An Effective Method of Preparing Sections of *Bacillus polymyxa* Sporangia and Spores for Electron Microscopy

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ABSTRACT

Bacillus polymyxa sporangia and spores were prepared for examination in the electron microscope by methods whose critical features were apparently: judicious use of vacuum, to encourage complete penetration of the embedding medium; the use of epoxy resins as embedding media; and cutting of the thin sections with a diamond knife. Electron micrographs of material prepared in this manner exhibit undeformed sporangial sections. Some of the structures revealed have been shown before, though perhaps less distinctly; other structures are revealed here for the first time. While this single study does not pretend to elucidate all the complexities of sporulation in bacteria, these and similar images should make this possible, and some mention of the preparatory techniques that lead to them seems advisable at this time.

INTRODUCTION

Bacterial spores and their development have been studied in the electron microscope by a number of investigators (Robinow, 1953; van den Hooff and Aninga, 1956; Hashimoto and Naylor, 1958; Tokuyasu and Yamada, 1959). An earlier study of *B. polymyxa* was made by Dondero and Holbert (1957). This latter work left much to be desired in the way of illustrating spore structure in the bacterium studied, but certain features of the electron micrographic evidence indicated that considerably more useful information was potentially available. Accordingly, some additional experiments were done, using the bacterial cells fixed and dehydrated for the original study but employing other embedding matrices, in particular, several epoxy resins.

As an additional variation on the original techniques, a diamond knife (obtained from the Instituto Venezolano de Neurología y Investigaciones Cerebrales, Caracas, through the courtesy of Dr. Fernández-Morán—see his report, 1956) was put to use in the sectioning.

The electron micrographs from these latter experiments were so much more satisfactory than any previously obtained that a formal study was begun in an attempt to determine if the results were reproducible. This report deals with the preliminary findings of that study.

Materials and Methods

The source of the *Bacillus polymyxa* cultures used in this investigation was strain 1312 from the N. R. Smith collection at the Institute of Microbiology, Rutgers, the State University. Cells were grown for 6 days at 28°C. on a 2:1 mixture of soil extract agar to potato starch agar, harvested by scraping the surface of the agar with a glass slide, and placed directly into screw-top vials containing 3 to 5 ml. 2 per cent OsO_4 (unbuffered). The vials were capped, shaken gently to disperse the viscous material with the fixative, and incubated at 36°C. for 24 hours, during which period the vials were periodically agitated.

The OsO_4 solution was then decanted, and the sediment rinsed several times with 25 per cent ethyl alcohol, and resedimented after each rinse by centrifugation for about 5 minutes at 1000 g. Small bits of agar, which had been inadvertently scraped off by the slide, were filtered out by two layers of ordinary lens paper.

The material was dehydrated through an ethanol series, 50, 70, 95, and 100 per cents. The sediment was dispersed through the alcohol, allowed to stand at room temperature for about 30 minutes, recentrifuged as before, and the next higher concentration of alcohol was added; this procedure was finally repeated through

two changes of absolute alcohol. Although considerable sediment was lost with the various supernates, the final product was notably free from extraneous materials.

Two drops of accelerator (α-methylbenzyl dimethylamine) were placed in each embedding capsule and a small quantity of the final sediment lifted by means of a small spatula and scraped off onto the inner rim of the capsules. This was washed down into the capsules by another drop or two of accelerator. The capsules were then placed in an oven at 54°C. for 4 hours, following which the accelerator was decanted and all excess removed by carefully blotting with lens paper the sides and base around the material.

The capsules were then approximately half-filled with the epoxy resin[1] mixture recommended in the New York Society of Electron Microscopists kit,[2] but omitting the plasticizer:

Epoxy resin—Ciba 6005	10.0 ml.
Hardener—dodecenylsuccinic anhydride	10.0 ml.
Accelerator—α-methylbenzyl dimethylamine	0.5 ml.

The container of unused embedding agent and the capsules were placed in a vacuum desiccator and the chamber very cautiously evacuated by means of a mechanical pump. Unless great care was exercised at this stage, all the cells would have been lost by violent bubbling. However, if evacuation was halted by turning a stopcock in the line when spilling over appeared imminent, many of the bubbles broke. As soon as the volume reduced, evacuation was resumed, until all bubbling ceased. During this procedure, it was found, if the bubbles do not break when the capsules are brim full, it is better to bleed enough air into the chamber to reduce the volume to its original value, and then reapply the vacuum. The process removes the alcohol and assists impregnation of the cells with accelerator— the component most reluctant to penetrate. The capsules are finally filled almost to the brim with the vacuum-treated resin.

After standing at room temperature for 3 to 4 hours, the filled capsules were placed in an oven at 54°C. until very hard and dry—optimally for several days (although the blocks may be ready for sectioning after 18 to 24 hours). The epoxy resin so prepared is extremely brittle and hard, and its trimmed surfaces do not appear as smooth as those of methacrylate. Nevertheless, the resin block can be trimmed, and good sections can be obtained with a diamond knife.

The description above constitutes Procedure I of three procedures to be considered here. For results of this procedure, see Figs. 1 to 11 inclusive, 13, and 16.

For purposes of comparison, two other procedures were employed involving new embedding and sectioning treatments of material prepared originally for the earlier study. In both instances, the cells used had been grown on plain potato starch agar and harvested as in Procedure I.

In a Procedure to be known as II, sections were cut with the diamond knife from the methacrylate-embedded samples that had been prepared for the earlier work (Dondero and Holbert, 1957), following the preparation method of Birch-Andersen et al. (1953), except that fixation (at 37°C. in buffered saline and OsO_4) was prolonged to 18 hours. The appearance of such sections is shown in Fig. 12.

In a Procedure to be known as III, a portion of the buffered OsO_4-fixed and dehydrated bacteria, which had been preserved in absolute alcohol from the time of the earlier work, was embedded with judicious use of vacuum in the mixture noted below. The resin was then polymerized at 54°C. for 24 hours.

Epoxy resin—araldite 502 (Ciba)[3]	10.00 ml.
Hardener—dodecenylsuccinic anhydride	10.00 ml.
Plasticizer—dibutyl phthalate	00.75 ml.
Accelerator—tri (dimethylamino) methylphenol	00.40 ml.

Micrographs from sections of these bacteria are shown in Figs. 14, 15, 17 to 19.

The microtome used was a Sjöstrand model, geared down to approximately 25 R.P.M. Micrographs were taken at magnifications of 13,000 X to 15,000 X, and enlarged photographically to the indicated dimensions. The RCA EMU-2 microscope had been fitted with a wide field objective, 125 μ condenser aperture, and 25 μ objective aperture. Canalco equipment, i.e., externally adjustable condenser, objective aperture centering device, and electrostatic pole piece compensator, was used.

OBSERVATIONS AND DISCUSSION

This discussion is divided into two sections: I. Observations on the quality of the sections as revealed in the electron micrographic evidence; II. Observations on the sporulation process in bacteria based on the fine structure revealed in the micrographs presented.

I. In accounting for the improved images provided by the three procedures described above, several factors come to mind. Complete penetration of the embedding medium clearly increases preservation of existing cellular structures. A con-

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[1] An epoxy resin was first used as an embedding medium in 1956, by Glauert, Rogers, and Glauert.

[2] This kit is now obtainable at Cargille Sons, 194 Second Avenue, Little Falls, New Jersey.

[3] This resin sample and other components were kindly donated by the Smooth-On Manufacturing Co. of Jersey City. Mr. H. M. Silver, General Manager, suggested the basic formula.

comitant destruction or distortion of those structures may occur when the embedding plastic polymerizes, and in this regard the epoxy resins are less damaging because, where methacrylate shrinks on polymerization some 20 per cent in volume, the epoxies shrink only about 2 per cent. There is evidence, furthermore, that methacrylate frequently does not polymerize as uniformly as the epoxies, and this accounts in part for the common problem of explosion with effects that have been widely depicted. Such effects are notably absent after epoxy embeddings.

The advantages frequently ascribed to the diamond knife would seem to have been demonstrated in this investigation, where it appears that excellent sections of probably hard bacterial spores can be cut when they are embedded in hard and brittle plastic, and that better than usual sections can be cut with the diamond from methacrylate. Slowing down the Sjöstrand microtome should probably be regarded as another feature contributing to the success of the procedures.

II. The micrographs shown here are far from artifact-free, but at the same time, they do represent some real components of the B. polymyxa sporangium and spore. With respect to the single and lamellate layers of the spore coat, for example, they confirm previous and accepted findings. (See descriptions in legends). Some of those structural features shown here that have not been previously identified may also be real, but no definitive statement can be made on the basis of this single experience. It will suffice at this time, therefore, simply to draw attention to these with the proposal implied that their presence, nature, and function be subjected to further investigation.[4]

The star-like profile of B. polymyxa was first shown in a remarkable drawing of the spore in cross-section by A. Meyer (1897). Recently, van den Hooff and Aninga (1956) showed the stellate profile in electron micrographs, and Bradley and Williams (1957) revealed it with shadowed replicas. It represents a feature characteristic of B. polymyxa,[5] consisting of a set of ridges (usually 8 or 9) forming an integral part of the spore coat (Figs. 1 to 12), running parallel to the long axis of the

spore, and coalescing at its ends. Of interest in the present micrographs is the appearance of a dense, sharply limited material within the ridges of some spore sections (Figs. 12, 14, 17, 19) that may be independent of the outermost layer of the spore coat. In sections of cells fixed in unbuffered OsO_4 (Procedure I), this element is not revealed (Figs. 1 to 11, 13, and 16), and was presumably dissolved out.

The significance of the element marked U in Fig. 5 is unknown. The structure at Z in Fig. 10 is of some special interest in that it seems to represent differentiation of the innermost layer of the spore coat. As such, it is conceivable that the structure might be designed to function in the release during germination of the new vegetative cell from the spore coat.

The rough mottled appearance of the sporangial cytoplasm of B. polymyxa, visible most distinctly in this series in Figs. 5 to 7, deserves to be noted, since it has not been shown in the sporangia of other bacteria; its significance is not clear.

The relatively low density of the cortex poses a question as to its nature. Whether the density depicted is a reflection of a different degree of native hydration or whether it means that a part of this layer has been removed by fixation or dehydration cannot be decided. Its composition is obviously different from that of the coat and the spore cytoplasm and one is lead to wonder whether, through its intimate association with the spore, it may not contribute to the spore's survival in a nutritive manner. It is to be noted that in none of the sections are the spore core wall and spore coat in contact at a point. This contact is absent also in the spore micrographs of Chapman (1956), Hashimoto and Naylor (1958), and Tokuyasu and Yamada (1959), but it is present in micrographs of Robinow (1953).

Figs. 1 through 4 are arranged in an order that is thought to represent the natural sequence of stages in the development of the mature spore. This decision is based on what appears to be a progressive development of the ridges, differentiation of the various layers of the spore coat, the increasing definition of the mature cortex, and the gradual disappearance of the cytoplasm of the spore mother cell.

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[4] The author has recently undertaken such an investigation. (Ed.)

[5] Suggestions of similar spore coat contours obtain occasionally in micrographs of B. cereus and B. megaterium, but the regular-polygonal or stellate effect in these is far less pronounced.

Dondero for his assistance in growing the cultures used and for his valuable criticism. Appreciation is also extended to Dr. Vernon Bryson for editorial criticism and assistance. Grateful thanks is extended to Mary Koester Johnson for her assistance.

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EXPLANATION OF PLATES

All figures are electron micrographs of ultrathin sections of Bacillus polymyxa either in a late sporangial phase or free resting-spore stage. Abbreviations used are as follows:

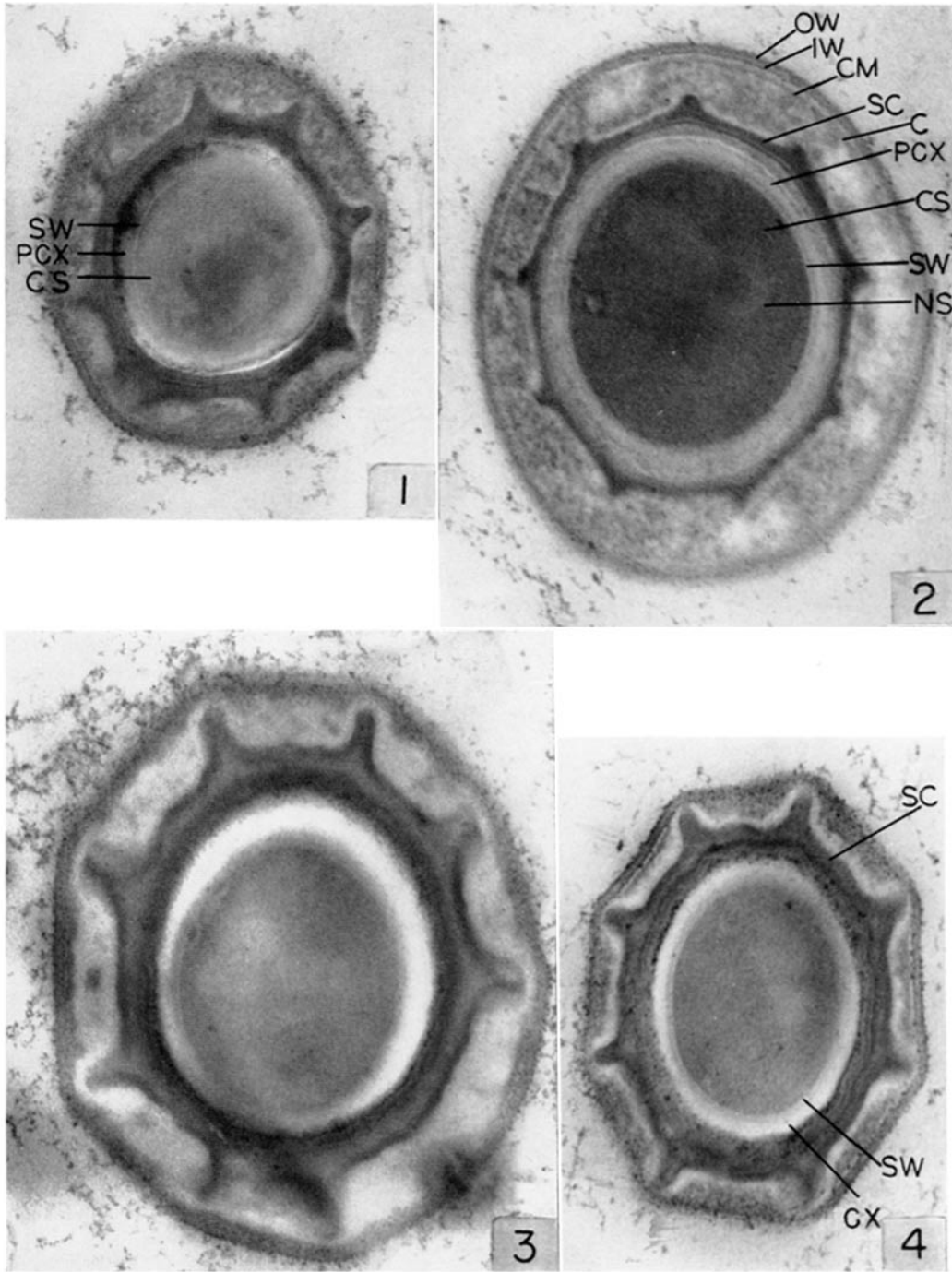|				
A	artifact	IW	inner cell wall
C	cytoplasm	NS	spore nucleoplasm
CA	innermost spore coat layer	OW	outer cell wall
CB	second coat layer	PCX	primordium of cortex
CC	third or middle coat layer	RS	ridge structure
CD	fourth coat layer	SC	sporangial spore coat
CE	outside coat layer	SW	spore core wall
CM	cytoplasmic membrane	U	unidentified structure
CS	spore cytoplasm	Z	accessory structure in spore coat
CX	cortex		

PLATE 205

FIG. 1. A cross-section of a sporangium. The spore is quite well formed. While the spore core wall (SW) is defined in the area at the left, the developing cortex (PCX) is not yet distinguishable in density and structure from the cytoplasmic material of the spore (CS). × 65,000.

FIG. 2. A cross-section of a sporangial cell in which spore is only partly formed. All of the sporangial cell membranes are still in evidence; outer cell wall (OW), inner cell wall (IW), cytoplasmic membrane (CM), sporangial cytoplasm (C), spore coat layers (SC), spore core wall (SW). Cortex (PCX) here is of lower density than that of the cytoplasm of the spore core, the spore cytoplasm (CS), and the spore nucleoplasm (NS). × 65,000.

FIG. 3. A cross-section of an almost mature spore within the sporangium. Note the lower density of the cortex (in part due to artificial separation from spore coat) and the conformity of the sporangial cell wall to the shape of the spore ridge tips. × 65,000.

FIG. 4. A cross-section of a sporangium with a fairly mature spore enclosed. All spore coat layers (SC) are distinguishable and the cortex (CX) is apparently structureless. The spore core wall (SW) is well defined. × 65,000.
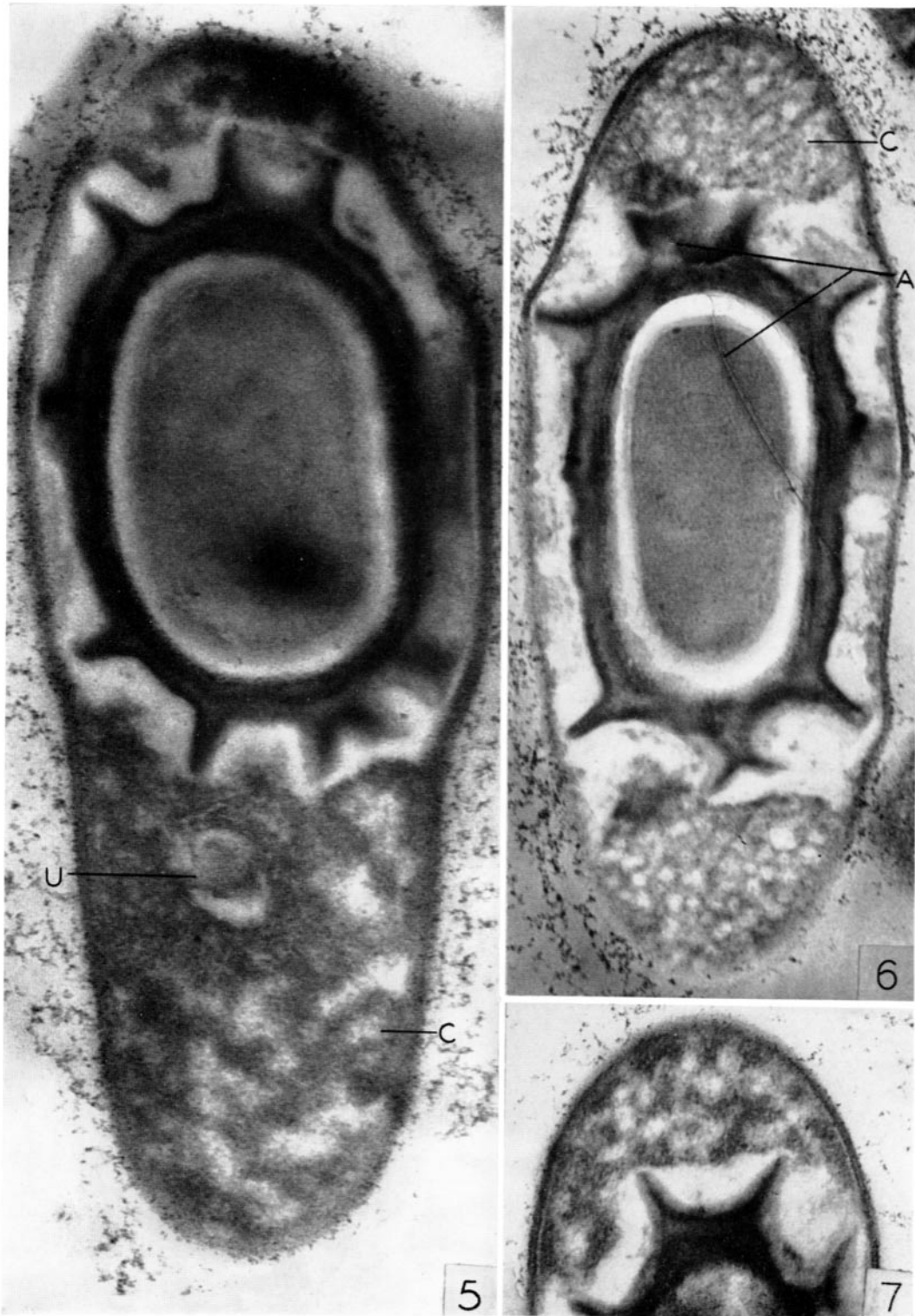
(Holbert: Preparation of sporangia and spores)

FIG. 5. A longitudinal section of a clavate sporangium. The spore is developing at the end of the mother cell. Note the mottled appearance of the sporangial cytoplasm (C). U is a structure of unidentified origin and function. × 65,000.

FIG. 6. A longitudinal section of an almost mature spore developing in a spindle-shaped sporangium. Note appearance of cytoplasm (C). A, an artifact. × 65,000.

FIG. 7. A part of a slightly oblique longitudinal section of a sporangium showing similar cytoplasmic characteristics to that in Figs. 5 and 6. × 65,000.

(Holbert: Preparation of sporangia and spores)

Plate 207

Figs. 8 and 9. Cross-sections of *Bacillus polymyxa* spores prepared by Procedure I. All parts are well defined throughout the entire spore. × 65,000.

Fig. 10. A longitudinal section of a spore. The spore cytoplasm (CS) is quite homogeneous in appearance; the nucleoplasm (NS) is of lower electron density than the cytoplasm (CS); the spore core wall (SW) is evident around the entire core; the cortex (CX) surrounds the entire spore core apparently protecting the potential vegetative cell; the innermost spore coat layer (CA) is thin and osmiophilic; the next layer (CB) consists of four lamellae; the middle layer (CC) is of higher electron density than CB and displays little or no evidence of lamellae when prepared by Procedure I; the fourth layer (CD) is less dense than CC and appears homogeneous; the outermost layer (CE) is of higher electron density than CD, this layer also covers the ridges which resemble spines in a section. A structure (Z), the nature and function of which is unknown, but may be associated with germination, lies at the end of the spore embedded outside the cortex but inside the lamellar layer and possibly inside of CA. × 65,000.

Fig. 11. A cross-section of a spore with all structures evident. × 65,000.

Fig. 12. A cross-section of a spore prepared by Procedure II. Note that what appears to be a ridge supporting structure (RS) is evident; this is not resolved in sections prepared by Procedure I. CA is much more osmiophilic than the other layers. The lamellae in CB are not so well defined as in the spores prepared by Procedure III, however they are discernible. × 65,000.
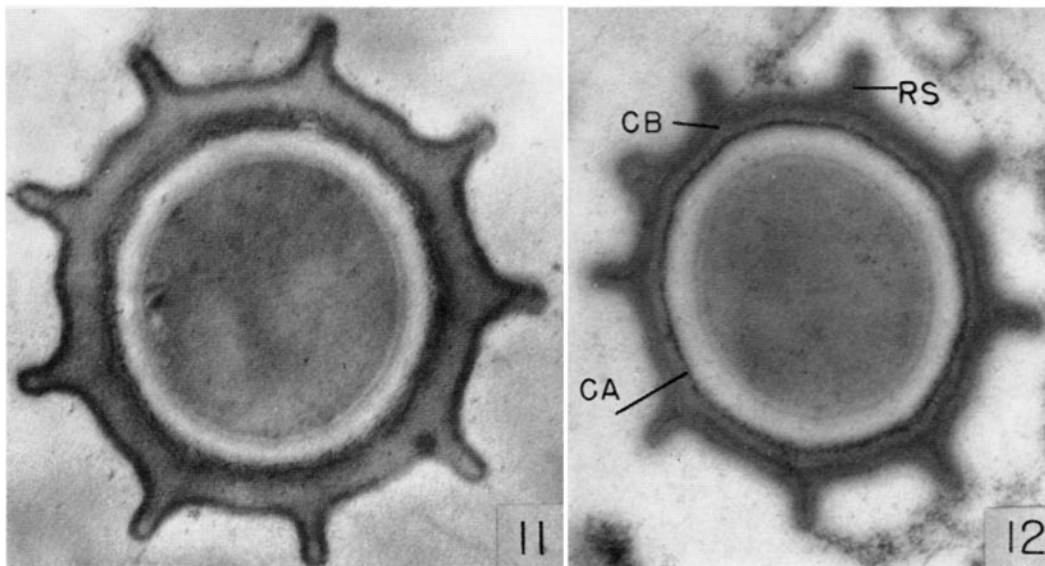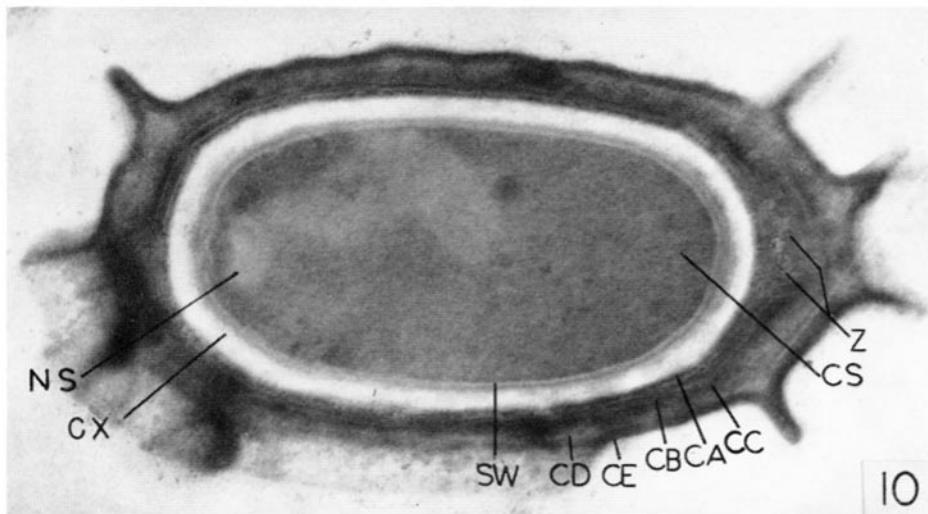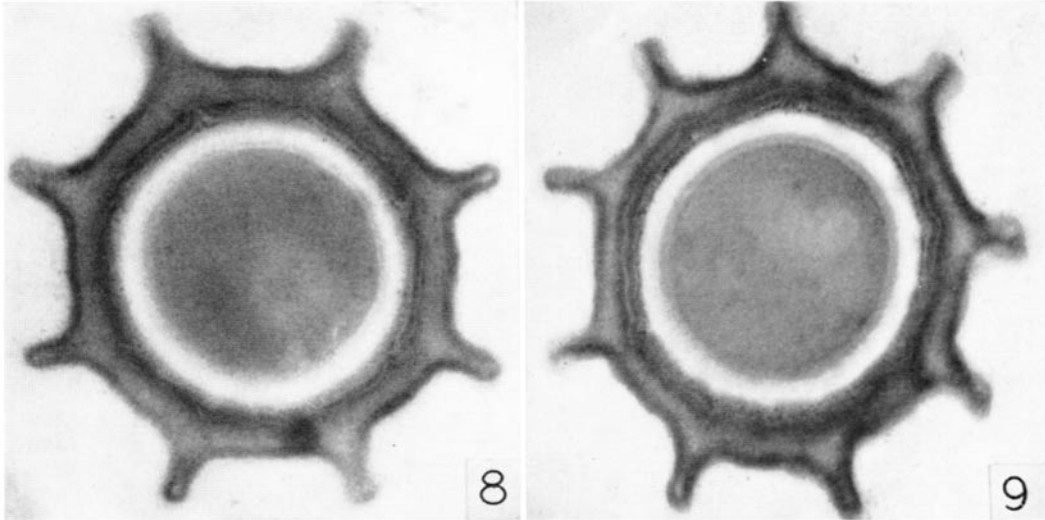
PLATE 208

FIG. 13. Part of the cross-section of the spore in Fig. 9 further enlarged to demonstrate various structures more clearly. $\times$ 145,000.
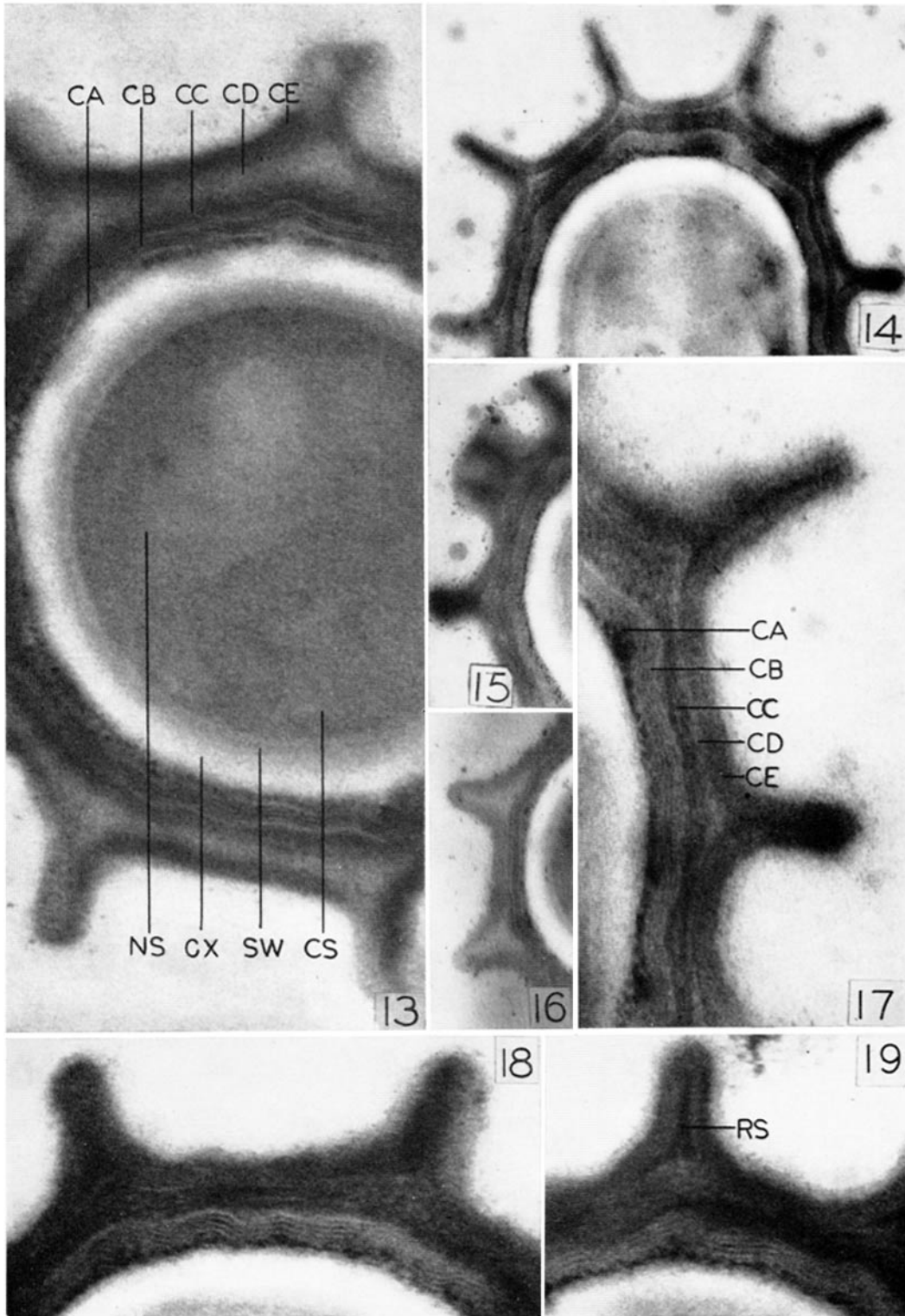
FIG. 14. A part of an oblique section of a spore prepared by Procedure III. Because of the oblique plane in which this section was cut, the various layers are spread out to more than their cross- or longitudinal section dimensions, thus enhancing the appearance of the respective layers for demonstration purposes. $\times$ 75,000.

FIG. 15. A part of a cross-section of a spore prepared by Procedure III, as was that in Fig. 14. This demonstrates structure resolution of the coat similar to that in Fig. 14. Note granules in the embedding material. $\times$ 75,000.

FIG. 16. A part of a spore section prepared by Procedure I displaying essentially the same structure as found in the section in Fig. 15, except that the densities within the ridges are absent. $\times$ 65,000.

FIG. 17. A part of the section in Fig. 14 enlarged to show the structure more clearly. At this magnification, it can be discerned that dense CC is also lamellar in structure. Note the innermost layer of the spore coat (CA), which appears to contain fibrillae. $\times$ 165,000.

FIGS. 18 and 19. Two parts of the spore coat from a spore prepared by Procedure III shown at high magnification to demonstrate the complex design of the spore coat. Note that RS resolved in the section prepared by Procedure III in Fig. 19 is comparable to that seen in the section prepared by Procedure II shown in Fig. 12. $\times$ 165,000.

(Holbert: Preparation of sporangia and spores)