodogram of A. Note that in several cases the core is reduced in size but remains sharply separated from the minerally rich area that surrounds it. Note also asymmetric shrinkage of the spore content, apparently related to stage of germination.*

FIG. 6. *A. (61-A, 16). Microculture similar to that of Fig. 1, after incubation for 2 hr at 35 C on the broth described under 3A. B. (61-A, 36). Spodogram of A. Note 2 to 4 mineral aggregates in each cell.*

Note: The numbers and letters in parentheses after the numbers and letters indicating the figures identify the corresponding negatives in the author's library. Figures 5 and 6 have the same scale of magnification.

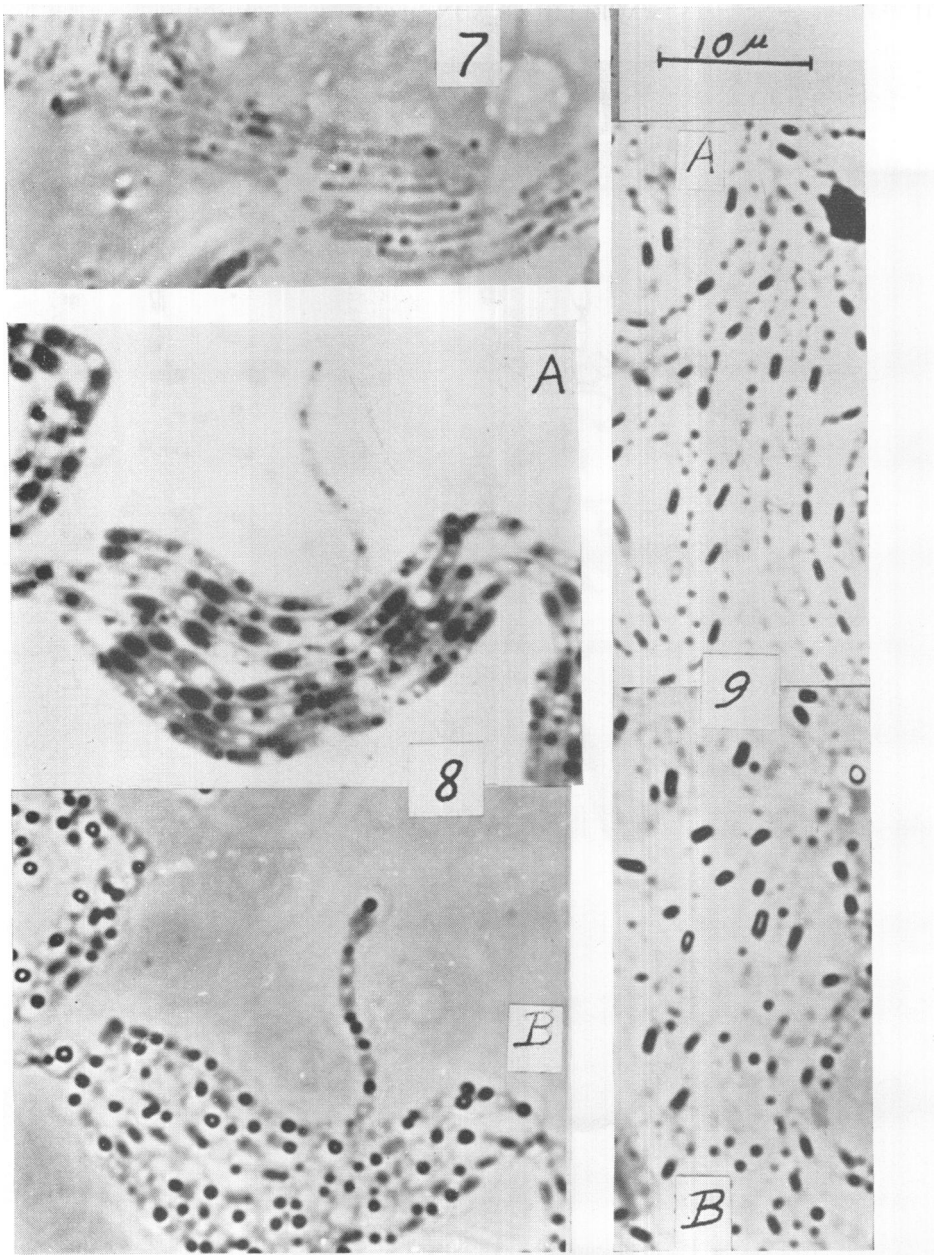

FIG. 7. (*61-B, 5*). *Spodogram of a microculture similar to that of Fig. 1, after incubation for 2 hr at 35 C on the broth described under 3A. Here growth has gone further than in the microculture of Fig. 5, and the mineral aggregates are larger and more clearly visible.*

FIG. 8. *A.* (*61-C, 15*). *Microculture on collodion, supported by an agar medium containing 2% agar, 0.2% glucose, and 0.2% sodium acetate. Age: 16 hr + 30 min at 30 C. Fixed for 2 min with gaseous formaldehyde and mounted in 0.1% methylene blue solution of pH 5. Note the numerous nuclei and forespores. B.* (*61-C, 26*). *Spodogram of A. Note that the spodograms of the forespores show uniform distribution of mineral matter, and those of compound nuclei concentration in a peripheral ring.*

FIG. 9. *A.* (*61-B, 24*). *Microculture similar to that of Fig. 8. Age: 23 hr + 30 min at 30 C. Fixed and mounted as in Fig. 8A. Note the numerous forespores and nuclei. B.* (*61-B, 35*). *Spodogram of A. Note that the spodograms of most forespores show uniform distribution of mineral matter. However, at this cultural age two show a distribution similar to that of the completed spore.*

Note: The numbers and letters in parentheses after those which indicate the figures identify the corresponding negatives in the author's library. Figures 7 to 9 have the same scale of magnification.

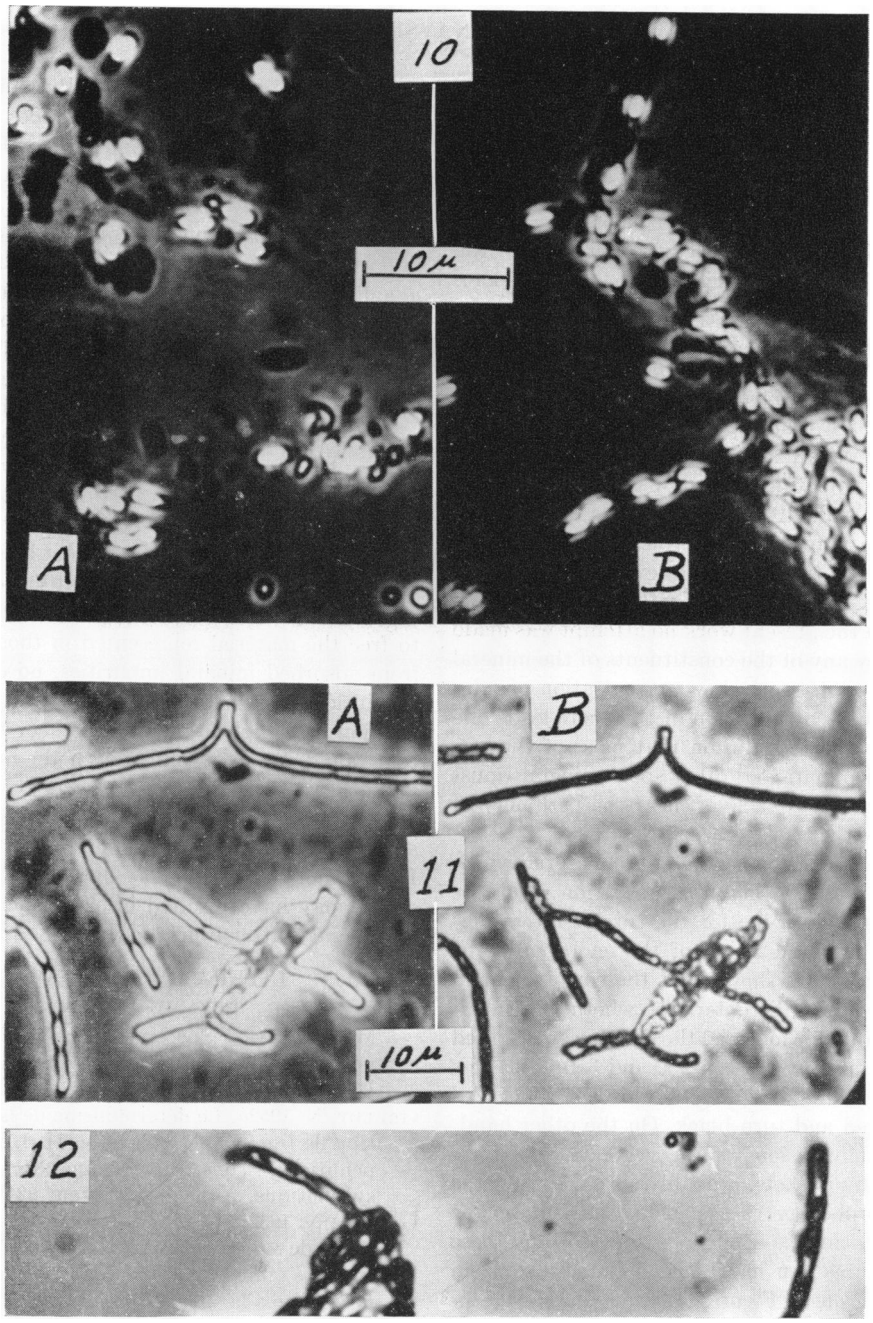

FIG. 10. *A*, (*61-G, 25*). *Microculture similar to that of Fig. 1. Age: 3 days at 30 C + 14 days at 3 C. Untreated. B.* (*61-G, 17*). *Microculture similar to that of Fig. 1. Age 2 days at 30 C + 56 days at 3 C. Heated, dry, to 300 C. Both A and B observed in dark phase-contrast mounted in air. There is no noticeable difference between A and B in the optical properties of the spores.*

FIG. 11. *A.* (*61-H, 27*). *Microculture on collodion supported by beef infusion, glucose agar. Age: 6 hr at 30 C. Floated on water, then air-dried. Observed mounted in air. B.* (*61-H, 32*). *The same cells as those of A after exposure dry, to 300 C. Note the evidence of charring of the vegetative cells.*

FIG. 12. (*61-H, 26*). *Microculture similar to that of Fig. 11 and similarly treated. Note the charred cells. The intracellular bodies in some of the cells may be nuclei.*

Note: The numbers and letters in parentheses after the numbers and letters which indicate the figures serve to identify the corresponding negatives in the author's library. Figures 10 and 12 have the same scale of magnification.

it tends to occupy the peripheral position it occupies in the completed spore (*see* Fig. 8 and 9).

DISCUSSION

The results of the present work are highly illuminating, particularly with respect to the organization of the nucleus and the endospore. It now seems clear that the bacterial nucleus, like the nucleus of higher plants and animals (*see* Scott, 1943), is a locus of mineral concentration. It has also been recently shown (Powell, 1957; Tinelli, 1955) that the endospore contains 12 to 15% of calcium dipicolinate which is excreted during germination; it is now generally believed (Curran, et al., 1943; Grelet, 1952*a*, *b*) that this salt plays a role in the optical properties and resistance of the spore. However, no one seems to be sure of its location within the spore. Although in the present work no attempt was made to identify any of the constituents of the mineral layer, the apparently low concentration of minerals in the central region of the spore leads one to the obvious conclusion that most of the calcium dipicolinate and other spore minerals must be localized in that layer, near the periphery of the spore.

Knowledge of the nature of the material which occupied the core before incineration would be of considerable value for the correct interpretation of the organization of the endospore. The spore spodogram shows that the core is poor in mineral matter. To determine whether or not it is rich in organic material the spores were exposed to temperatures between 250 and 350 C. In this range, organic substances that are not volatilized are charred and turn black. On the other hand, the lipoid inclusions of the vegetative cells are replaced by pockets, probably air pockets, of the same morphology as the inclusions, but of low refractive index. Spores subjected to these temperatures can hardly be distinguished from untreated ones. Figures 10 and 11 show, respectively, how spores and vegetative cells appear before and after exposure to 300 C. Even the cytoplasm of the vegetative cell appears to be more deeply charred than the core of the spore. Therefore, the content of the core must be either poor in both mineral and organic matter, or much of its organic content is of the type that is volatilized by the charring temperatures. In Fig. 12, some of the vegetative cells show internal structures that may represent nuclei.

The question comes to mind: In which structure or structures of the spore does mineral matter accumulate? Measurement made on the microphotographs of 13 dry spores and their spodograms at 10^4 magnification gave, for the bright, central parts of the spores, when observed in dark phase-contrast, an average width of 1.44 μ, and, for the over-all width of the spodograms, an average of 1.40 μ. Similar measurements made on 18 spores that were heated to 100 C and rapidly cooled to room temperature just before photographing, to reduce the errors of differences in the degree of shrinkage, gave, respectively, 1.59 and 1.47 μ. This means that the spodograms are confined to the bright, central part of the spore and do not include the outer coat. The inner coat may or may not be included. A glance at Fig. 4 to 6 suggests that this coat may be minerally rich. Since no precaution was taken to free the material represented in those figures from adsorbed mineral impurities, no definitive conclusion can be drawn. However, demonstration of a high mineral content of the inner coat would not be in conflict with the report of a low ash content of the spore coat by Yoshida et al. (1957), since the value reported by these authors applies to both the inner and outer coats. Furthermore, mechanical disruption of the spores may have resulted in a considerable loss of mineral matter from the coats.

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