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# Inhibition of the Development of the Spore Septum and Membranes in *Bacillus cereus* by β-Phenethyl Alcohol

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## ABSTRACT

REMSEN, C. C. (Syracuse University, Syracuse, N.Y.), D. G. LUNDGREN, AND R. A. SLEPECKY. Inhibition of the development of the spore septum and membranes in *Bacillus cereus* by β-phenethyl alcohol. J. Bacteriol. **91**:324-331. 1966.—The effect of phenethyl alcohol (PEA) upon the initial stages of sporulation in *Bacillus cereus* was studied with an electron microscope. PEA (0.35%) completely inhibited the development of the spore septum and forespore membranes. Some of the treated cells did form the axial filament of chromatin material regarded as the first stage in sporulation, but this was delayed by 4 to 5 hr compared with untreated cells. The definite effect upon these membrane systems lends support to the belief that the primary site of PEA inhibition may be upon the bacterial membrane. The fine-structure details observed during the initial stages of sporulation in untreated cells were in agreement with the structure published for other *Bacillus* species.

Growth inhibition of bacteria by β-phenethyl alcohol (PEA) was shown to be a selective and reversible inhibition of deoxyribonucleic acid (DNA) synthesis (1). Further proof for this explanation was the demonstration that PEA had a striking effect on the replication of bacteriophage T2 (10). This inhibitor has subsequently proved useful in studying various aspects of bacterial viruses (6, 13) and processes involved in bacterial conjugation (2, 8, 15). PEA and related compounds have also been shown to inhibit bacterial sporulation and germination (18; Slepecky and Celkis, Bacteriol. Proc., p. 14, 1964). This effect occurs at a substantially lower concentration than that required to inhibit growth, and does so under conditions where no demonstrable inhibition of DNA synthesis is occurring. Addition of PEA at various times of growth of bacilli indicated that forespore formation was prevented.

Additional sites for PEA inhibition that have been suggested are DNA replication (19), messenger ribonucleic acid (RNA) formation (16), and permeability processes (11). The suggestion of Treick and Konetzka (19) that the primary site of PEA inhibition may be a bacterial membrane appears most attractive for explaining the varied responses to this compound. Membranes may serve to initiate DNA replication (7, 8), and are particularly important during bacterial sporulation (5).

This study was undertaken to determine whether bacterial membrane changes associated with forespore development could be detected during the inhibition of sporulation by PEA.

## MATERIALS AND METHODS

A spore suspension of *Bacillus cereus* ATCC 4342 harvested from a glucose-glycine-glutamic acid-salts medium (GGGS) (12) was heat shocked for 15 min at 65 C, and 1 ml was inoculated into each of 16 Klett flasks (300-ml) containing 50 ml of GGGS medium. The cultures were incubated on a rotary shaker (187 rev/min) at 30 C. PEA to give a final concentration of 0.35%, an amount shown in preliminary experiments to be needed to inhibit sporulation of this organism, was added to eight flasks at 15 hr. The cultures, at 15 hr, were at the end of logarithmic growth as indicated by turbidity measurements made by use of a Klett Summerson photoelectric colorimeter with a green filter. The other eight flasks represented control (untreated) cultures. At 16 to 22 hr and at 37 hr, flasks were removed from the shaker and total counts were made in a Petroff-Hausser counting chamber, cell types were distinguished by phase-contrast microscopy, and 20 ml of culture was removed and frozen at −20 C. The frozen cells were

subsequently used for electron microscopic observations.

A 2-ml amount of a 1% osmium tetroxide solution was added to the frozen culture samples, and the cultures were allowed to thaw at room temperature. The thawed samples were centrifuged, and the supernatant fluid was decanted before fresh 1% osmium tetroxide was added to the pellet; the pellet was shaken and the cell suspension was stored overnight. Fixed cell specimens were prepared for embedding in Epon 812 by use of the agar-block technique of Kellenberger, Ryter, and Séchaud (9). Sections were cut on a MT-2 Porter-Blum microtome, transferred to carbon-coated grids, and post-stained with lead hydroxide by the method of Watson (20). Sections were examined in an EMU-2D electron microscope with an objective aperture of 25 $\mu$. Micrographs were made by the through focus technique at an initial magnification of 7,000 to 9,000 times, and were enlarged photographically.

## RESULTS AND DISCUSSION

The control (untreated) cultures contained some forespores at 20 hr, as evidenced by phase darkening toward the pole of the cell, and some sporangia were observed as refractile bodies by about 22 hr. At 37 hr, 95% of the cells were sporangia. No sporangia were observed in the PEA-treated flasks even after 109 hr of incubation. Through 37 hr, PEA-treated cells were indistinguishable from the normal vegetative cells (under phase optics) and no detectable lysis had occurred, as indicated by total cell counts. Beyond 37 hr, the PEA-treated cells underwent lysis.

Figure 1 is an electron micrograph of an untreated 17-hr cell in the axial filament stage of sporulation, just prior to enclosure of a part of the nucleus toward the end of the cell; the future spore nucleus is shown enclosed by the spore septum in Fig. 2. The spore septum (single arrows) arises from the cytoplasmic membrane. The double-layered membrane system (sps) is marked by opposing arrows, and will subsequently proliferate into a double-layered forespore enclosure, as is partly seen in the sections shown in Fig. 3. Early forespores are seen in Fig. 4 and 5, in which the forespore membranes are marked by arrows; between the membranes is the more dense primordial cortex. The forespore nucleus initially is quite discernible and is generally seen as a single body with uranyl acetate staining (Fig. 2–4); however, with forespore maturation, the nucleus appears scattered and less discrete, and is difficult to resolve (Fig. 5 and 6). Figure 6 represents a mature forespore (fs) with a more advanced cortex (cx). The stage of forespore development seen in Fig. 5 and 6 probably represents that stage associated with some cytoplasmic darkening in vegetative cells, as seen under the phase microscope. Figure 13 shows a normal sporangium (37 hr) with a fully developed cortex and exosporium. We have made no attempt to characterize the fine structure of these later sporulation stages.

PEA completely inhibited the formation of forespore membranes. Figures 7–10 represent cells harvested at 16, 18, 19, and 20 hr, respectively, and in no cases were forespore membranes seen. The cytoplasm of these cells resembled that of vegetative cells containing the storage granules of poly-$\beta$-hydroxybutyrate (14), whereas by 22 hr little poly-$\beta$-hydroxybutyrate was noted; therefore, it seems that the normal degradation of polymer was not effected by PEA. By 22 hr, some of the treated cells contained the axial filament of chromatin (Fig. 11 and 12), the first stage of sporulation following the cessation or slow-down of growth; however, no spore septa or forespore membranes were subsequently formed. In treated cells, membranous organelles (presumably mesosomes) were seen at 22 hr (Fig. 12), indicating that either the PEA inhibition showed some apparent selectivity for the two different membrane systems, that is, forespore membranes and mesosomes, or that these mesosomes were formed during growth (before addition of PEA). In the sections studied, we were unable to resolve any mesosome affiliations with chromatinic material.

Figure 14 shows a 37-hr treated cell which has nuclear bodies at the poles but no forespore membranes; considerable contrast is noted between these cells and the control (Fig. 13). Thus, a comparison of the treated and untreated cells clearly shows that the effect of PEA is on early forespore membrane formation. This result lends some support to the idea of Treick and Konetzka (19) that the primary site of PEA may be upon bacterial membranes. Further, our fine-structure studies of early sporulating cells indicate that in our strain of *B. cereus* sporulation events are similar to those reported for other bacilli (3, 4, 17).

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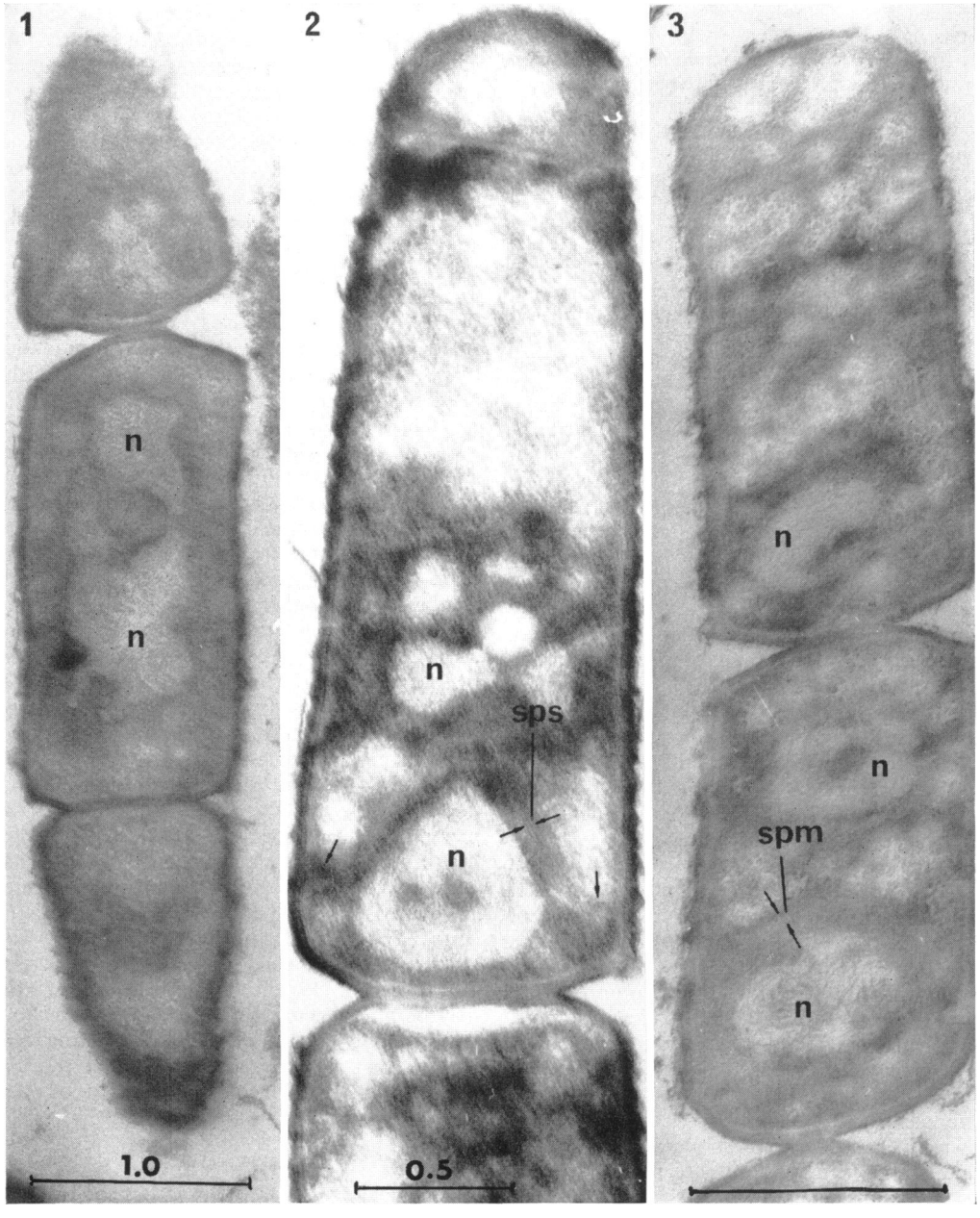


FIG. 1. *Thin section of Bacillus cereus. Control 17-hr cell in the axial filament stage of sporulation just prior to enclosure of a part of the nucleus (n). × 31,000.*

FIG. 2. *Thin section of Bacillus cereus. Control 18-hr cell showing a developed spore septum (sps) marked by opposing arrows. The spore septum (single arrows) arises from the cytoplasmic membrane. Enclosed nuclear body (n) is shown, as is the chromatinic body (n) in the nondifferentiated part of the cell. × 43,000.*

FIG. 3. *Thin section of Bacillus cereus. Control 19-hr cells with a double-layered forespore membrane (spm) enclosing the nuclear material (n) in the lower cell. Opposing arrows show the double membrane. The top cell is in the same stage, but its forespore membrane is not resolved in this section.*

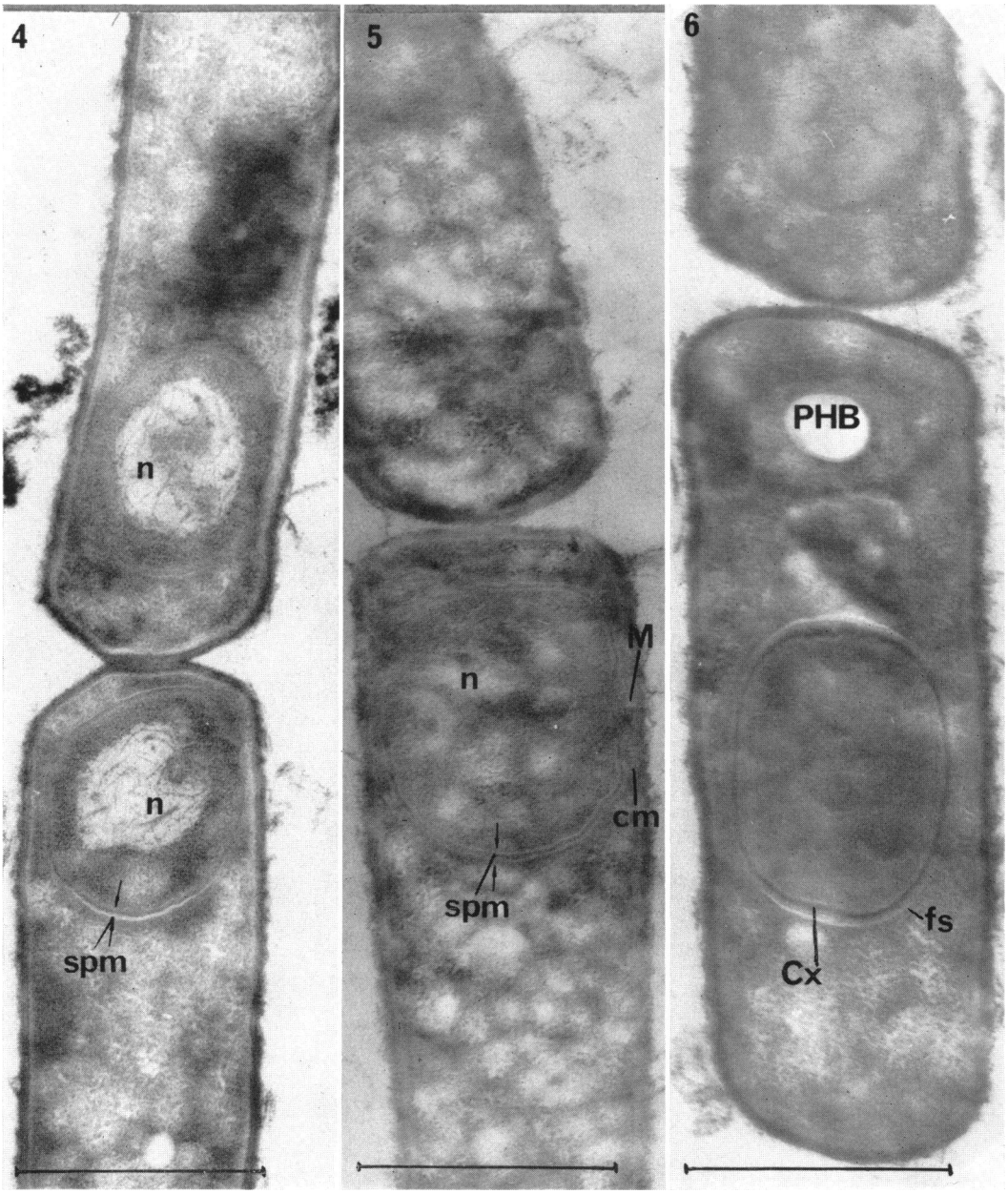


FIG. 4. *Thin section of Bacillus cereus. Control 20-hr cells showing a completed double-layered forespore membrane (spm) marked by opposing arrows (bottom cell). Enclosed within the forespore is the spore nuclear material (n). The top cell is in the same stage, but in this particular section these structures are not clearly resolved.* × *35,200.*

FIG. 5. *Thin section of Bacillus cereus. Control 20-hr cell with a completed forespore membrane (spm) marked by opposing arrows. A mesosome (M) is shown associated with both the cytoplasmic membrane (cm) and the forespore. The nuclear material (n) became difficult to discern at this stage of development, which is slightly more advanced than that seen in Fig. 4. Again, the top cell is poorly resolved in this section.* × 35,100.

FIG. 6. *Thin section of Bacillus cereus. Untreated 22-hr cell showing a more advanced forespore (fs) and cortex (cx) with the latter still electron-dense but considerably broadened. A storage granule of poly-β-hydroxybutyrate (PHB) is seen in the cell.* × *37,300.*

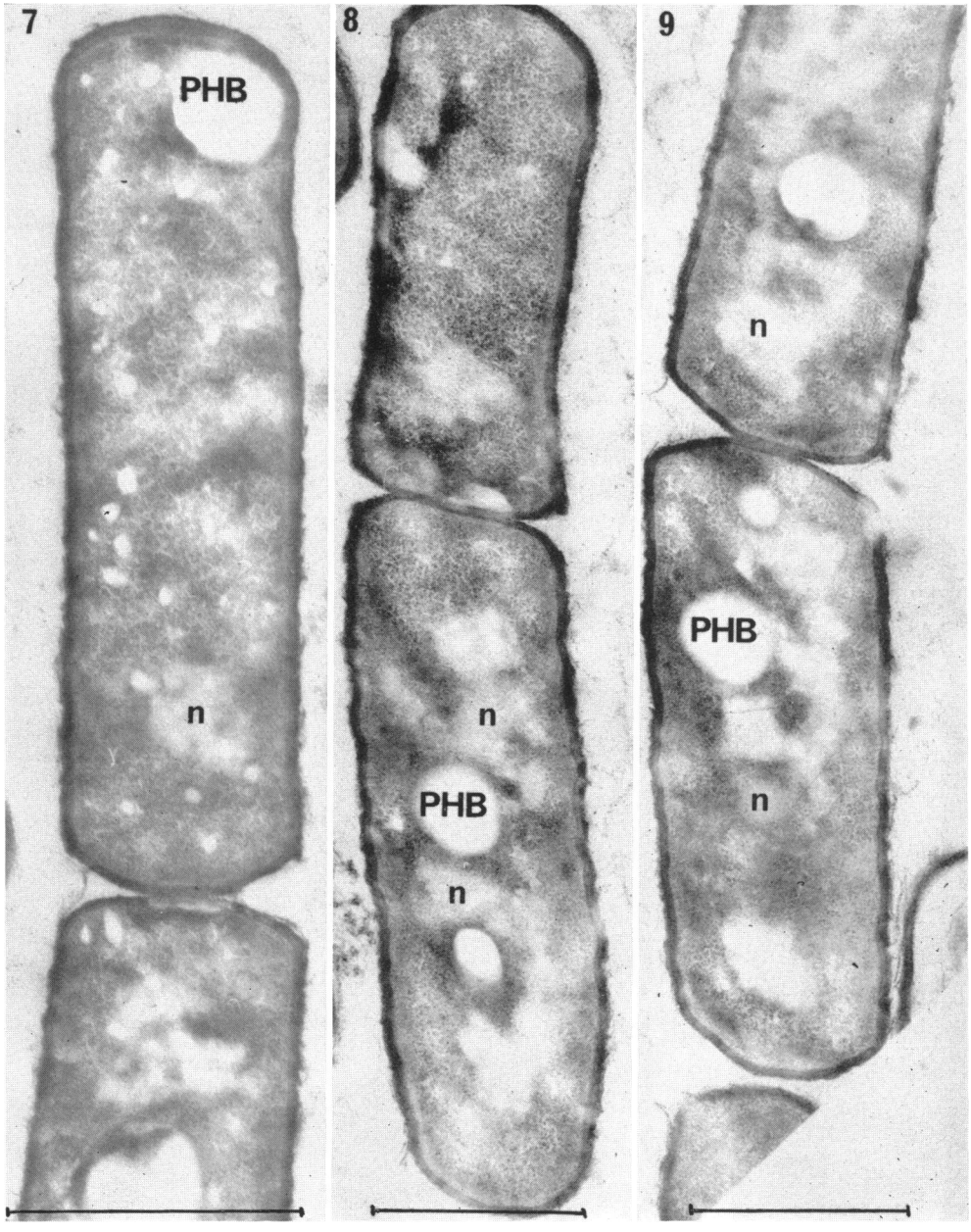


FIG. 7. *Thin section of Bacillus cereus. Treated 16-hr cell showing no nuclear aggregation or sign of differentiation. Poly-β-hydroxybutyrate (PHB) granules are found in the cell.* × *39,600.*

FIG. 8. *Thin section of Bacillus cereus. Treated 18-hr cells showing a nonaggregated nucleus and some PHB granules. No evidence of spore septa is seen in the cells.* × *29,700.*

FIG. 9. *Thin section of Bacillus cereus. Treated 19-hr cells again without any spore septa.* × *29,700.*

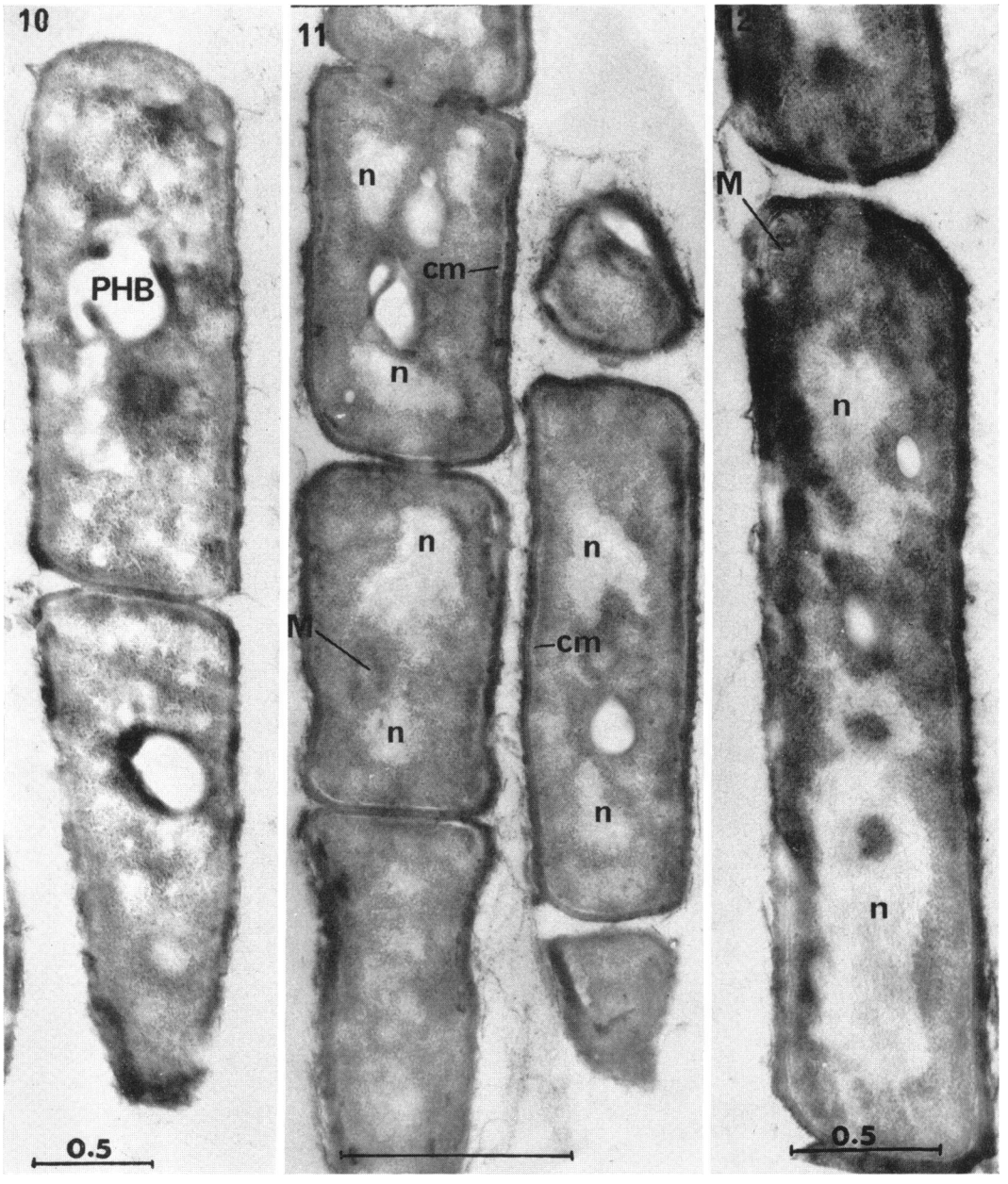


FIG. 10. *Thin section of Bacillus cereus. Treated 20-hr cells 1 hr older than in Fig. 9, but again no spore septa are seen. PHB granules are still found.* × *36,900.*

FIG. 11. *Thin section of Bacillus cereus. Treated 22-hr cells showing well-developed nuclear areas (n); in one instance a mesosome (M) is noted. In another of the cells, the cytoplasmic membrane (cm) is well resolved.* × *32,400.*

FIG. 12. *Thin section of Bacillus cereus. Treated 22-hr cell where the nuclear area (n) resembles the axial filament stage common to sporulating cells. A mesosome (M) is found in the upper corner of the cell, but again no spore septum is seen.* × *42,300.*

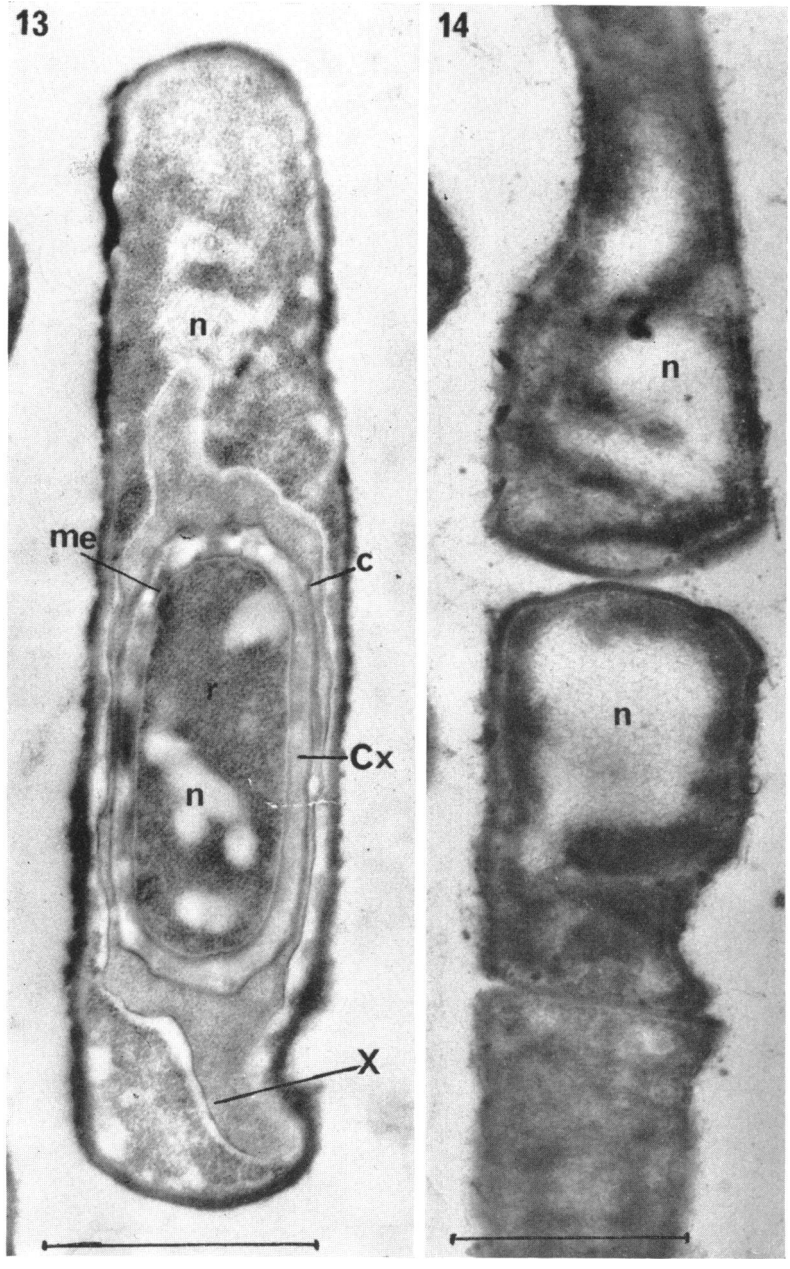


FIG. 13. *Thin section of Bacillus cereus. Control 37-hr cell in the terminal stage of spore formation. The spore at this stage shows a well-developed exosporium (x) and cortex (cx), as well as spore coats (c) and an inner cortical membrane (me). Nuclear areas (n) are visible within the spore, and a well-defined nuclear body is still visible in the vegetative portion of the cell. The spore cytoplasm has a granular consistency; presumably these granules are ribosomes (r).* × *36,000.*

FIG. 14. *Thin section of Bacillus cereus. Treated 37-hr cells showing nuclear material (n) rather heavily stained in distorted cells. No spore septa or forespore membranes were seen at this latter hour.* × *31,000.*

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