Fine Structure of *Bacillus subtilis*

I. Fixation

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ABSTRACT

The fine structure of *Bacillus subtilis* has been studied by observing sections fixed in $KMnO_4$, OsO_4, or a combination of both. The majority of examinations were made in samples fixed in 2.0 per cent $KMnO_4$ in tap water. Samples were embedded in butyl methacrylate for sectioning.

In general, $KMnO_4$ fixation appeared to provide much better definition of the boundaries of various structures than did OsO_4. With either type of fixation, however, the surface structure of the cell appeared to consist of two components: cell wall and cytoplasmic membrane. Each of these, in turn, was observed to have a double aspect. The cell wall appeared to be composed of an outer part, broad and light, and an inner part, thin and dense. The cytoplasmic membrane appeared (at times, under $KMnO_4$ fixation) as two thin lines.

In cells fixed first with OsO_4 solution, and then refixed with a mixture of $KMnO_4$ and OsO_4 solutions, the features revealed were more or less a mixture of those revealed by each fixation alone.

A homogeneous, smooth structure, lacking a vacuole-like space, was identified as the nuclear structure in a form relatively free of artifacts.

Two unidentified structures were observed in the cytoplasm when *B. subtilis* was fixed with $KMnO_4$. One a tortuous, fine filamentous element associated with a narrow light space, was often found near the ends of cells, or attached to one end of the pre-spore. The other showed a special inner structure somewhat similar to cristae mitochondriales.

INTRODUCTION

Thread- or string-like structures in vacuoles of varying sizes in the central regions of bacteria have been described as nuclear structures (1–3). Other reports have shown that the morphology of such structures varies as the composition (4) or pH (5) of the fixative is changed. Robinow (6) has discussed the possibility that these effects are artifacts and has noted the difficulties involved in preparing bacterial sections for examination by electron microscopy (1).

In the present study, $KMnO_4$, OsO_4, or mixed solutions were used to fix *B. subtilis* under a wide variety of conditions. A homogeneous nuclear structure without a vacuole was observed almost invariably, but occasionally, filamentous networks with varying degrees of coarseness were found in less dense regions, to be referred to as vacuoles.

In sections of several strains of bacteria, a cytoplasmic membrane has been observed (7–9). A membrane was also recognized in the present strain, and with the $KMnO_4$ fixation, it sometimes appeared double.

A comparison of the characteristics of $KMnO_4$ and OsO_4 fixations showed that the main difference resides in the clearer definition of structural limits provided by the former solution.

Methods

Cells of *B. subtilis*, cultured on agar-agar media for 2 to 20 hours at 37°C., were suspended directly in

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the fixatives. The majority of fixations were made in the following ways: (*a*) For 4 to 6 hours at 2°C. with 2.0 per cent, or sometimes 1.5 per cent $KMnO_4$ in tap water. (*b*) For 4 hours, starting at 2°C., and ending at 15–25°C., with 2.0 per cent $KMnO_4$ in a buffered sucrose solution. (*c*) For 4 hours, under the same rising temperature conditions as (*b*) with 1.0 per cent OsO_4 in buffered sucrose. (*d*) For 8 hours at 2°C., with 0.2 per cent OsO_4 in distilled water and then for 14 hours more at 2°C. with a distilled water solution of 0.2 per cent OsO_4 and 0.5 per cent $KMnO_4$. The buffered sucrose solution used in (*b*) and (*c*) was that described by Caulfield (10), or a variant of it modified slightly to adjust tonicity. The pH and tonicity values of the fixative in each method were: (*a*) pH 6–6.5; 0.1–0.13 M; (*b*) pH 7.2–7.6; 0.34 M; (*c*) pH 7.2–7.6; 0.34 M; (*d*) pH 6–6.5; 0.01–0.04 M.

Other fixatives tried were 2 per cent $KMnO_4$ in buffered saline (11); 0.6 per cent $KMnO_4$ in veronal buffer (12); various concentrations of $KMnO_4$ or OsO_4 in tap and distilled water; and mixed solutions of $KMnO_4$ and OsO_4 in tap and distilled water. The fixing time was changed from 0.5 to 24 hours, and the temperature was varied from 2 to 37°C.

Method (*a*), above, appeared to be the essentially optimum for $KMnO_4$ fixation—at least for the present bacterial strain.

The samples were washed, dehydrated in increasing concentrations of ethanol, bathed in three changes of butyl methacrylate, and then in methacrylate plus catalyst which was polymerized at 45°C. for 24 hours. Sections were cut with a Porter-Blum microtome set at $\frac{1}{40}$ μ and later examined with either an Hitachi HS-5 or an HS-6 electron microscope.

OBSERVATIONS

Two envelopes are usually observable around the bacterial cell fixed with $KMnO_4$ or OsO_4 (Figs. 1 and 2). The inner envelope is the thinner of the two, and its thickness is substantially uniform—about 50 to 70 A (Figs. 1 and 2). The outer envelope has a wider range of thickness—about 100 to 150 A, is lighter in appearance than the inner envelope, and often has an irregular contour (Figs. 1, 6, 14, and 15). The free surface of the outer envelope appears better defined when fixed with $KMnO_4$ (Figs. 1, 6, 9, 11, 12, 14, and 15) than when fixed with OsO_4 (Fig. 2).

When the cytoplasm is artificially separated from these envelopes, during sectioning or by expanding sections with chloroform vapor, a thin membrane is found adhering to the cytoplasm (Figs. 3 to 5) and it becomes obvious that the inner envelope really consists of two subunits, one about 40 A thick with an affinity for the outer envelope, and the other about 30 A thick that adheres to the cytoplasm. This innermost membranous structure often appears to be double after $KMnO_4$ fixation (Figs. 6 and 14). These three components of the surface structure seem to represent the outer and inner layers of the cell wall, and the cytoplasmic membrane (7–9, 13, 14).

When *B. subtilis* is fixed with OsO_4, its cytoplasmic matrix shows a poorly defined granularity of 100 to 200 A (Figs. 2 to 5), whereas with $KMnO_4$ fixation the matrix possesses a fine and clearly uniform granularity of about 30 to 50 A (Figs. 1, 6, 9, 13, 14, 15). This granularity appears more sharply defined when carbon supporting films are used (Figs. 1, 6, 9, 13, 14, 15) than when formvar films are used (Figs. 7, 8, 11, 12).

A slightly less dense region with some granularity and no limiting membrane is often found in the central portion of the cell (Figs. 1, 3, 4, 7 to 10). At times, with the $KMnO_4$ fixation, some degree of granularity can be observed so that the region is not easily distinguishable from the cytoplasmic matrix (Figs. 9 and 10). Such regions seem to correspond to sites of nuclear material (1, 3, 15). They can be seen also in sporulating cells (Fig. 3). It should be noted that in some instances the periphery of these regions does appear slightly more dense than the surrounding cytoplasm (Figs. 3, 4, 7 to 10).

In the central portions of other cells, dense thread- or string-like structures can be seen within less dense, vacuole-like spaces (Figs. 11 and 12). They are rather similar to the nuclear structures reported by other observers (1, 2, 7). These structures are revealed in cells of *B. subtilis* fixed with a solution at pH 7.4, but when the pH of the fixative is 6.2, these structures are not discernible. Instead, fine filamentous networks are visible in the vacuoles (Fig. 13). Others have reported similar findings (3, 5). The relationship of these fibrous structures of variable coarseness to structures of homogeneous texture described above will be discussed later.

Besides the nuclear structures, two other elements are found in the cytoplasm. One is a tortuous, fine filamentous component accompanied by a narrow light space. It is often found near the ends of cells (Figs. 6 and 14), or, at times,

attached to one end of a pre-spore (Fig. 15). The other is round in profile, about 70 mμ in diameter, and consists of a circular limiting membrane and a matrix of medium density comparable to the cytoplasm proper (Fig. 16). Its internal structure, however, appears to differ from that of cytoplasm, but does resemble that of mitochondria. These two structures seem to be kinds of organelles of the bacterium rather than artifacts.

When the bacterial cells are fixed first with dilute OsO_4 solution and then with a mixed solution of OsO_4 and $KMnO_4$, dense granular components of about 100 A diameter are found in a rather agranular cytoplasmic matrix (Figs. 17 and 18). The same granular components can also be recognized around the nuclear material (Fig. 17), and sometimes they seem to aggregate into a round structure similar to the nuclear body (Fig. 18). The boundary of the outer layer of the cell wall (*cwo*) appears better defined by this method than by OsO_4 solution alone (Figs. 17 and 18).

DISCUSSION

As noted above, the surface structure of the bacterium seems to consist of the outer and inner layers of the cell wall, and the cytoplasmic membrane. The latter can be identified by its position adherent to the cytoplasm (7–9, 13, 14). The cell wall can be identified as such by its comparatively loose connection with the cytoplasm (7–9, 13, 14).

The cell walls of *Spirillum serpens* and *Bacillus cereus* have been reported to a single-layered (7, 8), and that of *Escherichia coli* as a three-layered wall (9). The cell wall of *B. subtilis* differs from these in that it is double-layered, and has a broad outer layer that may vary as much as 100 to 150 A in thickness and a peripheral boundary that is often irregular.

As for the apparently redundant cytoplasmic membrane, evident in $KMnO_4$-fixed cells, further confirmation would seem to be necessary before it can be accepted as a real element of fine structure.

The dense thread-like element and filamentous network in the central vacuoles of these cells do not appear at the same time in a single fixation. Each appears separately, as described above, depending on the pH of the fixative. This observation agrees qualitatively with the results of Kellenberger and Ryter (5). In contrast to these variable elements, the homogenous vacuole-free nuclear structure can be observed invariably under a wide variety of fixing conditions: pH 6–7.6; tonicity 0.01–0.34 M; temperature 2–37°C; $KMnO_4$ or OsO_4 fixatives. One may conclude then that when a fixative penetrates into cells sufficiently rapidly, the composition and pH of that fixative may not critically influence the appearance of the cellular structures. While in practice an ideally rapid penetration of the fixative may not occur, one may assume that the structures of some cells are probably only minimally altered.

This consideration supports the conclusion that, at least as far as the present bacterial strain is concerned, the homogeneous element is the nuclear structure as it appears when least altered by the fixation process. The other observed elements would, therefore, be artificial aggregates or precipitations of the homogeneous element. Such a nuclear structure has not been reported before in the literature and it may be that the specific bacterial strain used in these studies allows relatively rapid penetration of fixatives into its cytoplasm, even under a variety of conditions. The cytoplasmic membrane is known to be the main barrier to the passage of substances into or out of the cytoplasm (16); hence the specific reaction of a particular strain to any one fixative may be chiefly a function of this membrane.

In micrographs of many bacterial cells neither the homogeneous nuclear material nor the thread-like structures with vacuoles have been observed in the uniformly granular cytoplasmic matrix. Chapman and Kroll (7) reported that cells of *Spirillum serpens* from a young culture did not show any nuclear structure, whereas cells from older cultures showed dense thread-like nuclear structures in vacuoles. Whether the present observations reflect the improbability of finding such a nuclear structure in any single section of a cell, or whether at some growth phase the nuclear material takes a diffuse form that is difficult to recognize after ordinary fixations, is unknown at the present time. The electron microscope study of cellular and more especially nuclear structures in bacteria seems to require the development of special staining methods to supplement

simple impregnation of fixatives. When, for example, cells of *B. subtilis* from a 5-hour culture were fixed for 4 hours at 2°C. in a 1.5 per cent $KMnO_4$ solution saturated with phosphotungstic acid, such a fixation revealed in almost all cell profiles very dense granules attached to, or accumulated at the inner layers of cell walls or in central regions corresponding specifically to nuclear sites (Fig. 19).

As noted above, two other elements, besides the nuclear system, are sometimes found in the cytoplasm. One of these consists of tortuous filamentous structures accompanied by a narrow light space. Two are present at the upper and lower ends of the cell profile in Fig. 14 (marked *x*). When the profile is examined closely, several light regions can be recognized and these are somewhat similar to the nuclear structures shown in Figs. 1, 7 to 10, or 13. If they represent the nuclear material, then it would seem probable that they are in the process of nuclear division, since they are spread over such a large portion of the cell profile. The tortuous filaments (*x*) may then have some role in nuclear division (6). It is worth noting that the round body in Fig. 16 somewhat resembles the mitochondria-like structures of mycobacteria described by Shinohara, Fukushi, and Suzuki (17, 18).

One big difference between $KMnO_4$ and OsO_4 fixatives is that $KMnO_4$ reacts with carbohydrates such as sucrose or agar-agar, particularly at high temperature, to form brownish precipitants, while OsO_4 does not. For instance, in the cell in Fig. 11 fixed with the $KMnO_4$ + sucrose mixture dense precipitants can be seen attached to its walls and in the background.

At high temperature, OsO_4 fixes bacteria well (including the present strain (1, 19)), but $KMnO_4$ does not seem to give good results since it makes the cells brittle. This is true with $KMnO_4$ fixatives of various composition, and even at fixing temperatures as low as 2°C. The relatively high concentrations of $KMnO_4$ (2 per cent) used in these studies were necessary to compensate for the reduced reactivity of the fixative at low temperatures.

Using $KMnO_4$ fixation and epoxy resin embedding, Robertson found (20) that the membrane of the Schwann cell, including the mesaxon, appeared to consist of two equally dense thin lines and a light interspace, and on this basis he considered the hypothesis of Sjöstrand and Rhodin (21) that this membrane consists of two layers of different composition, namely protein and lipide to have been disproved. Robertson's observations should be interpreted with caution, however, since the characteristics of $KMnO_4$ fixation have not been sufficiently studied. In fact, one pertinent feature of the present study is the demonstration that $KMnO_4$ fixation preserve structures of different chemical composition (*e.g.*, peripheral boundary of the outer layer of the cell wall; granularity of the cytoplasm, etc.) apparently better than does OsO_4. This suggests that $KMnO_4$ reacts with a variety of substances, as yet unknown.

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Bibliography

1. Maaløe, O., and Birch-Anderson, A., On the organization of the "nuclear material" in *Salmonella typhimurium*, *in* Bacterial Anatomy, 6th Symposium of the Society for General Microbiology, Cambridge, University Press, 1956.
2. Birch-Anderson, A., Maaløe, O., and Sjöstrand, F. S., *Biochim. et Biophysica Acta*, 1953, **12**, 395.
3. Chapman, G. B., and Hillier, J., *J. Bact.*, 1953, **66**, 362.
4. Toda, T., Koike, M., Yuta, Y., Takeya, K., and Miyauchi, K., lecture at the meeting of Japan Society of Bacteriology, held at Okayama, April, 1958.
5. Kellenberger, E., and Ryter, A., *Experientia*, 1956, **17**, 420.
6. Robinow, C. F., The chromatin bodies of bacteria, *in* Bacterial Anatomy, 6th Symposium of the Society for General Microbiology, Cambridge, University Press, 1956.
7. Chapman, G. B., and Kroll, A. J., *J. Bact.*, 1957, **73**, 63.
8. Murray, R. G. E., *Canadian J. Microbiol.*, 1957, **3**, 531.
9. Kellenberger, E., and Ryter, A., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 323.
10. Caulfield, J. B., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 827.
11. Rhodin, J., Correlation of Ultrastructural Organization and Function in Normal and Experimentally Changed Proximal Convoluted Tubule Cells of the Mouse Kidney, Karolinska Institutet, Stockholm, Aktiebolaget Godvil, 1954, 1.
12. Luft, J. H., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 799.

13. Weibull, C., *J. Bact.*, 1953, **66,** 688.
14. Robinow, C. F., and Murray, R. G. E., *Exp. Cell Research*, 1953, **4,** 390.
15. Hillier, J., Mudd, S., and Smith, A. G., *J. Bact.*, 1949, **57,** 319.
16. Weibull, C., *Exp. Cell Research*, 1955, **9,** 139.
17. Shinohara, C., Fukushi, K., and Suzuki, J., *J. Bact.*, 1957, **74,** 413.
18. Shinohara, C., Fukushi, K., and Suzuki, J., *J. Electron Micr.*, 1958, **6,** 47.
19. Hannay, C. L., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 1001.
20. Robertson, J. D., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 1043.
21. Sjöstrand, F. S., and Rhodin, J., *Exp. Cell Research*, 1953, **4,** 426.

EXPLANATION OF PLATES

All figures are electron micrographs of sections of *B. subtilis*, or its parts, in vegetative or sporulation phases. The type of fixation used in each figure is indicated in the explanation by reference to the section on methods or by direct exposition. Abbreviations used in the explanation are as follows: *nm*, nuclear material; *cwo*, outer layer of cell wall; *cwi*, inner layer of cell wall; *cm*, cytoplasmic membrane; *im*, superimposed structure of *cwi* and *cm*; *dm*, dense material around nuclear material; *X*, tortuous filamentous structure of unknown nature; *Y*, mitochondria-like structure; *dg*, dense granule found in cytoplasm; *ns*, probable site of nuclear material; *sx*, spore cortex; and *ps*, pre-spore.

PLATE 47

FIG. 1. A part of a vegetative cell of 18 hours culture, fixed with 1 per cent $KMnO_4$ solution in tap water for 4.5 hours at 2°C. The outer broad envelope may be the outer layer of the cell wall (*cwo*) and the inner thin dense envelope (*im*) is presumed to represent the superimposition of the inner layer of the cell wall and the cytoplasmic membrane. Two structures of smooth (non-granular) appearance at the central portion of the cytoplasm seem to be sites of the nuclear material (*nm*). Note the well defined boundary of the outer layer of the cell wall and the homogeneous granularity of the cytoplasm. × 122,000.

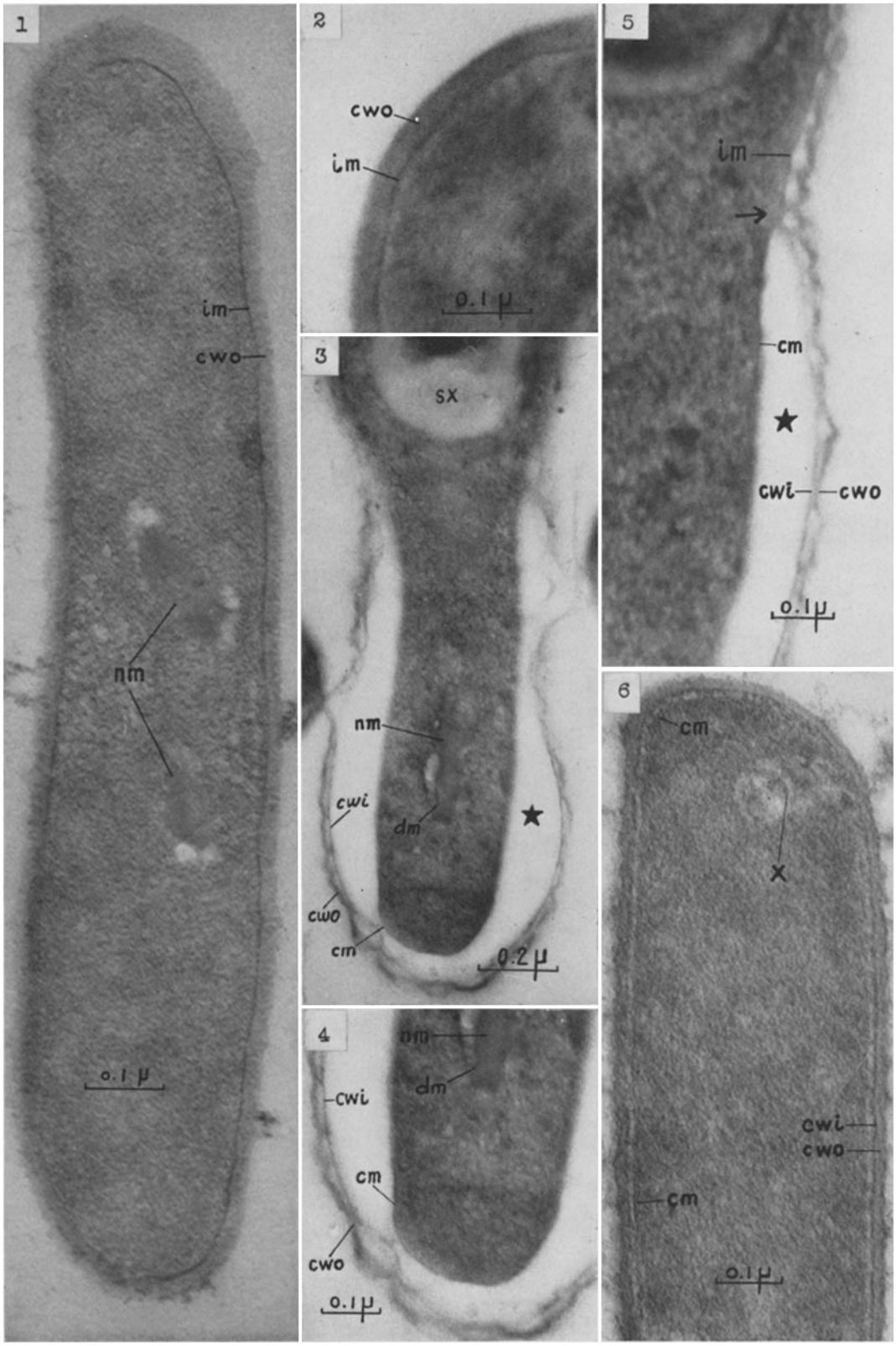
FIG. 2. A part of a vegetative cell of 18 hours culture, fixed with OsO_4 ((*c*) procedure). The boundary of the outer layer of the cell wall is not defined so well as the one in Fig. 1 and the cytoplasm appears rather agranular. × 140,000.

FIG. 3. Two-thirds of a sporulating cell of 18 hours culture, fixed with OsO_4 (*c*). The outer and inner layers of the cell wall (*cwo* and *cwi*) separated artificially from the cytoplasm, probably during sectioning or the expansion of the section with the chloroform vapor, since the separation space indicated with a star mark shows a lighter electron density than the surrounding background. At a point on the naked surface of the cytoplasm, a thin membrane is observed which may be the cytoplasmic membrane (*cm*). Smooth nuclear material (*nm*) can be observed with a small amount of vacuole-like space, possibly caused artificially during sectioning. × 63,000.

FIG. 4. An enlargement of a part of the cell in Fig. 3. The cytoplasmic membrane (*cm*) is obvious. × 92,000.

FIG. 5. A part of a sporulating cell of 18 hours culture, fixed with OsO_4 (*c*). The inner layer of the cell wall (*cwi*) and the cytoplasmic membrane (*cm*) join each other to form an apparently single membrane (*im*, see Figs. 1 or 2) at the position indicated with an arrow. The space resulting from the separation of the inner layer of the cell wall and the cytoplasmic membrane is indicated with a star mark. × 98,000.

FIG. 6. A part of a vegetative cell of 17 hours culture, fixed with $KMnO_4$ (*a*). The cytoplasmic membrane (*cm*) appears to be double in places. The structure indicated with the mark *X* seems to be not an artifact, but rather a real structure of unknown nature. × 105,000.



(Tokuyasu and Yamada: Fine structure of *B. subtilis*. I)

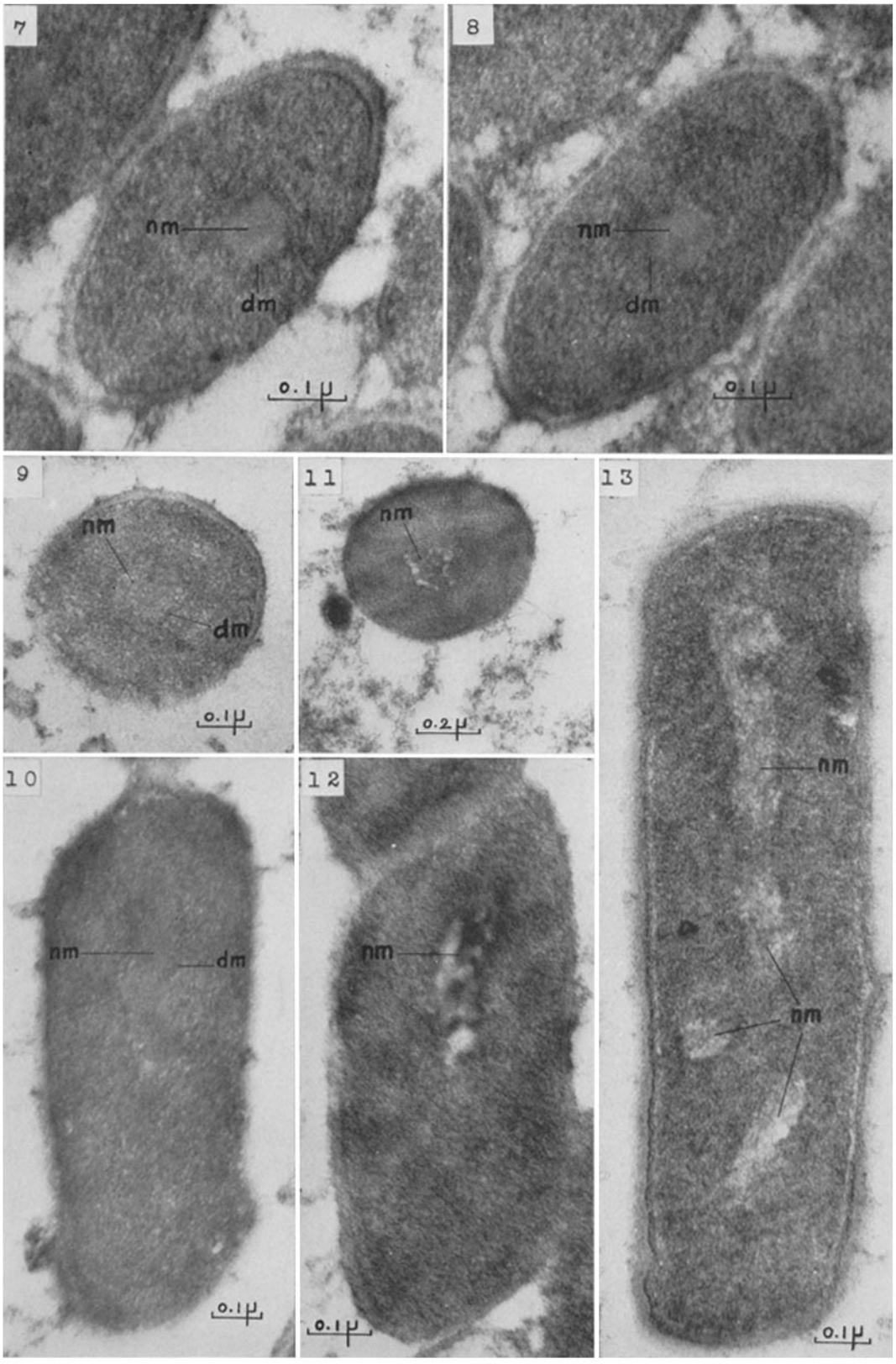
PLATE 48

FIGS. 7 and 8. Oblique sections of vegetative cells of 18 hours culture, fixed with $KMnO_4$ (*b*). The central, relatively agranular regions may be sites of nuclear material (*nm*). A dense material (*dm*) is observable around these regions. Both figures, × 122,000.

FIGS. 9 and 10. Cross- and longitudinal profiles of nuclear material in vegetative cells of 17 hours culture, fixed with $KMnO_4$ (*a*). The profiles (*nm*) are not so distinct as in the cases of Figs. 7 and 8 and are recognizable, mainly because of the surrounding dense material (*dm*). Fig. 9, × 88,000; and Fig. 10, × 80,000.

FIGS. 11 and 12. Cross- and longitudinal sections of vegetative cells of 18 hours culture, fixed with $KMnO_4$ (*b*; pH 7.4). Dense thread-like structures are observed in vacuoles. These configurations of nuclear material (*nm*) seem to have been produced artificially during fixation or embedding, from homogeneous nuclear structures such as shown in Figs. 1, 3, and 7 to 10. They probably do not express various phases of the nuclear system at different growth stages (see discussions in text). Dense particles outside the cell body in Fig. 11 seem to be artificial precipitants produced during the fixation. Fig. 11, × 50,000; and Fig. 12, × 103,000.

FIG. 13. A longitudinal section of a vegetative cell from a 15 hours culture, fixed with $KMnO_4$ (*a*, pH 6.2). Fine networks are observed in vacuoles and such nuclear structures probably represent artifacts (compare with those in Figs. 11 and 12 and note the difference of pH at fixatives of two cases). × 88,000.



(Tokuyasu and Yamada: Fine structure of *B. subtilis*. I)

PLATE 49

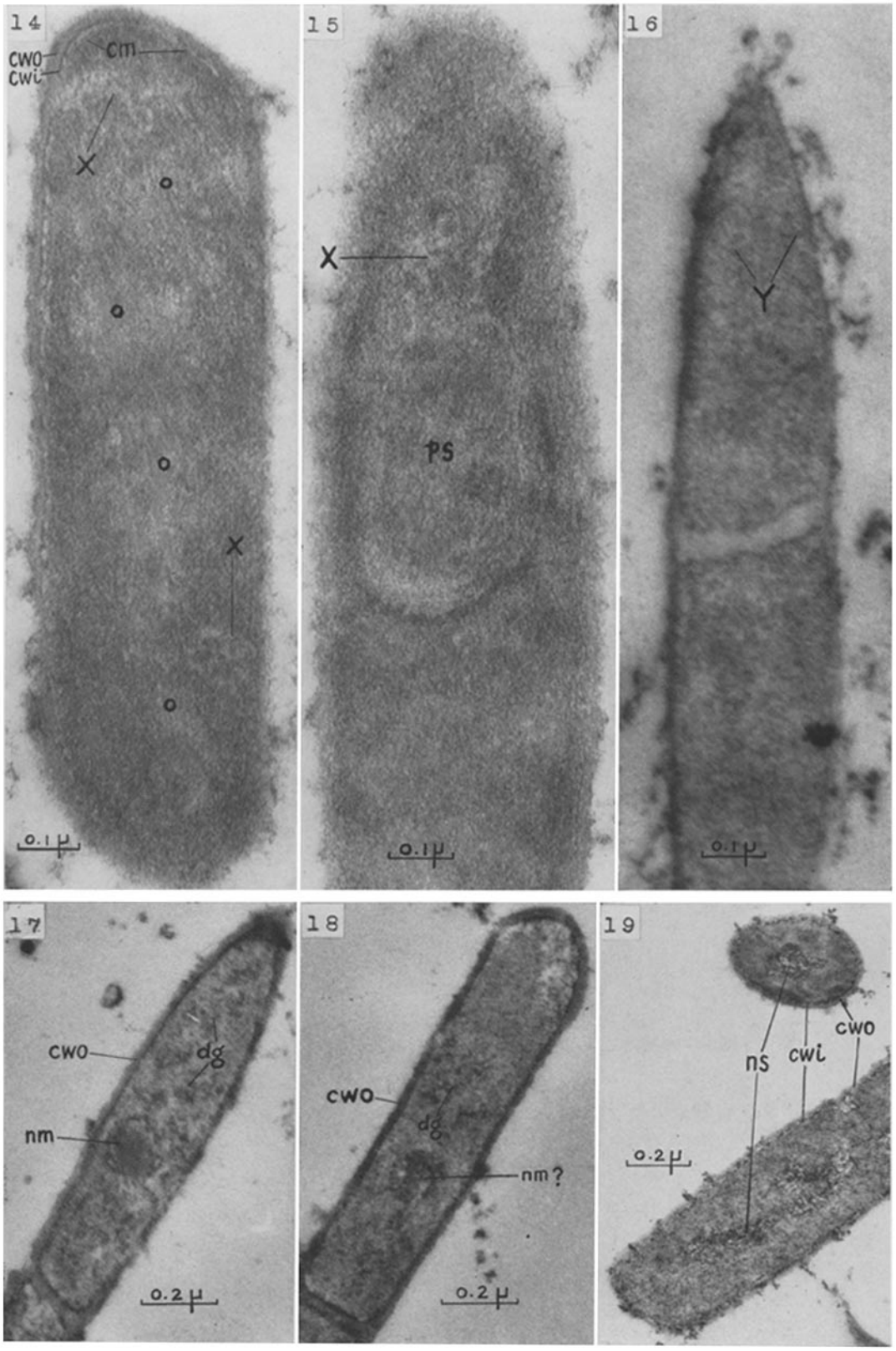
FIG. 14. A vegetative cell of 17 hours culture, fixed with $KMnO_4$ (*a*). The double-membranous nature of the cytoplasmic membrane (*cm*) is recognizable in places. Tortuous filamentous structures (*X*) with associated narrow light spaces are observed near the ends of the cell. The one near the upper end may represent a longitudinal section, while that at the lower end a cross-section through the structure. Regions marked with *o* are lighter than the rest of the cytoplasm and possibly are sites of nuclear material. × 98,000.

FIG. 15. A part of a sporulating cell of 17 hours culture, fixed with $KMnO_4$ (*a*). A structure similar to that marked *X* in Figs. 6 and 14 is found attached to the pre-spore (*ps*). × 102,000.

FIG. 16. Two connecting vegetative cells of 18 hours culture, fixed with $KMnO_4$ (*b*). Two round structures (*Y*) are found near the distal end of the upper cell; they show inner structures somewhat similar to the so called cristae mitochondriales. × 100,000.

FIGS. 17 and 18. Vegetative cells of 18 hours culture, fixed at first with OsO_4 and subsequently with the mixture of OsO_4 and $KMnO_4$ solutions (*d*). Dense granular components (*dg*) are found throughout the cytoplasmic matrix which appears rather agranular. In Fig. 17, these components are also recognized around the nuclear material (*nm*). It is unknown whether the dense round structure (*nm?*) in Fig. 18 is the site of the nuclear material or an aggregate of the granular components. The boundary of the outer layer of the cell wall (*cwo*) appears less clearly defined than in the case of the $KMnO_4$-fixation but better defined than in the case of the OsO_4-fixation. Both figures, × 61,000.

FIG. 19. Vegetative cells of 5 hours culture, fixed with a 1.5 per cent $KMnO_4$ tap water solution saturated with phosphotungstic acid for 4 hours at 2°C. Note dense depositions at the inner layer of the cell wall (*cwi*) and the central region corresponding to the nuclear site (*ns*). × 50,000.



(Tokuyasu and Yamada: Fine structure of *B. subtilis*. I)