Cytology of Spore Germination in
Clostridium pectinovorum[1]

JUDITH F. M. HOENIGER AND C. L. HEADLEY

Department of Microbiology, School of Hygiene, University of Toronto, Toronto, Ontario, Canada

The process of spore germination in *Clostridium pectinovorum* has been followed by phase-contrast and electron microscopy. Unlike most other *Bacillaceae*, germination of this species takes place within the sporangium. Under phase-contrast, the spore darkens and swells slightly, and then the vegetative rod slips out through the end opposite the collar-like extension of the sporangium. In thin sections, a spore from an early stage in germination consists of a central protoplast, core membrane, germ cell wall, cortex, and two coats. Within a short period, the cortex disintegrates and the young cell develops. It possesses a large fibrillar nucleoplasm and several mesosomes. Subsequently, the young cell elongates, becomes somewhat deformed, and then emerges through a narrow aperture in the inflexible coats of the spore, finally rupturing the sporangium. Free vegetative cells of *C. pectinovorum* resemble in their structure other gram-positive rods.

The process of germination of bacterial spores has fascinated microbiologists ever since Cohn first described it in *Bacillus subtilis* in 1877 (2). Germination is a complicated sequence of events, now believed to occur in several stages. These stages have been called by Murrell (13) "initiation and outgrowth" and by Keynan and Halvorson (7) "activation, germination (proper), and outgrowth." We prefer the latter terminology. Although many recent investigations have tended towards the physiological and biochemical aspects of the process (13, 24), much interest continues to be shown in its cytological sequence. Most of the structural studies have been carried out with *Bacillus* species (1, 6, 8, 9, 11, 12, 22). However, there have been a few investigations of the ultrastructure of germination in *Clostridium* spores (17, 25, 26). These studies did not have the advantage of currently available fixation techniques or newer electron microscopes of high resolving power. Therefore, we have investigated by more refined techniques the germination of *C. pectinovorum*, the sequence of spore formation of which has been illustrated so well by Robinow (21) and Fitz-James (3).

MATERIALS AND METHODS

The observations reported in this study were made on a strain of *C. pectinovorum* obtained from C. F.

Robinow, University of Western Ontario, London, Canada, who first isolated the organism from the soil covering the roots of a potted azalea and from a sample of peat moss.

To obtain a culture of mature spores, the organism was grown at 37 C for 48 to 72 hr in Fitz-James' (3) liquid medium (YACG broth, pH 7.8) which contains yeast extract (0.5%), sodium acetate (0.5%), and glucose (2%). In one experiment, the organism was grown for a period of 6 to 8 days to yield pancake-shaped colonies between two layers of the same medium solidified with 2% agar.

Giemsa staining was used to outline the sporangium within the clostridial cell. Following the suggestion of C. F. Robinow, pancake-shaped colonies, grown as indicated above, were excised from between the two layers of agar and placed in a test tube; they were fixed in acetic-alcohol (one part glacial acetic acid to three parts absolute alcohol), rinsed with 70% alcohol and distilled water, and then hydrolyzed in 1 N HCl at 60 C for 12 min. The cellular deposit was spread on grease-free cover glasses, allowed to dry, stained with Giemsa for 1 to 1.5 hr at 37 C, and mounted in dilute Giemsa (19, 20). The preparations were photographed with a Zeiss Universal microscope using the same optics, illumination, and filters as described previously (5).

For all other experiments involving phase-contrast and electron microscopy, refractile spores were centrifuged and the pellet was suspended in trypsin (1 mg/ml) made up in Sorensen's 0.066 M phosphate buffer (pH 7.8); the pellet was held overnight at 37 C to free the spores of debris. The cleaned spores were then washed three times in 0.85% NaCl.

Germination was effected by first heat-activating

the washed spores in physiological saline at 60 C for 1 hr, and then inoculating 1 ml (10^8 to 10^9 spores/ml) into 20 ml of YACG broth. Samples for cytological study were taken at 10- to 15-min intervals for 1.5 to 2 hr. The same process, but without heat-activation, was used for preparing samples of resting spores.

For phase-contrast microscopy, 0.5 ml of the cell suspension was mixed with 0.5 ml of 28% gelatin in YACG broth (10). Preparations were mounted and examined with Zeiss phase-contrast optics as previously described (5).

For electron microscopy, the cells were fixed with osmium tetroxide by the Ryter-Kellenberger (R-K) procedure (23), or with glutaraldehyde followed by osmium tetroxide in a modified version of Glauert and Thornley's method (4; M. J. Thornley, *personal communication*), as described previously (5). All samples were treated with uranyl acetate (0.5%), dehydrated in a graded series of acetones, and embedded in Vestopal W. Sections were cut on a Porter-Blum MT-2 ultramicrotome with a glass knife, picked up on Formvar-coated grids reinforced with carbon, and stained first with uranyl acetate (1%, made up in distilled water) for 5 min and then with Reynolds' (18) lead citrate solution (diluted 1:75) for 8 min. The preparations were examined with a Philips EM 200 electron microscope operating at 60 kv with double condenser illumination and an objective aperture of 40 μm.

RESULTS

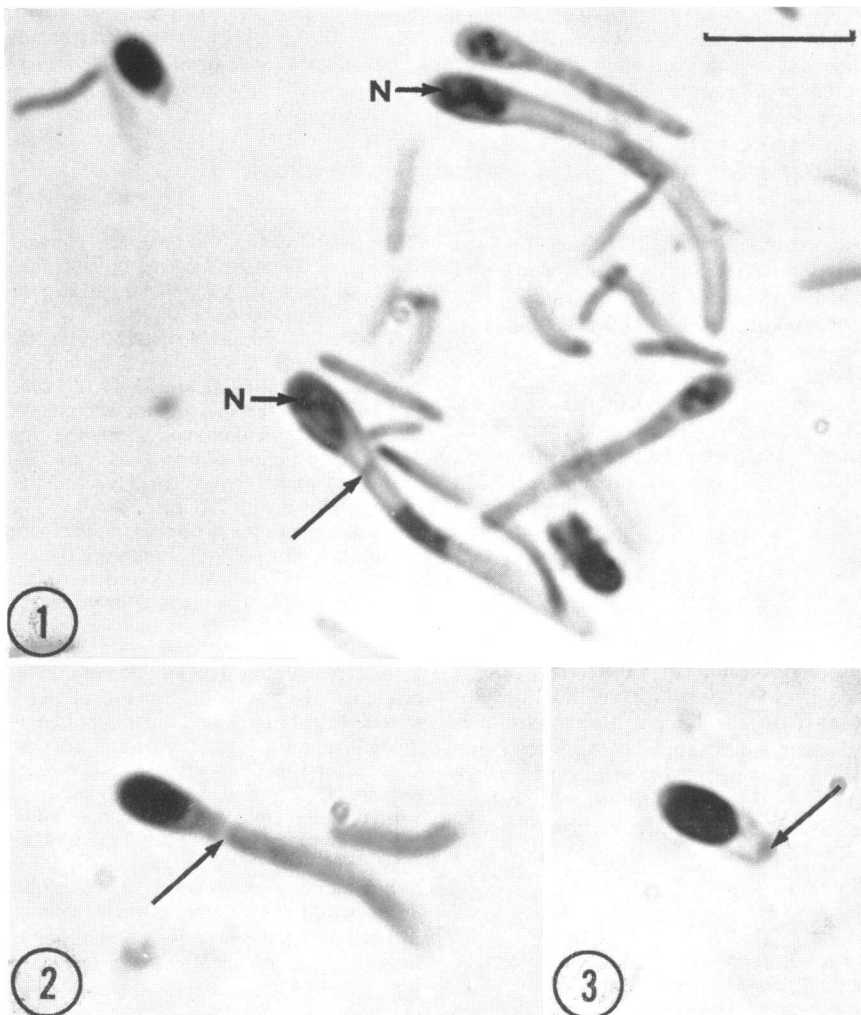Germination of the spore cultures of *C. pectinovorum*, like other species studied so far (7, 12), is

FIG. 1–3. *Ordinary light micrographs of sporangium of C. pectinovorum stained with Giemsa. Arrows indicate border where sporangium will break away (Fig. 1) or has broken away (Fig. 2 and 3) from the shaft of the clostridium. "N" points to the S-shaped nucleus in developing spores. Marker represents 5 μm.*

asynchronous. The events described below represent a germination sequence based on a logically consistent process of morphological changes and not necessarily on culture age.

Light microscopy. C. pectinovorum is an unusual organism in that during sporulation the sporangium proper is not the entire clostridial cell but only a terminal part thereof (21). This terminal portion is delimited from the rest of the vegetative cell or shaft by a collar or sleeve extending from the clostridial bulge. The delimitation of the sporangium is illustrated in the Giemsa-stained preparations of Fig. 1 to 3 where the arrows point to the lower end of the collarlike remains of the sporangium. Within the developing spore, the stained nuclear material (N) has an S-shaped configuration (Fig. 1) reminiscent of Fig. 20b in Robinow (21). In Fig. 2, the break between sporangium and vegetative shaft is evident. Figure 3 shows a free spore with its collar.

Figures 4 to 8 demonstrate the progress of germination in heat-activated spores, as observed with phase-contrast. The initially refractile spore darkens and enlarges slightly (Fig. 4). The vege-

tative rod begins to emerge from the darkened spore (Fig. 5) and, as it slips out (Fig. 6 and 7), leaves behind the empty coat, here seen at two different levels of focus. In Fig. 7, the collar or sleeve of the sporangium is clearly visible at the end opposite to that from which the rod is emerging. Figure 8 shows two cells in a slightly later stage of germination; after they have emerged completely, they swim away rapidly, as described previously by Robinow (21). It should be noted that Fig. 4 to 8 are from different preparations. We were unsuccessful in all attempts to observe germination continuously in a single field of spores. Therefore, we were unable then to estimate the rate of darkening or the degree of synchrony in germination of the heat-activated spores.

Electron microscopy. Thin sections of the resting spores (Fig. 9) are usually poorly impregnated by the fixing and embedding reagents. As a result, one can distinguish only with difficulty the following components. At the center lies the spore protoplast (14) or core (21), which is delimited by a thin core membrane. Beyond this layer lies the rather wide, electron-lucent cortex, surrounded
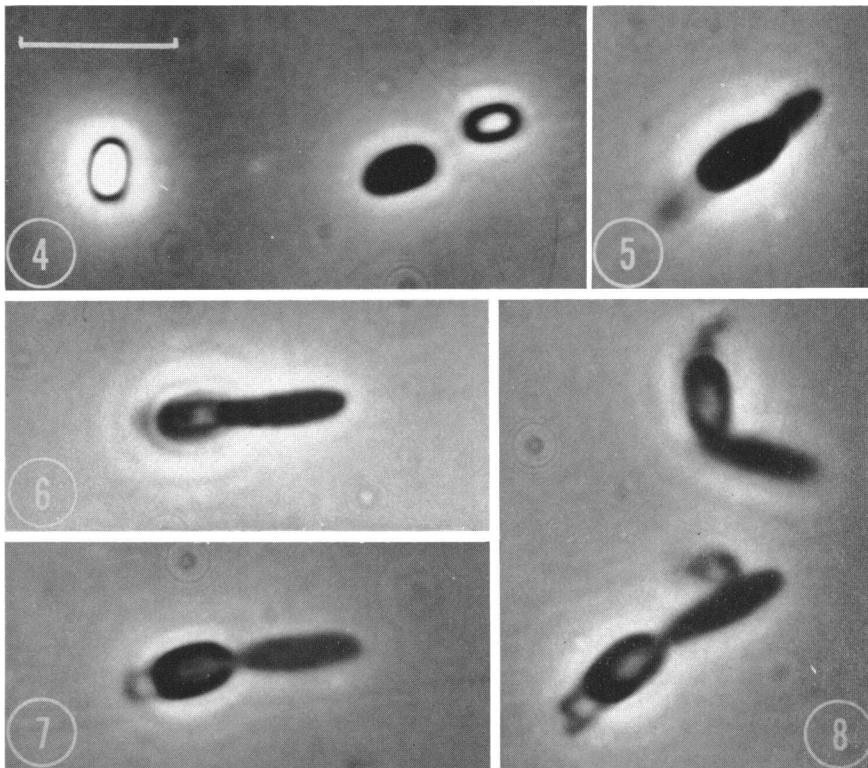

FIG. 4–8. *Series of phase-contrast photomicrographs of gelatin (14%) mounts of germinating spores showing loss of refractility and progressive emergence of vegetative cell. Marker represents 5 μm.*
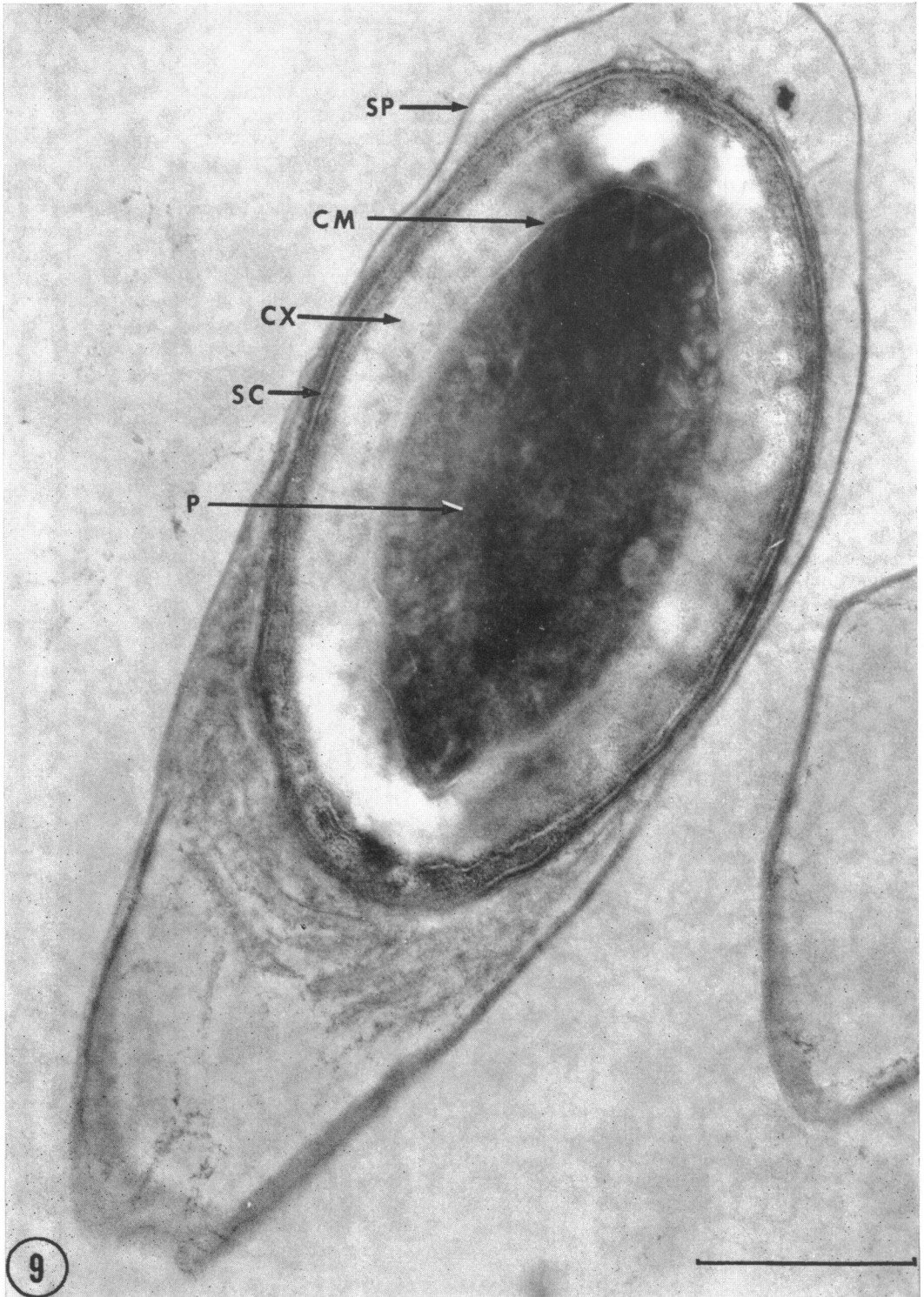
FIG. 9. *Electron micrograph of a longitudinal section of a resting spore (prior to heat activation) in which the protoplasmic core (P), core membrane (CM), cortex (CX), and spore coat (SC) are visible. The spore itself lies within the sporangium (SP). Marker represents 0.5 μm.*

1838

in turn by a many-layered spore coat. Enclosing all is the wall of the sporangium (i.e., the remains of the wall of the mother bacterium), which extends beyond the spore to form a collar. Part of the collar is filled with several layers of membranous material similar to those observed by Fitz-James (3).

An early stage in germination from a heat-activated sample is shown in Fig. 10. The components discerned in the resting spore can now be seen more clearly, as well as several others not readily observed before. These are the protoplast, core membrane, germ cell wall (14), cortex, inner coat, outer coat, and sporangium (its collar filled with many layers of membranes). Vesicular structures can be distinguished at the periphery of the protoplasmic core.

A conspicuous feature of germination in all spores is the breakdown of the cortex (11). In *C. pectinovorum*, this occurs in stages (Fig. 11); the outermost portion disintegrates first, leaving a clear space within the spore coat, and the innermost portion disintegrates later. Within 0.5 hr of the onset of germination, the young cell is well developed (Fig. 12 and 13). As shown in Fig. 12, it possesses a distinctly fibrillar nucleoplasm surrounded by a cytoplasm packed with ribosomes, a thin plasma membrane (which has a typical "unit" membrane structure, in contrast to the core membrane observed at earlier stages), and a relatively thick (3 pm) cell wall. In Fig. 13, lamellated mesosomes are present. As can be seen in both figures, the cortex has broken down almost completely, although the spore coats and sporangium are still intact.

Figures 14 and 15 show advanced stages of cortical disintegration. The young cells have elongated and have undergone some deformaticn in the process. In Fig. 15, lamellated mesosomes are present within the protoplast.

Within 1 hr after the commencement of germination, the vegetative rod has further elongated and ruptured the spore coat (Fig. 16). Subsequently, it also breaks through the sporangium (Fig. 17). The cells in Fig. 16 and 17 were prefixed with glutaraldehyde, a procedure which preserved an unusual, dense, ovoid structure visible in both micrographs.

Figure 18 illustrates a stage similar to that in Fig. 17, except that the preparation, like most others in this paper, had been made by the R-K method. The emerging vegetative cell has a fibrillar nucleoplasm with which a large vesicular mesosome is associated. A somewhat longer rod (3.0 by 0.6 μm) is emerging from the upper spore in Fig. 19. The lower cell in this micrograph was apparently cut tangentially. Note that in all the germinating cells of Fig. 16 to 19, the rod is compressed at the point where it escapes through a narrow opening in the inflexible spore coat.

Figure 20 shows a portion of a fusiform vegetative cell which has already emerged from the spore. This organism has a rather thin cell wall (25 nm), within which lies a typical tripartite plasma membrane enfolded at the lower left to form a simple intrusion. The most conspicuous features of the cell are the two fibrillar nuclear bodies and the large, vesiculo-tubular mesosome which lies near the lower of the nuclei. Mesosomes were observed in a number of vegetative cells of *C. pectinovorum*, usually close to the nucleoplasm but also lying free in the cytoplasm towards the ends of the cells or at the point of formation of the septum in dividing cells.

DISCUSSION

The main features of the germination process in *C. pectinovorum*, as observed by both phase-contrast and electron microscopy, can be summarized in terms of the three stages described by Keynan and Halvorson (7). During the first (activation) stage of the process, fine structural studies (Fig. 10) reveal a clear delineation in the several membranes surrounding the spore protoplast and the appearance of vesicular structures. The latter may be the vestiges of mesosomes formed during sporulation (3), or they may be newly differentiated structures.

During the second (true germination) stage, the layers surrounding the protoplast further lose the indistinctness which characterizes them during the resting stage. This is clearly viewed as loss of refractility, swelling, and degeneration of the cortex. It is generally agreed that cortical lysis involves an imbibition of water (11). Chemical studies initiated by Powell and Strange (16) revealed that dissolution of the cortex coincides with the release of spore mucopeptide, calcium ions, and dipicolinic acid. In *C. pectinovorum*, there is an unusual stratification of cortex degeneration; lysis proceeds from the outside inwards.

The third stage, outgrowth, commences with the differentiation of a new cell within the spore coat, and indeed is the longest process in the sequence of germination. That it is an actively synthetic period is shown by the abundance of ribosomes, the rearrangement and enlarging of the nucleoplasm, the development of mesosomes, and the thickening of the cell wall, which is derived from the germ cell wall. It is conceivable that hydrostatic pressures produced by materials of the disintegrating cortex account for the distortion of the elongating young cells. W. Van Iterson (*Handbook of Molecular Cytology, in press*) has reached a similar conclusion regarding the cause of deformation in young ger-
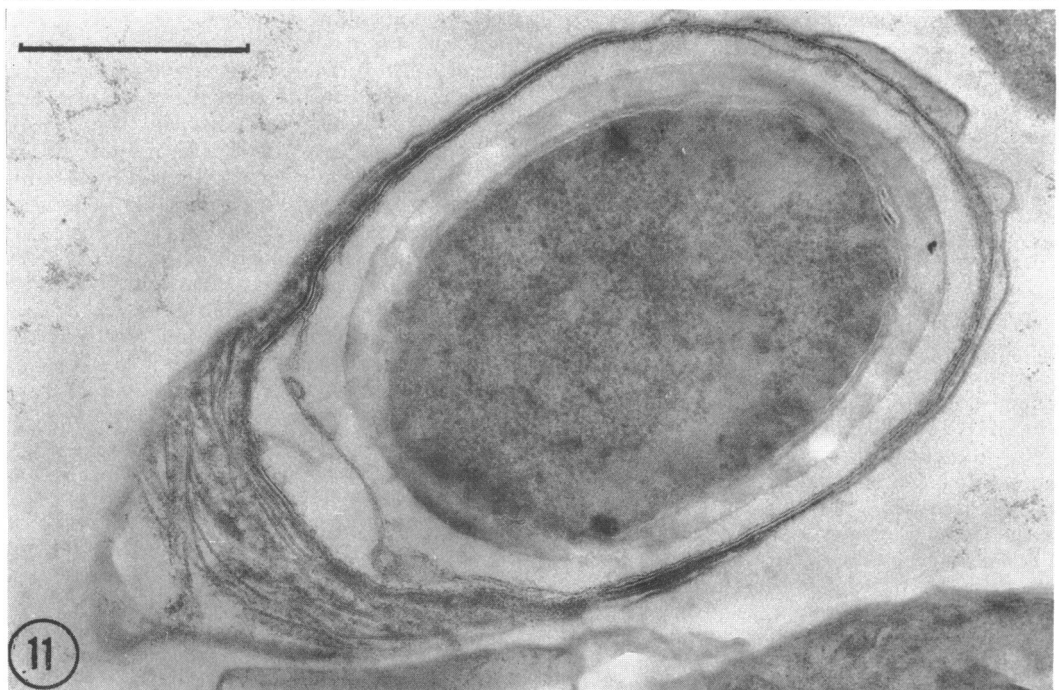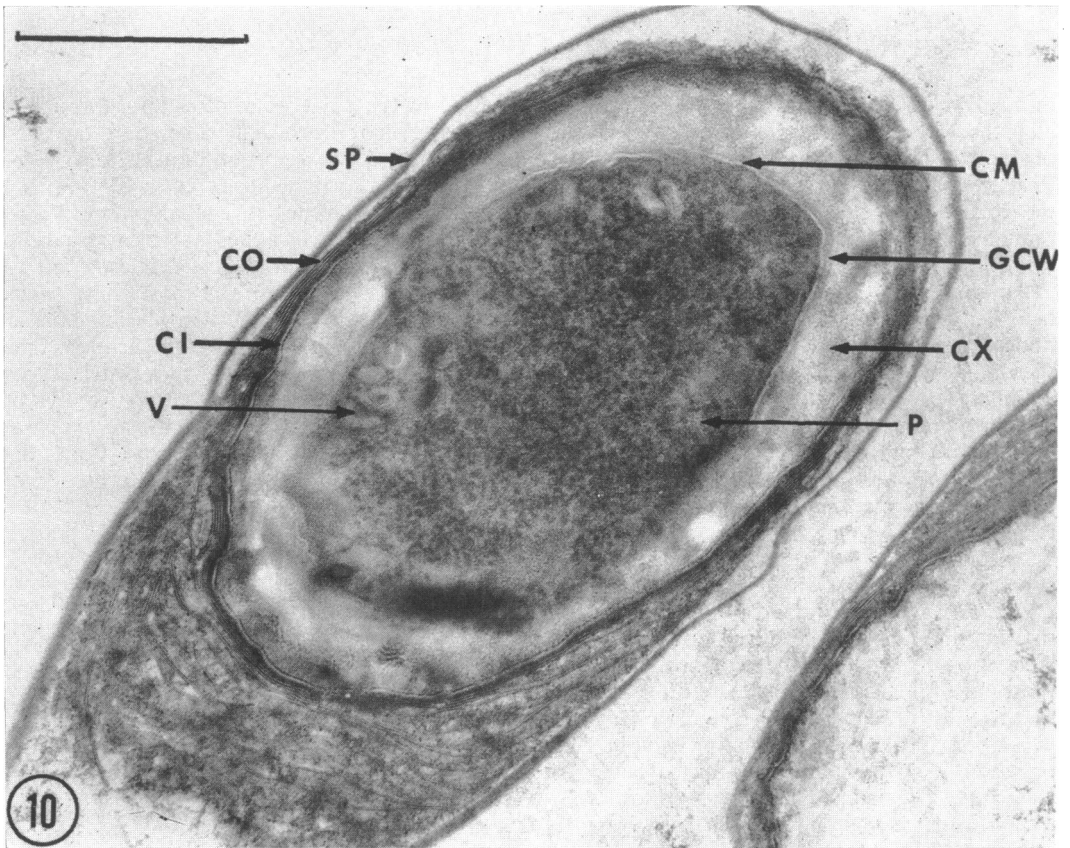
FIG. 10. *Section of spore in an early stage of germinative transformation showing protoplast (P), core membrane (CM), germ cell wall (GCW), cortex (CX), inner coat (CI), outer coat (CO), and vesicular structures (V); the entire spore is enclosed by the sporangium (SP), the terminal collar of which is filled with layers of membranous material. R-K fixation. Marker represents 0.5 μm.*

FIG. 11. *Germinating spore in which the outermost portion of the cortex has already dissolved. R-K fixation. Marker represents 0.5 μm.*
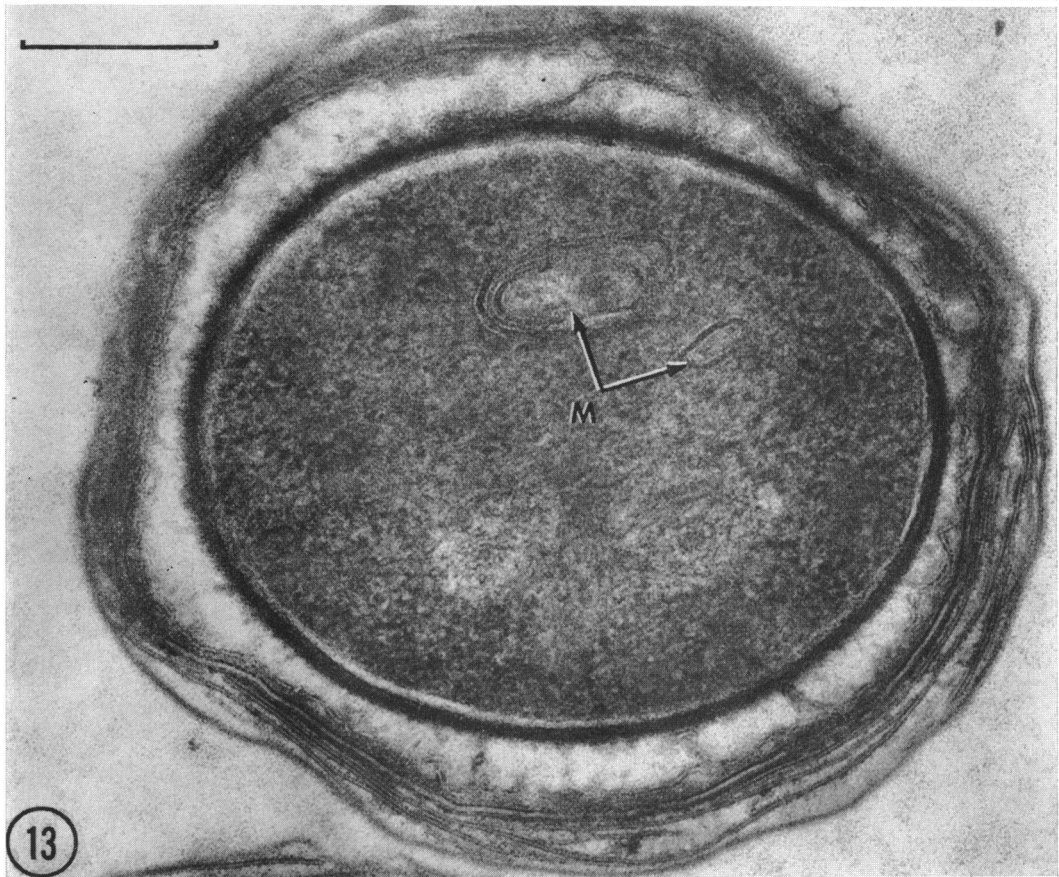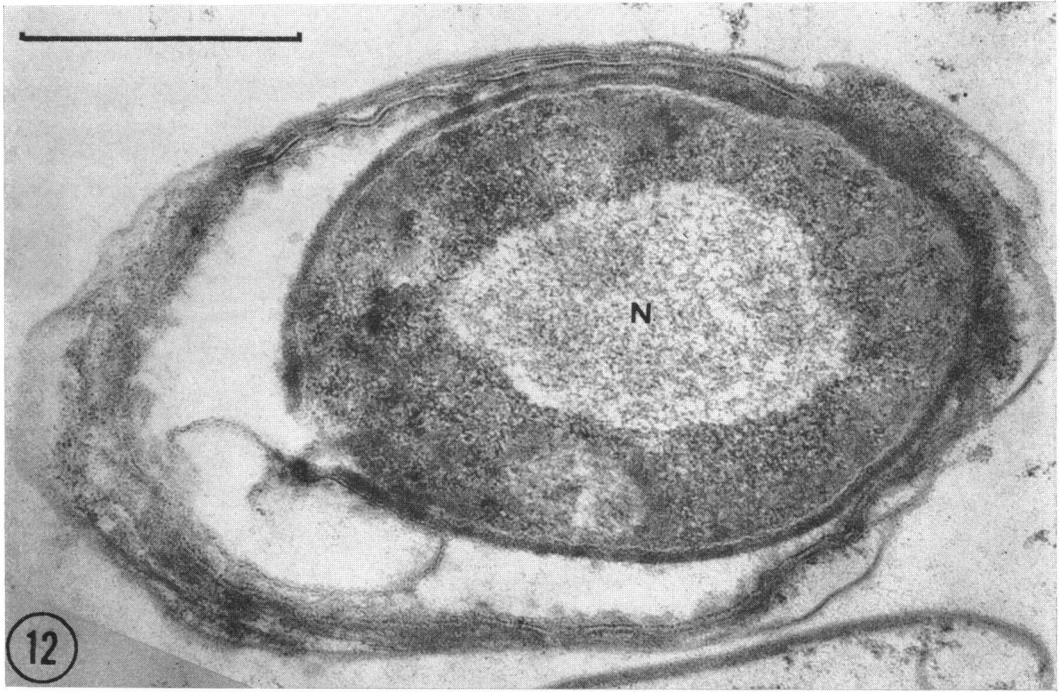
FIG. 12 and 13. *Young, developing cells after 0.5 hr of germination. The cell in Fig. 12 contains a large fibrillar nucleoplasm (N); that in Fig. 13, lamellated mesosomes (M). R-K fixation. The marker in Fig. 12 represents 0.5 μm; that in Fig. 13, 0.25 μm.*
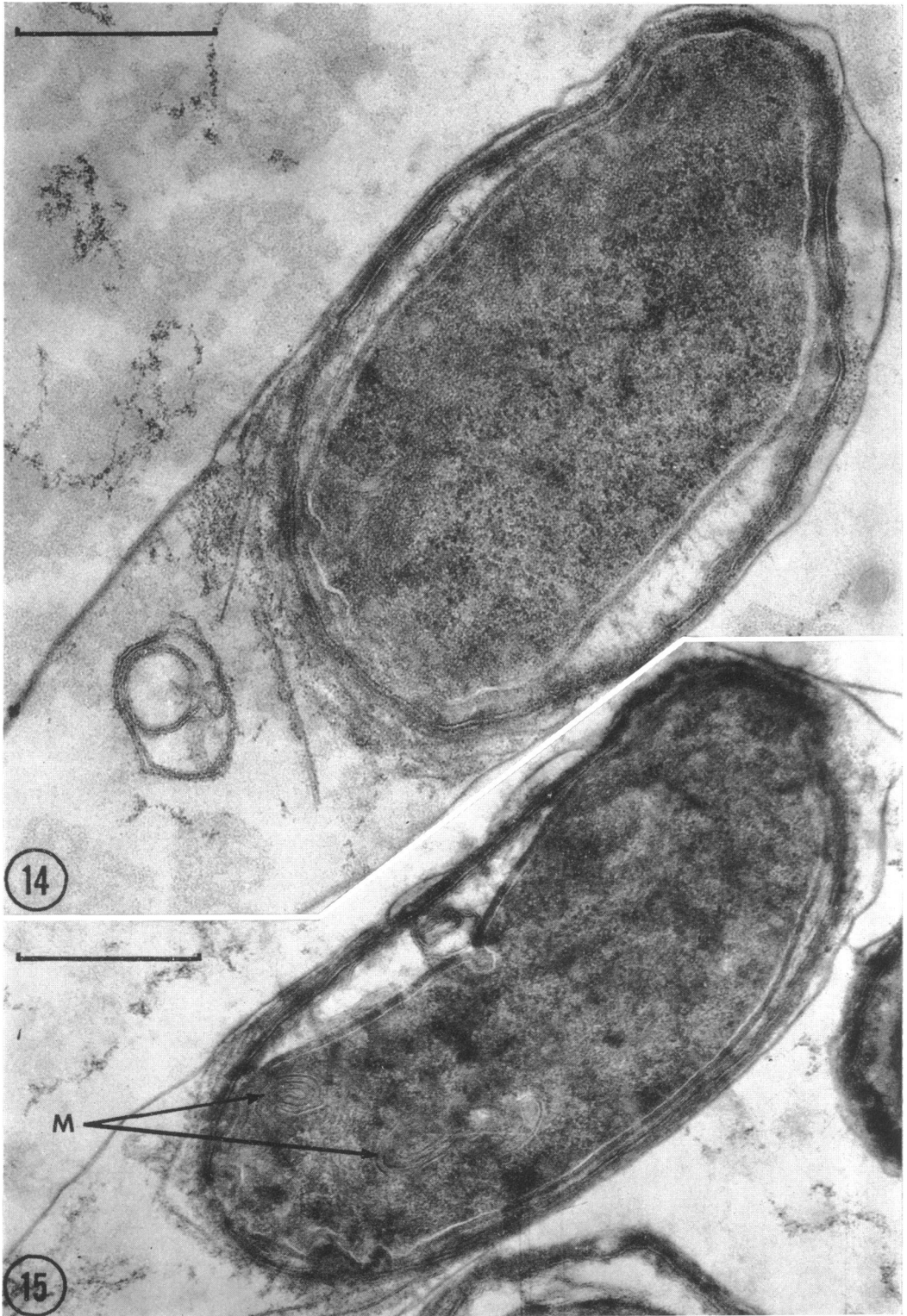
FIG. 14 and 15. *Deformed vegetative cells developing within the spore coat. The cell in Fig. 15 contains several mesosomes. R-K fixation. Markers represent 0.5 μm.*
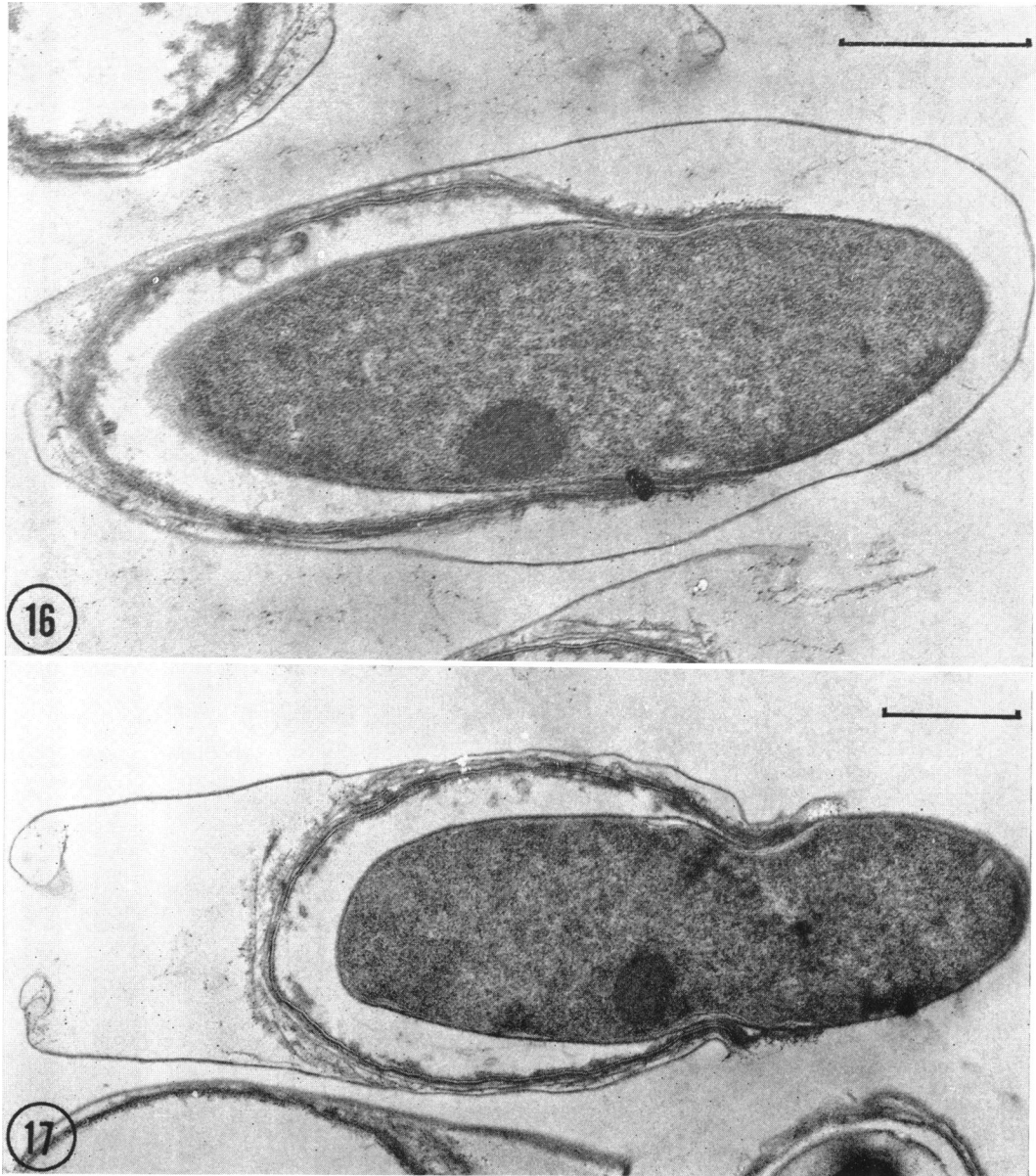
1842

FIG. 16 and 17. *Elongated rod in Fig. 16 has ruptured the spore coat, whereas that in Fig. 17 has broken through both the spore coat and the sporangium. Note the large, dense bodies in both cells. Glutaraldehyde prefixation. Markers represent 1 μm.*

minating rods of *B. subtilis.* Uptake of water by these materials may also provide the propulsive mechanism for emergence from the sporal resi-due which, in the living state, takes place with explosive rapidity. The cell always emerges at the pole opposite the sporangial collar and involves progressive rupture of the inner coat, the outer laminated coat, and, finally, the sporangium.

Noteworthy in emergent cells prefixed with glutaraldehyde (Fig. 16 and 17) is a large, dense body. Similar bodies have now been seen in several gram-positive and gram-negative bacteria (D. E. Bradley and R. G. E. Murray, *personal communication*), but their function and chemical composition is totally unknown. To our knowl-edge, Fig. 16 and 17 are the first published elec-tron micrographs of these bodies.
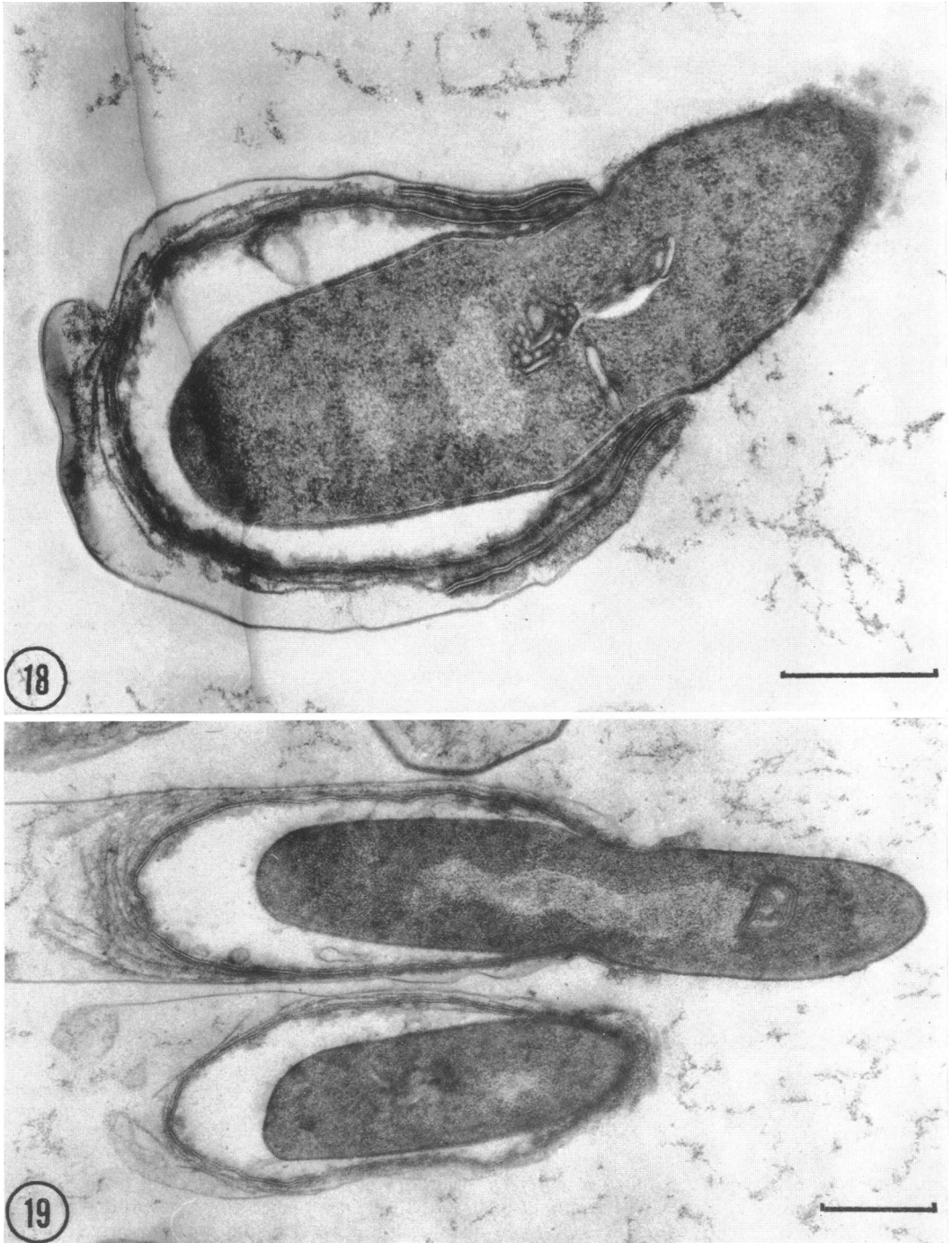
A striking feature of germination in *C. pectino-*

FIG. 18 and 19. *Vegetative rods emerging from fractured, inflexible spore coats. R-K fixation. Markers represent 0.5 μm.*

FIG. 20. *Portion of a free vegetative cell containing two nuclei and a conspicuous mesosome. R-K fixation. Marker represents 0.25 µm.*

1845

vorum, in contrast to most *Bacillus* species, is that emergent rods already have the dimensions of mature vegetative cells. Thus, measurements of more than 25 representative specimens of each cell type produced the following values. The emerging rods measure 2.31 ± 0.44 (mean ± SD) by 0.76 ± 0.08 μm; mature, free vegetative cells measure 2.61 ± 0.61 by 0.71 ± 0.11 μm. Neither length nor width of the two cell types is significantly different at the 5% probability level.

The germination process in *C. pectinovorum* spores differs in several respects from that in other *Bacillaceae* examined so far. During the activation stage, the coat does not become mottled, nor the cytoplasm more dense, as is the case with *B. anthracis* (12). During germination proper, the spore of *C. pectinovorum* swells hardly at all, and thus differs from most other spores, especially those of *B. megaterium* (9, 21). Further, the cortex does not become spongy, as Rousseau, Fléchon, and Hermier (22) observed with *B. subtilis*. Again, the spore coat is so rigid that the vegetative rod is compressed as it emerges through the narrow aperture at the end of the spore opposite the membrane-filled collar-like remains of the sporangium. This situation is to be contrasted with the ready escape of various representatives of *Bacillus*, in which the spore coat cracks open and folds back (8, 21) or splits widely either at the middle or near the end (9, 21).

In one very important respect, the germination of several *Clostridium* spores differs significantly from that of all *Bacillus* spores. Spores of *Bacillus* species are released from the mother cell or sporangium prior to germination (1, 8, 9, 11, 12, 21, 22). Spores of *C. tetani* (15), *C. butyricum* (17), and *C. pectinovorum* are not so released; in fact, they germinate within the sporangium.

LITERATURE CITED

1. Chapman, G. B., and K. A. Zworykin. 1957. Study of germinating *Bacillus cereus* spores employing television microscopy of living cells and electron microscopy of ultrathin sections. J. Bacteriol. 74:126–132.
2. Cohn, F. 1877. Untersuchungen über Bacterien. IV. Beiträge zur Biologie der Bacillen. Beitr. Biol. Pflanz. 2:249–276.
3. Fitz-James, P. C. 1962. Morphology of spore development in *Clostridium pectinovorum*. J. Bacteriol. 84:104–114.
4. Glauert, A. M., and M. J. Thornley. 1966. Glutaraldehyde fixation of gram-negative bacteria. J. Roy. Microscop. Soc. 85:449–453.
5. Hoeniger, J. F. M., P. F. Stuart, and S. C. Holt. 1968. Cytology of spore formation in *Clostridium perfringens*. J. Bacteriol. 96:1818–1834.
6. Hunnell, J. W., and Z. J. Ordal. 1961. Cytological and chemical changes in heat killed and germinated bacterial spores, p. 101–112. *In* H. O. Halvorson (ed.), Spores II. Burgess Publishing Co., Minneapolis, Minn.
7. Keynan, A., and H. Halvorson. 1965. Transformation of a dormant spore into a vegetative cell, p. 174–179. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.
8. Knaysi, G., R. F. Baker, and J. Hillier. 1947. A study, with the high-voltage electron microscope, of the endospore and life cycle of Bacillus mycoides. J. Bacteriol. 53:525–537.
9. Knaysi, G., and J. Hillier. 1949. Preliminary observations on the germination of the endospore in *Bacillus megatherium* and the structure of the spore coat. J. Bacteriol. 57:23–29.
10. Mason, D. J., and D. M. Powelson. 1956. Nuclear division as observed in live bacteria by a new technique. J. Bacteriol. 71:474–479.
11. Mayall, B. H., and C. F. Robinow. 1957. Observations with the electron microscope on the organization of the cortex of resting and germinating spores of *B. megaterium*. J. Appl. Bacteriol. 20:333–341.
12. Moberly, B. J., F. Shafa, and P. Gerhardt. 1966. Structural details of anthrax spores during stages of transformation into vegetative cells. J. Bacteriol. 92:220–228.
13. Murrell, W. G. 1961. Spore formation and germination as a microbial reaction to the environment, p. 100–150. *In* G. G. Meynell and H. Gooder (ed.), 11th Symp. Soc. Gen. Microbiol., Cambridge University Press, Cambridge.
14. Murrell, W. G. 1967. The biochemistry of the bacterial endospore, p. 133–251. *In* A. H. Rose and J. F. Wilkinson (ed.), Advances in microbial physiology, vol. 1. Academic Press, Inc., New York.
15. Neumann, H. 1929. Die Isolierung und die Entwicklung von Anaërobiersporen. Zentr. Bakteriol. Parasitenk., Abt. I. Orig. 115:100–108.
16. Powell, J. F., and R. E. Strange. 1953. Biochemical changes occurring during the germination of bacterial spores. Biochem. J. 54:205–209.
17. Propst, A., and J. R. Möse. 1966. Sporulation und Germination beim *Clostridium butyricum* M55. Elektronenmikroskopische Untersuchung. Zentr. Bakteriol. Parasitenk. Abt. I. Orig. 201:373–396.
18. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17:208–212.
19. Robinow, C. F. 1942. A study of the nuclear

apparatus of bacteria. Proc. Roy. Soc. (London) Ser. B **130:**299–324.

20. Robinow, C. F. 1944. Cytological observations on *Bact. coli, Proteus vulgaris* and various aerobic spore-forming bacteria with special reference to the nuclear structures. J. Hyg. **43:**413–423.

21. Robinow, C. F. 1960. Morphology of bacterial spores, their development and germination, p. 207–248. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 1. Academic Press, Inc., New York.

22. Rousseau, M., J. Fléchon, and J. Hermier. 1966. Étude au microscope électronique de la germination de la spore chez *Bacillus subtilis*. Ann. Inst. Pasteur **111:**149–160.

23. Ryter, A., and E. Kellenberger. 1958. Étude au microscope électronique de plasmas contenant de l'acide désoxyribonucléique. I. Les nucléoides des bactéries en croissance active. Z. Naturforsch. **13b:**597–605.

24. Sussman, A. S., and H. O. Halvorson. 1966. Spores. Their dormancy and germination. Harper and Row, New York.

25. Takagi, A., T. Kawata, and S. Yamamoto. 1960. Electron microscope studies on ultrathin sections of spores of the Clostridium group, with special reference to the sporulation and germination process. J. Bacteriol. **80:**37–46.

26. Takagi, A., T. Kawata, S. Yamamoto, T. Kubo, and S. Okita. 1960. Electron microscopic studies on ultrathin sections of spores of *Clostridium tetani* and *Clostridium histolyticum*, with special reference to sporulation and spore germination process. Japan. J. Microbiol. **4:**137–155.