Fine Structure of *Bacillus subtilis*

II. Sporulation Progress

By KIYOTERU TOKUYASU*, Ph.D., and EICHI YAMADA, M.D.

(*From the Department of Anatomy, School of Medicine, Kurume University, Kurume, Japan*)

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ABSTRACT

The sporulation process in *Bacillus subtilis* has been studied principally with $KMnO_4$ fixation, but also, for the purpose of comparison, with OsO_4 and mixtures of both fixatives.

At a very early stage, the pre-spore is seen to consist of what seems to be the nuclear material and granular substance, surrounded by a layer of dense material destined to become the innermost layer of the spore coat. At a subsequent stage, a light interspace is observed that is destined to become the spore cortex.

The mature spore shows a very complex structure. The spore coat is composed of three layers, the middle layer of which consisted of 5 to 8 lamellae of thin membranes and interspaces, both about 20 to 25 A thick. Between the inner layer of the spore coat and the spore cortex, a thin membrane with an affinity to the cortex can be observed. The spore coat is enclosed within two envelopes, one loosely surrounding the core, and the other adhering to it. The process of spore maturation has been studied in detail.

Certain peculiar cellular structures have been observed that seemed to represent features of abnormal sporulation processes.

INTRODUCTION

The sporulation process has been studied by many investigators (1–6) and considerable data has been presented, but the relationship between the nuclear material and the pre-spore still remain to be fully clarified. The objectives of the present study were to examine in greater detail spore structure and the process of spore coat formation.

Methods

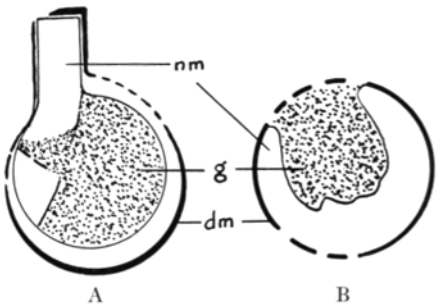
Cells of *B. subtilis*, cultured on agar-agar media for from 8 hours to 9 days at 37°C., were suspended directly in fixatives. The principal conditions of fixation were as follows: (1) $KMnO_4$, as in the first Method of Part I (7); (2) OsO_4, as in the third Method of Part I; (3) Mixed solution, as in the fourth Method of Part I. Other methods of fixation used were (*a*) for 30 minutes at 2°C. in 0.5 per cent $KMnO_4$ in tap water, and (*b*) first for 8 hours at 2°C. in 0.2 per cent OsO_4 in distilled water and then for 14 hours more at 2°C. in 1.5 per cent OsO_4 in distilled water. Method (*a*) was tried as a means of estimating the penetration velocity of $KMnO_4$ into the pre-spore. Method (*b*) was used as a means of checking on artifacts induced by the mixed fixation ((3) above).

The procedures of embedding, sectioning, and examination were the same as those in Part I of the report on this study.

OBSERVATIONS

$KMnO_4$ fixation was used for the majority of observations on the sporulation process. At what is apparently its earliest phase, the pre-spore has a rod-like profile with a denser and more granular bulge at one end (Fig. 1 and Text-fig. 1 *A*). The rod-like profile resembles the nuclear material of the vegetative cell (Fig. 10) in Part I (7)) and the bulging part is similar to the cytoplasm proper. The pre-spore is fringed with a light narrow space and poorly defined dense material. In the next phase, the bulging part

* Present address: Section of Scientific Instruments, Taga Works, Hitachi Products, Ltd., Hitachi-shi, Ibaragi-ken, Japan.



TEXT-FIG. 1 is a schematic representation of profiles of two pre-spores at very early stages. *A* is from Fig. 1, and *B* is from Fig. 5. Both profiles are composed of what is considered to be nuclear material (*nm*), and the granular substance (*g*), and are fringed with the dense material (*dm*), though they have different forms.

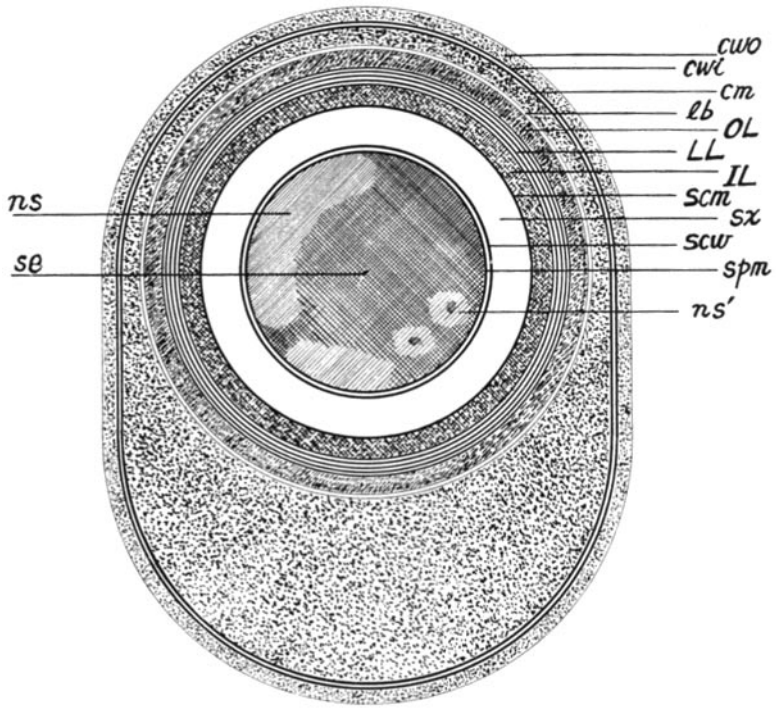
seems to increase in volume (Fig. 2) and the whole profile gradually approaches an oval shape (Figs. 3 and 4). There is also a round form of the apparently earliest phase of the pre-spore that consists of a crescent-shaped agranular area enclosing a round granular region.

The profile, represented in Fig. 5 and Text-fig. 1 *B*, also seems to approach the oval shape (Figs. 6 to 8). During this process, the light interspace between the pre-spore and mother cell cytoplasm becomes clear and in the space two membranes form, one tightly attached to the pre-spore and the other loosely surrounding the outside of the former (Figs. 3 and 7). In Fig. 3, the latter membrane appears to be in contact with an invaginating part of the cell wall. At the oval-profiled stage, the whole pre-spore appears uniformly granular and of somewhat lower density than the mother cell cytoplasm.

The so called nuclear site (2, 3) appears in the pre-spore at the next stage (Figs. 9 to 11). The light narrow space between the pre-spore and mother cell cytoplasm widens and starts to form the structure of the spore cortex (Fig. 11). Two membranes surrounding the pre-spore, now to be called the spore core, become distinct (Figs. 9 to 11). The relationship of these two membranes to the spore core seems to correspond to the relationship of the cell wall and the cytoplasmic membrane to the vegetative cell and, accordingly, they may be called "spore core wall" and "spore cytoplasmic membrane." It is also at this stage that another thin membrane can be recognized at the boundary between the spore cortex and the mother cell cytoplasm the "spore cortex membrane" (Figs. 9 and 11). In the nuclear site of the pre-spore, a network of fine filaments is sometimes observed (Fig. 11), but it is not known whether the material is a real structure or an artifact.

At the next stage, the granularity of the spore core decreases and the nuclear sites of mostly crescent or round profiles become quite clearly discernible (Figs. 12 to 15). Later, at other sites, round light regions, each with a dense particle can be recognized (Figs. 12 to 15). The spore cortex thickens more and takes definite shape (Figs. 12 to 15). The spore core wall of about 40 A surrounds the spore core but is separated from the spore cytoplasmic membrane of about 30 A by a narrow light interspace of about 40 A (Figs. 12, 14, and 15). The spore cortex membrane becomes more distinct than before and shows the thickness of about 50 A (Figs. 12 to 15). This membrane seems to adhere preferentially to the cortex since under the artificial stresses of sectioning it is detached from the mother cell (Fig. 13, 15 to 17).

After this phase the irregular dense material around the pre-spore (shown in Figs. 1 to 9) undergoes a marked transformation. At first, the band increases its thickness in places (Fig. 12) and finally forms a clearly defined dense layer around the spore cortex (Figs. 13 to 17). Almost at the same time, another layer with the same degree of granularity and density as mother cell cytoplasm appears outside the dense layer, *i.e.*, inside the cell cytoplasm. A thin light space of about 50 A forms the boundary between the outer layer and mother cell cytoplasm (Figs. 13 to 17). Between the inner dense and outer light layers, a dense boundary of about 50 A appears (Figs. 14 and 15) and outside the dense boundary, fine membranes develop, gradually forming a laminated structure (Figs. 16 and 17). The appearance of this structure and the disappearance of the dense boundary seem to occur simultaneously. Each lamellar membrane is about 20 to 25 A thick and the interspace between each two membranes is of about the same order of thickness. It is interesting to note that the process of spore coat formation at the proximal part appears before the process at the distal side (Figs. 13 to 17). When the spore is mature, the number of lamellar membranes reaches five to eight (Figs. 18 and 19), and the lamellae finally forms a definite layer between the outer and inner layers (refer to Text-fig. 2).



TEXT-FIG. 2. Schematic representation of the structure of an almost mature spore in the mother cell. *ns*, nuclear site; *ns'*, round light profile with a dense particle; *se*, spore core; *sx*, spore cortex; *cwo*, outer layer of the cell wall; *cwi*, inner layer of the cell wall; *cm*, cytoplasmic membrane; *lb*, light boundary between the outer layer of the spore coat and the cytoplasm of the mother cell; *OL*, outer layer of the spore coat; *LL* lamellar layer of the spore coat; *IL*, inner layer of the spore coat; *scm*, spore cortex membrane; *scw*, spore core wall; and *spm*, spore cytoplasmic membrane.

When fixed with OsO_4 the stages of sporulation appear as with $KMnO_4$-fixation (Figs. 20 to 23), but a considerable difference is apparent in that the $KMnO_4$-fixation gives much more clearly defined details. This is obvious when cellular structures of equivalent sporulation stages revealed by the two kinds of fixation are compared. The coat of the mature spore, *e.g.*, fixed with OsO_4, rarely shows the lamellar layer found on occasion with $KMnO_4$ fixation (Fig. 24). The dimensions of the lamellae under the two kinds of fixations are substantially equivalent. The spore cortex generally appears denser when fixed with OsO_4 (Figs. 22 and 23) than when fixed with $KMnO_4$ (Figs. 12 to 19).

When the bacteria are fixed at first with OsO_4 and subsequently with the mixture of OsO_4 and $KMnO_4$ fixatives, the lamellar structure in the spore coat appears equally as well defined as with the $KMnO_4$ fixation (Fig. 25). The dimensions of lamellar membranes or interspaces agree well with the results of fixation with $KMnO_4$. This mixed fixation gives the spore cortex the same degree of density as does OsO_4 and in addition, imparts to it a somewhat lamellar appearance (Fig. 26).

To check the possibility that the long fixation time in the mixed solution (22 hours), might have induced artifacts, cells were fixed at first with a dilute OsO_4 solution and subsequently with a dense OsO_4 solution together with the mixed fixation. They showed the features common to the usual fixation with 1 per cent OsO_4 solution (Fig. 23).

Various peculiar structures can be seen in a number of cells. Occasionally, a single cell appears to have two sporulation sites (Fig. 27). However, on close examination a faint partition can be detected at the narrowest part of the cell suggesting that, probably, each of the potential daughter cells had begun to sporulate before cell division was completed, and so the divisional

process was discontinued. In some cells, besides a typical sporulation site, two small dense particles can be seen that are partially surrounded by a membranous structure and this membranous structure appears to be in continuity with the spore coat (Fig. 28). In one cell in the present study, two small areas of cytoplasm are surrounded with several concentric membranous layers as if they were miniature sporulation sites (Fig. 29). In some cells, membranous structures which seem to be in continuity with the spore coat from loops in the cytoplasm and the regions within the loops seem to show a density somewhat lower than the residual cytoplasm (Fig. 30). In the profile of another cell, no sporulation site is observed, but tortuous membranous structures fill the cytoplasm (Fig. 31). All of these peculiar structures appear to represent various forms of abnormal sporulation.

DISCUSSION

The agranular regions in the earliest pre-spores are quite possibly nuclear sites and the granular areas may at least possess some cytoplasmic components. The dense material that faintly fringes the pre-spore at the beginning, and later transforms to the dense inner layer, or possibly even to the middle lamellar layer of the spore coat, may originate from the dense peripheral material around the nuclear site reported in Part I (7) (compare Figs. 7 to 10 in Part I with Figs. 1 and 5 in this article).

The cell wall in Fig. 3 invaginates at the point marked with an arrow and appears attached to the spore core wall. There can be no positive explanation of this configuration at the present, but it suggests perhaps the evidence that the spore core wall is derived from the inner layer of the cell wall at some stage of sporulation. The published data concerning the chemical composition of spore coats is scarce. It has been stated that they contain amounts of nitrogen and lipide in excess of those in the cell wall (8), but it seems probable that the resistance of the spore to heat or chemical treatments may be due largely to the lamellar layer since that structure develops in conjunction with the maturing of the spore. Vegetative cells or even sporulating cells with immature pre-spores appear to be fixed much faster than mature spores with lamellar layers in their spore coats (Fig. 9, fixed with 0.5 per cent $KMnO_4$ for 30 minutes at 2°C.). Hashimoto and Naylor in studies on the sporulation of *C. sporogenes* (6), considered "the inner membrane and the region of low electron density" (probably equivalent to the spore cortex and the spore core wall of the present strain) as a probable "effective barrier to dyes." This problem remains to be elucidated in future.

The similarity in dimensions of lamellae when fixed with $KMnO_4$, OsO_4, or the mixtures seems to eliminate the possibility of the lamellae representing the product of Liesegang phenomena during the diffusion of fixatives into the spore coat. Whether or not the lamellar appearance of the spore cortex when fixed with the mixed fixative is an artifact is unknown at present. It is rather strange that only the mixed fixation, neither $KMnO_4$ nor OsO_4 fixation alone, reveals such structures. Mayall and Robinow (5) reported a similar structure in the spore cortex of *B. megaterium* with the aid of lanthanum nitrate.

Hannay (4) reported that "there is some evidence that the spore walls are composed of concentric lamellar of high electron opacity separated by layers of less opaque material" (in the explanation of Figs. 13 and 14) in his study of the partially laminated parasporal body of *B. laterosporus*. Although his description is rather difficult to confirm on the basis of his published micrographs, the structure may quite possibly correspond to the lamellar layer in the spore coat of the present strain.

The fine structure of an almost mature spore revealed by the present study is schematically represented in Text-fig. 2.

BIBLIOGRAPHY

1. Chapman, G. B., *J. Bact.*, 1956, **71,** 348.
2. Robinow, C. F., *J. Bact.*, 1953, **66,** 300.
3. Fitz-James, P. C., *J. Bact.*, 1953, **66,** 312.
4. Hannay, C. L., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 1001.
5. Mayall, B. H., and Robinow, C. F., *J. Appl. Bact.*, 1957, **20,** 333.
6. Hashimoto, T., and Naylor, H. B., *J. Bact.*, 1958, **75,** 647.
7. Tokuyasu, K., and Yamada, E., *J. Biophysic. and Biochem. Cytol.*, 1959, **5,** 123.
8. Yoshida, N., Izumi, Y., Tani, I., Tanaka, S., Takaishi, K., Hashimoto, T., and Fukui, K, *J. Bact.*, 1957, **74,** 94.

Explanation of Plates

All figures are electron micrographs of sections of *B. subtilis*, or its parts, in the sporulation phase. The type of fixation used in each figure is as follows: Figs. 1 to 19, 27, and 29 to 31—$KMnO_4$ fixation (with 2 per cent $KMnO_4$ solution in tap water for 4 hours at 2°C., except Fig. 9); Figs. 20 to 24—OsO_4 fixation (with 1 per cent OsO_4 solution for 4 hours at 2°C., except Fig. 23); and Figs. 25, 26, and 28—mixed fixation with the OsO_4 and $KMnO_4$ solutions. (Refer to the text for more details of the fixation.)

Abbreviations used in the explanation are as follows: *nm*, what is considered to be the nuclear material; *g*, granular substance found in the pre-spore at a very early stage of sporulation; *dm*, dense material around the pre-spore; *ls*, light narrow space between the pre-spore and the dense material found at an early stage of sporulation; *ps*, pre-spore; *cwo*, outer layer of the cell wall; *cwi*, inner layer of the cell wall; *scw*, spore core wall; *spm*, spore cytoplasmic membrane; *scm*, spore cortex membrane; *ns*, what is considered to be the nuclear site in the spore core; *se*, spore core; *sx*, spore cortex; *nw*, network of fine filaments found in the nuclear site; *ns'*, round light profile with a dense particle in it; *OL*, outer layer of the spore coat; *IL*, inner layer of the spore coat; *lb*, light boundary between the outer layer of the spore coat and the cytoplasm of the mother cell; *db*, dense boundary that appears between the outer and inner layers of the spore coat at a stage of sporulation; *L*, lamination of thin membranes that appears outside the dense boundary; *LL*, layer of lamellae of thin membranes between the outer and inner layers of the spore coat; *sc*, spore coat; *dp*, dense round particle surrounded with a lamellar structure in the cytoplasm of the mother cell; *tm*, tortuous membranous structure in the cytoplasm of the mother cell.

PLATE 50

FIG. 1. A very early form of pre-spore in a cell of 17 hours culture. The pre-spore consists of a rod-like glassy structure (*nm*) and a dense and granular bulging part (*g*), and it is surrounded with a light space (*ls*) and a dense material (*dm*). The smooth less granular structure may represent the site of nuclear material. × 98,000.

FIGS. 2 to 4. A probable sequence of sporulation following Fig. 1. Cells of 17 hours culture. The thin light space (*ls*) between the pre-spore (*ps*) and the cytoplasm of the mother cell becomes clear, and, in the space, the spore core wall (*scw*) and spore cytoplasmic membrane (*spm*) develop gradually. In Fig. 3, the cell wall invaginates at the place indicated with an arrow and there, the inner layer of the cell wall (*cwi*) appears to be in contact with the spore core wall. Fig. 2, × 89,000; Fig. 3, × 103,000; and Fig. 4, × 94,000.

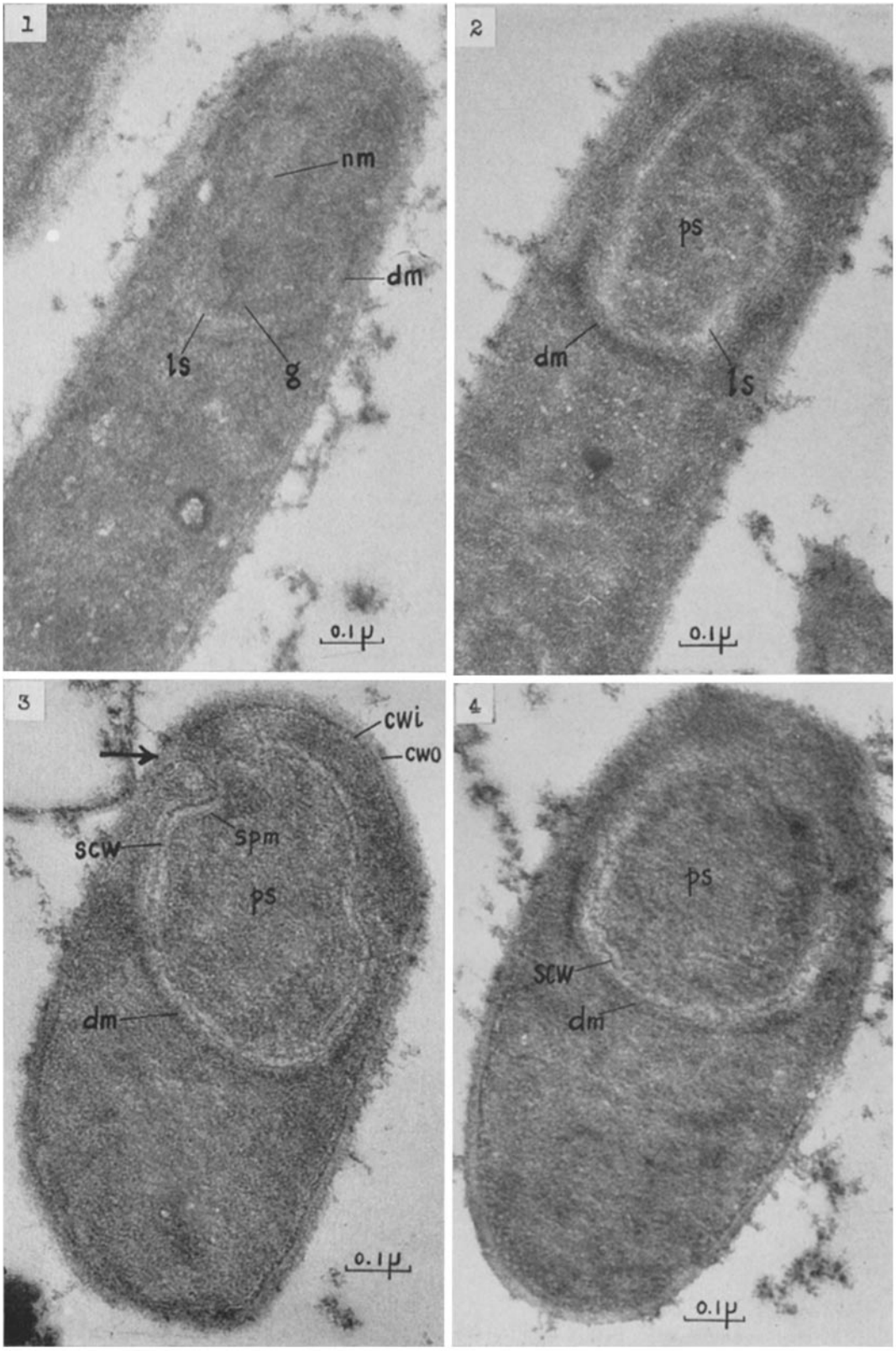


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PLATE 51

FIG. 5. A type of very early pre-spores different from the one of Fig. 1, in a cell of 17 hours culture. The profile of the pre-spore seems to consist of a crescent light region (*nm*) and a round area of granular substance (*g*) being surrounded with the former, and it is fringed with the dense material (*dm*). The crescent region may represent the site of nuclear material. × 68,000.

FIGS. 6 to 8. Cells of 17 hours culture. The round profile of the pre-spore in Fig. 5 may develop to the bigger ovoid shape in Fig. 7 or 8 through such a form as shown in Fig. 6. Note that a light space is observed around the pre-spore in Fig. 6, but no membranous structure is observed in the space. Note also that the spore core wall (*scw*) and the spore cytoplasmic membrane (*spm*) can be observed in the light space of Fig. 7. In the profile of Fig. 8, such membranous structures are not observed clearly and it probably means that the section has been cut at an angle oblique to the surface of the pre-spore. Figs. 6 and 7, × 65,000; and Fig. 8, × 70,000.

FIGS. 9 and 10. Sporulating stages following those shown by cells in Figs. 4, 7, or 8. The nuclear site (*ns*), spore core wall (*scw*), and spore cytoplasmic membrane (*spm*) are clearly recognized in these figures. The spore cortex membrane (*scm*) is visible in Fig. 9, as is also a mass of nuclear material (*nm*) in the cytoplasm. The cell in Fig. 9 was from a 14 hours culture, fixed with 0.5 per cent $KMnO_4$ solution in tap water for 30 minutes at 2°C., × 74,000. The image in Fig. 10, is of a cell from a 17 hours culture, × 129,000.

FIG. 11. The light space shown in previous figures widens and takes a primitive shape of the spore cortex (*sx*). The spore cortex membrane (*scm*), spore core wall (*scw*), and nuclear sites (*ns*) are clearly visible. In a wide nuclear site, a network of fine filaments (*nw*) is recognized. 17 hours culture. × 123,000.

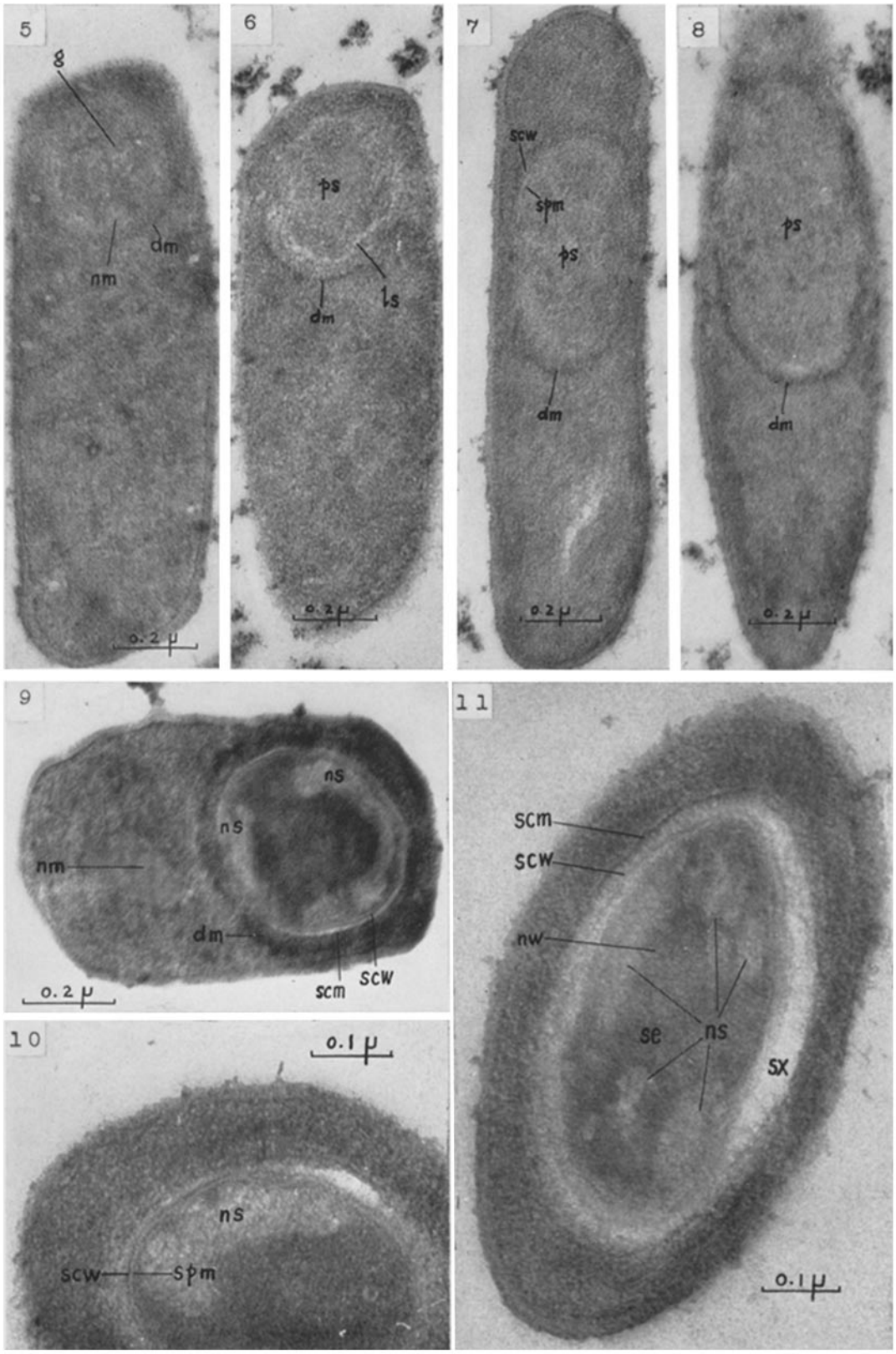

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PLATE 52

FIG. 12. At this stage the spore cortex becomes clear, but the dense material around the cortex does not yet show a definite layering. A spore cortex membrane (*scm*) is recognized at the outer periphery of the cortex. Besides typical nuclear sites (*ns*), round profiles, each with a dense particle (*ns'*), are found at this stage 17 hours culture. × 75,000.

FIG. 13. The dense material, such as is found around the spore cortex in Fig. 12, forms a clearly defined layer (inner layer; *IL*) and the region of cytoplasm adjacent to the layer becomes another layer (outer layer; *OL*), being separated by a light thin boundary space (*lb*) about 50 A thick from the residual cytoplasm of the mother cell. 17 hours culture. × 76,000.

FIGS. 14 and 15. A dense boundary (*db*) about 50 A thick appears between the inner dense (*IL*) and outer light (*OL*) layers. Such membranous structures as the spore cortex membrane (*scm*), spore core wall (*scw*), or spore cytoplasmic membrane (*spm*) are clearly observed at this stage. Note that the process of the formation of layers or boundaries at the proximal part appears before the corresponding process at the distal side. 17 hours culture. Fig. 14, × 87,000; and Fig. 15, × 88,000.

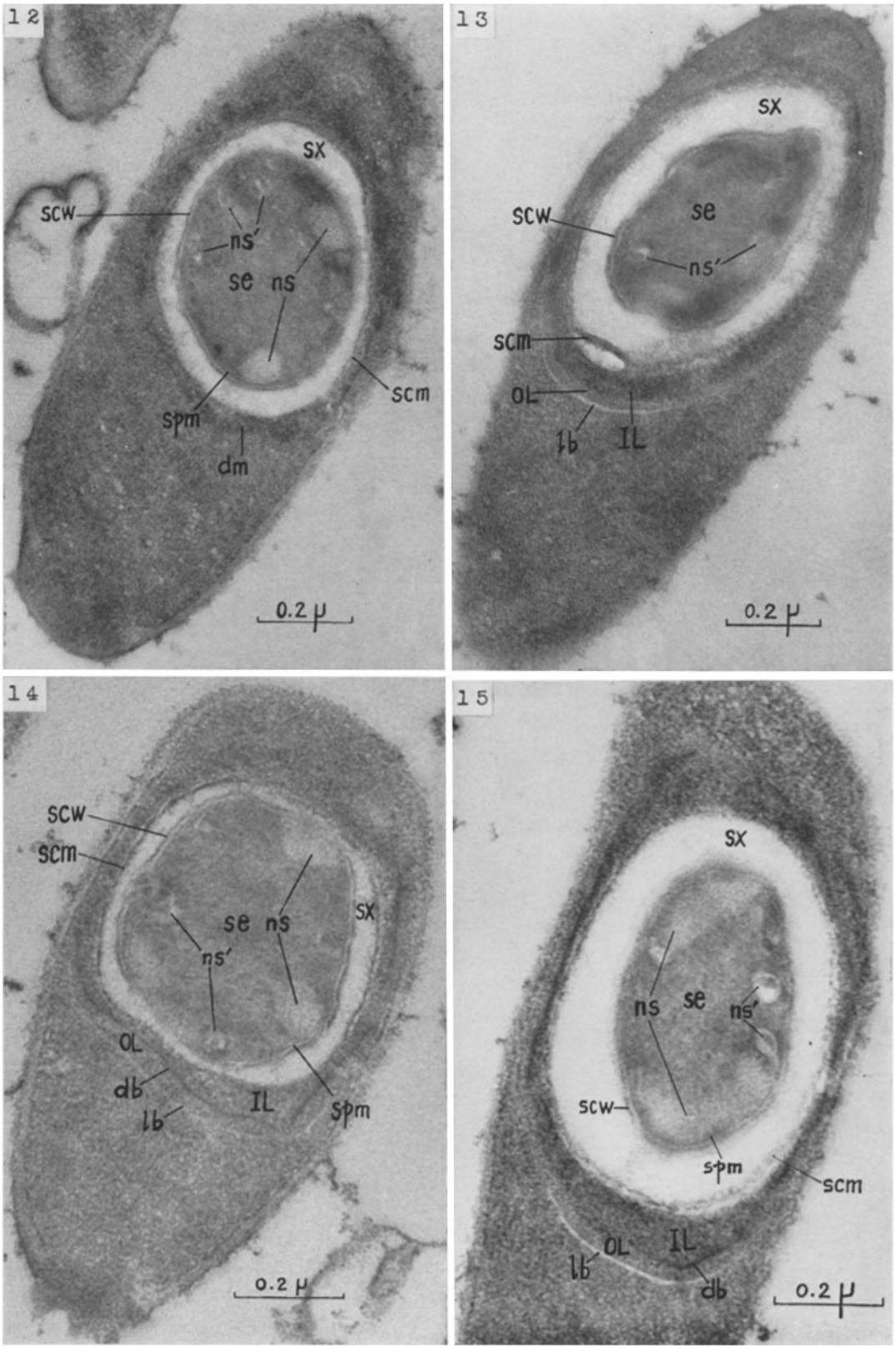

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PLATE 53

FIGS. 16 and 17. A lamination of fine membranes (*L*) is observed developing outside the dense boundary (*db*). The appearance of such lamellae and disappearance of the dense boundary seem to occur simultaneously. 17 hours culture. Fig. 16, × 99,000; and Fig. 17, × 97,000.

FIG. 18. An almost mature spore. The lamellae such as shown in Figs. 16 and 17 now form a layer (*LL*) between the inner and outer layers. Note that the dense boundary, marked with *db*, (a boundary as dense as that shown in Figs. 14 to 17) is not observable here. 17 hours culture. × 95,000.

FIG. 19. An enlargement of a part of a mature spore. The lamellae in the spore coat are readily seen. 17 hours culture. × 247, 000.

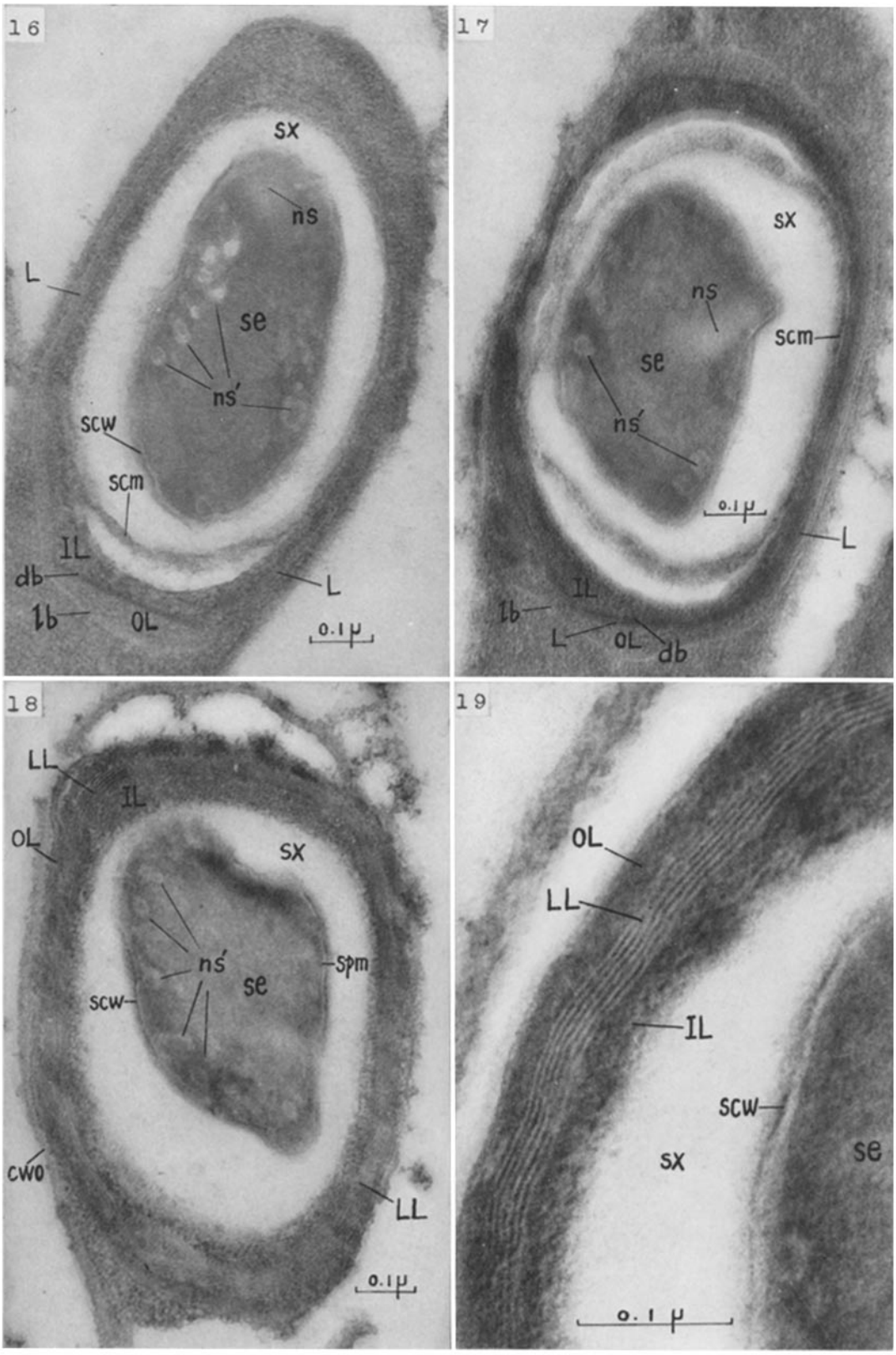

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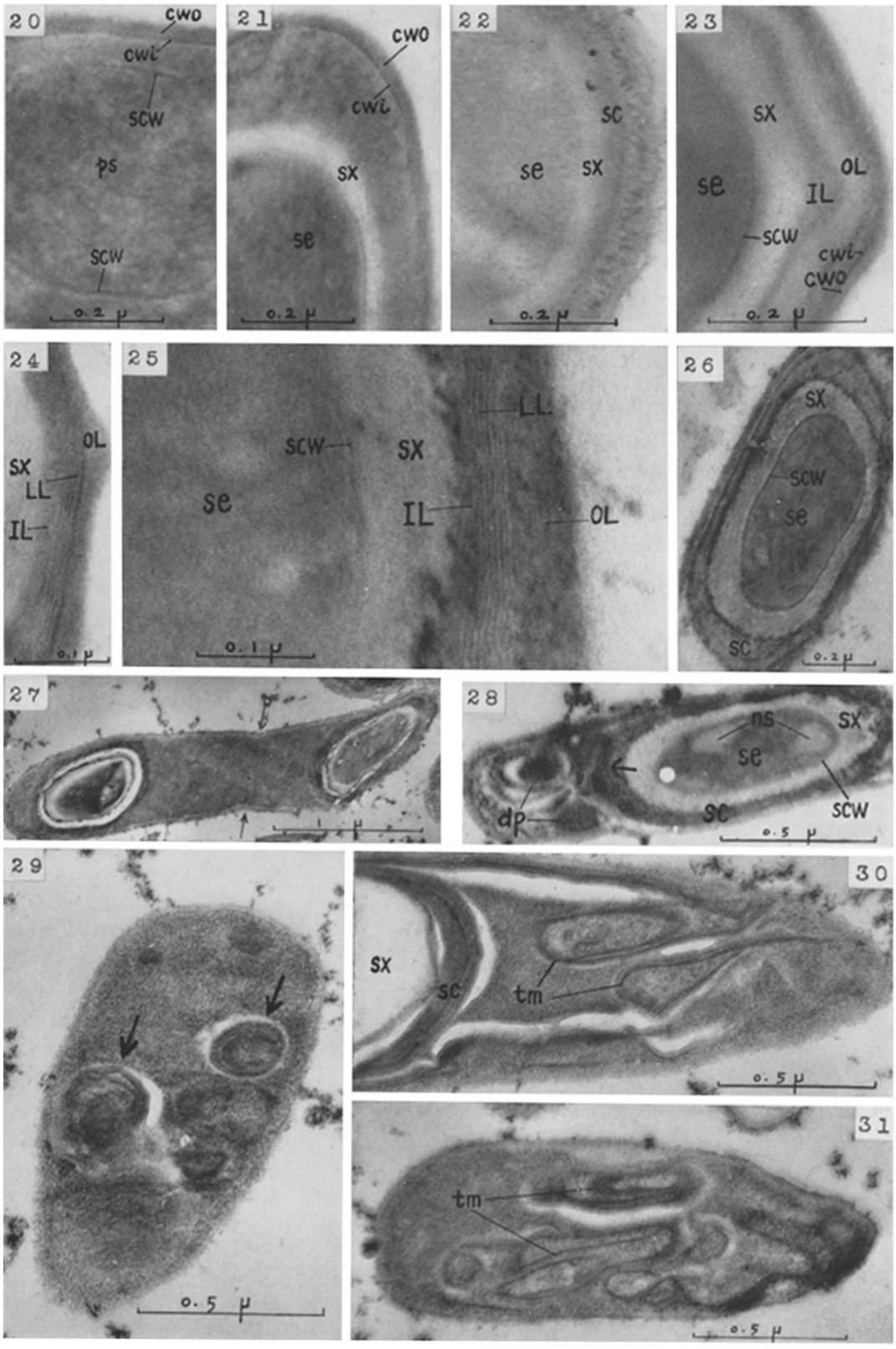
PLATE 54

FIGS. 20 to 23. A sequence in the sporulation process observed when fixed with OsO_4 in cells from 18 hours culture. The lamellae in the spore coat are not observable. The cell in Fig. 23 was fixed with 0.2 per cent OsO_4 solution in distilled water for 8 hours at 2°C. and further with 1.5 per cent OsO_4 in distilled water for more than 14 hours at 2°C. Figs. 20 and 21, × 90,000; Fig. 22, × 100,000; and Fig. 23, × 126,000.

FIG. 24. Occasionally, the lamellae in the spore coat are observed after OsO_4 fixation. The dimensions of the lamellae are in good agreement with those obtained with $KMnO_4$ fixation (see Fig. 19). 18 hours culture. × 150,000.

FIGS. 25 and 26. Cells of 18 hours culture, fixed at first with OsO_4 and subsequently with the mixture of OsO_4 and $KMnO_4$ solutions. The lamellar layer (*LL*) is clear in Fig. 25 and dimensions of lamellae agree well with those fixed with $KMnO_4$ or OsO_4 alone. The spore cortex (*sx*) in Fig. 26 shows a somewhat layered structure. Fig. 25, ×185,000; and Fig. 26, × 57,000.

FIGS. 27 to 31. Peculiar structures of probably abnormal sporulations. In Fig. 27, two sporulation sites are found in the seemingly single cell but a slight indication of division is observable at the plane marked with arrows. In Fig. 28, besides a typical sporulation site, two dense particles (*dp*) are observed partly surrounded with a lamellar structure, and the structure appears to be in continuity with the spore coat at the place marked with an arrow. Two round structures indicated with arrows in Fig. 29 appear to be like miniature spores. Tortuous membranous structures (*tm*) form loops in profiles of cytoplasm in Figs. 30 and 31, and regions limited with loops appear somewhat different from the rest of the cytoplasmic matrix. In Fig. 30, the tortuous membranes appear to be continuous with the spore coat (*sc*). Figs. 27 and 29 to 31, of cell from 17 hours culture; and Fig. 28, 18 hours culture. Fig. 27, × 24,000; Fig. 28, × 50,000; Fig. 29, × 59,000; Fig. 30, × 50,000; and Fig. 31, × 49,000.



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