FINE STRUCTURES OF INTRACYTOPLASMIC ORGANELLES OF MYCOBACTERIA

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ABSTRACT

The fine structure of the intracytoplasmic organelles of mycobacteria was studied by means of electron microscopy of ultrathin sections. A well-preserved nuclear apparatus was obtained by fixation with OsO_4 in acetate-veronal buffer, containing calcium and tryptone, or in collidine-HCl buffer, followed by uranyl-acetate treatment and embedding in araldite. A low density nuclear region was filled with fine fibrils, 30 A in diameter, in parallel or concentric arrangement. A membranous organelle, tentatively designated as "lamellar structure," consists of unit membranes in lamellar arrangement. The thickness of each lamella in this membranous organelle coincides with that of the three-layered cytoplasmic membrane Moreover, the continuity of this unit membrane with the cytoplasmic membrane was demonstrated.

INTRODUCTION

Electron microscope studies of ultrathin sections of mycobacteria have been reported by the present authors (1, 2), Bassermann (3), Brieger and Glauert (4), Shinohara *et al.* (5, 6), and Zapf (7, 8). According to these investigators, the nuclear apparatus was described as a nuclear vacuole with thread-like, coarse fibrillar, and/or granular nuclear material. In addition, a membranous structure, which was originally considered to correspond to mitochondria of the cells of higher organisms, has recently been reported to be composed of unit membranes thinner than cristae mitochondriales (2). This paper will present new findings on these organelles, obtained with an improved preparative technic. This work has been reported in preliminary form (9, 11).

MATERIALS AND METHODS

Bacteria and Cultivation: Mycobacterium Jucho strain (12–14) was used throughout the experiment. The cells were cultivated in 4 per cent glycerol broth at 37°C. for 16 hours with aeration.

Fixation: Fixation was carried out by the following four different methods.

(*1*) Following concentration by centrifugation, the cells were fixed by being suspended in 1 per cent OsO_4 solution in isotonic potassium-dichromate buffer (pH 7.4) (15) for 30 minutes at room temperature.

(*2*) One per cent OsO_4 solution in acetate-veronal buffer (pH 6.1) containing $CaCl_2$ in 0.01 M was prepared according to Ryter and Kellenberger (16). The culture was mixed with one-tenth of its volume of the fixative and centrifuged immediately. The cells collected were then fixed by being resuspended in the fixative solution containing one-tenth volume of tryptone medium and allowed to stand overnight at room temperature.

(*3*) The same procedure was employed, except the pH of the acetate-veronal buffer was changed to 7.4.

(*4*) After centrifugation the cells were fixed with 1 per cent OsO_4 solution in collidine-HCl buffer (pH 7.4) (17) overnight at room temperature. *Uranyl-Acetate Treatment:* The fixed cells were mixed well with melted 2 per cent agar. Small blocks of agar containing fixed cells were treated with 0.5

per cent uranyl-acetate solution in acetate-veronal buffer (16).

Embedding: The fixed cells were dehydrated in increasing concentrations of alcohol or acetone followed by embedding respectively in methacrylate or araldite (18).

Embedding in Araldite

Dehydration: 20 minutes in 30, 50, 70, and 90 per cent acetone solutions, followed by 30 minutes each in three changes of absolute acetone.

Impregnation: The specimens were immersed in a 1:3 mixture of freshly prepared embedding material (araldite, 10 ml.; hardener 964, 10 ml.; accelerator 964, 0.5 ml.; dibutyl phthalate 0.7 ml.) and absolute acetone for 30 minutes, followed by immersion for 30 minutes each in a 1:1 mixture and a 3:1 mixture at room temperature.

Embedding: The specimens were put into gelatin capsules filled with the embedding material and incubated at 30° to 35°C. overnight, at 45°C. for a day, and then at 60°C. for one or two days, until polymerization of the embedding material was completed.

Embedding in Methacrylate

Dehydration: 20 minutes in 70, 90, and 95 per cent alcohol solutions followed by 30 minutes each in three changes of absolute alcohol.

Impregnation: The specimens were immersed in a 1:1 mixture of *n*-butyl metacrylate monomer and absolute alcohol for 30 minutes, and then stored in an ice box overnight.

Embedding: The specimens were put into gelatin capsules filled with an 8:2 mixture of *n*-butyl methacrylate and methyl methacrylate monomer to which 2 per cent benzoyl peroxide had been added, and were kept in a 45°C. oven overnight until polymerization of embedding material was completed.

Sectioning

Sections were cut with glass knives using a Servall Porter-Blum microtome and were picked up on carbon-celloidin-coated copper mesh screens.

Electron Microscope

A JEM 5C electron microscope was used.

Explanation of Figures

KEY TO ABBREVIATIONS

CW, cell wall
CM, cytoplasmic membrane
N, nuclear apparatus
L, "lamellar structure"
P, polyphosphate granule

All of the figures are ultrathin sections of the cells of *Mycobacterium* Jucho strain. In each figure, the magnification mark indicates 0.1 μ.

FIGURE 1

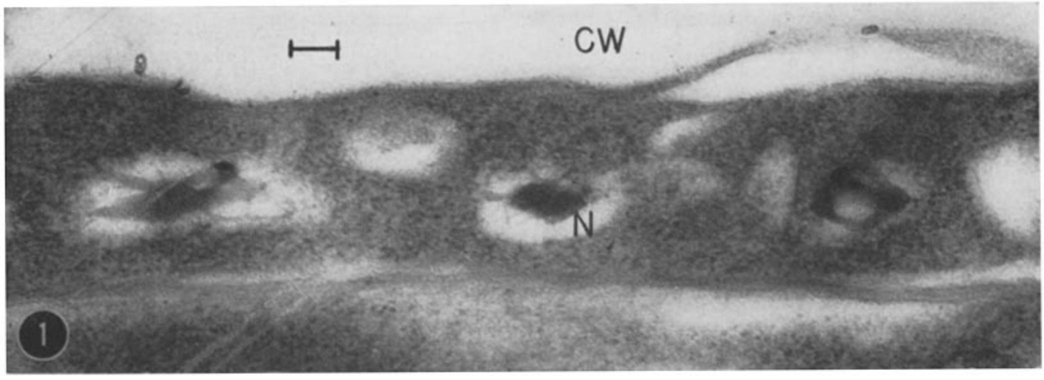
A cell fixed with 1 per cent OsO_4 in potassium dichromate buffer (pH 7.4) followed by embedding in methacrylate. The nuclear material is conglomerated in the nuclear vacuole. At the upper right corner the cell wall is detached from the cell body presumably as a result of an "explosion" during embedding. $\times$ 53,000.

FIGURE 2

A cell fixed with 1 per cent OsO_4 in potassium dichromate buffer followed by uranyl-acetate treatment and araldite embedding. The nuclear material is conglomerated in the nuclear vacuole. A lamellar structure is observable but its fine details are lost due to inadequate fixation. $\times$ 79,000.

FIGURES 3 and 4

Cells fixed with 1 per cent OsO_4 in acetate-veronal buffer (pH 6.1) followed by uranyl-acetate treatment and araldite embedding. The nuclear apparatus is filled with fine fibrils in parallel arrangement. No nuclear membrane is observable. The cell wall and the cytoplasmic membrane are composed respectively of two dense inner and outer layers and a less dense interspace. Fig. 3, $\times$ 74,000; Fig. 4, $\times$ 80,000.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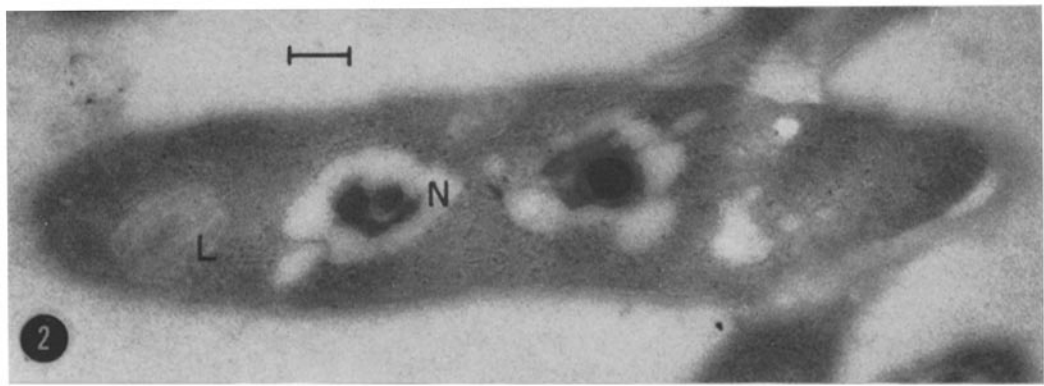
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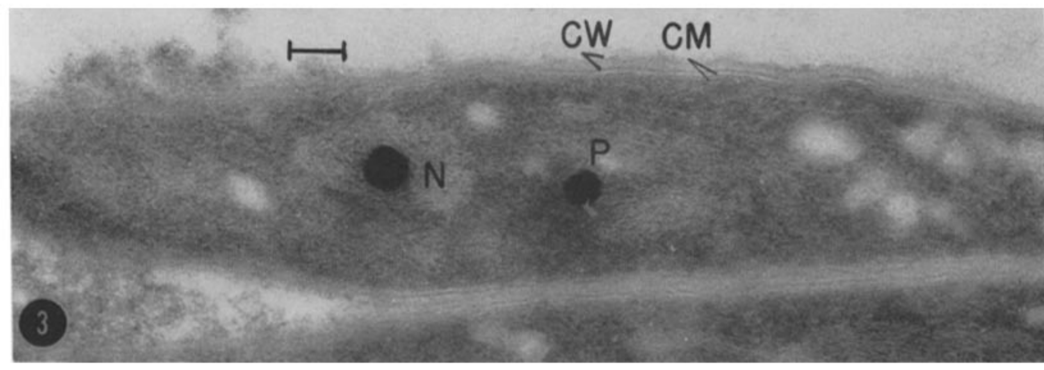
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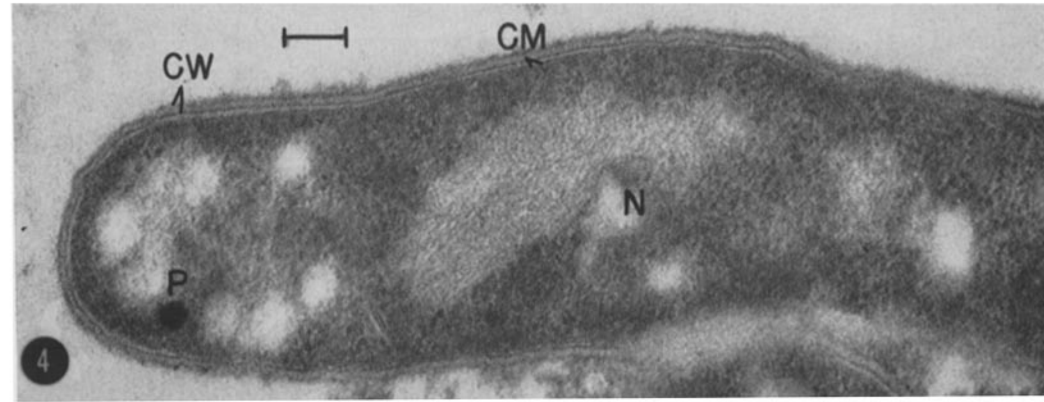
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OBSERVATIONS

The nuclear material was conglomerated in nuclear vacuoles in the specimens fixed with 1 per cent OsO_4 in potassium dichromate buffer and embedded in methacrylate (Fig. 1), although this method of preparation was found to be suitable for demonstrating the cell wall and other membranous systems (2). Fixation with the same solution followed by uranyl-acetate treatment and araldite embedding presented essentially the same result (Fig. 2). On the other hand, a nuclear structure similar to that shown in *Escherichia coli,* and accepted as the real structure of the bacterial nucleoid by Kellenberger *et al.* (19), was obtained by fixation with 1 per cent OsO_4 in acetate-veronal buffer followed by uranyl-acetate treatment and araldite embedding (Figs. 3 to 8). Fine fibrils, 30 A in diameter, fill the less dense nuclear regions, and parallel and/or concentric arrangements of the fibrils were often observed (Figs. 3 to 8). When the pH of the fixative was altered to 7.4, almost the same nuclear structure was obtained but the contour of the nuclear region appeared to become somewhat vague (Fig. 9). The more or less coarse coagulation of the nuclear material was found when uranyl-acetate treatment was omitted (Fig. 10). Fixation with 1 per cent OsO_4 in collidine-HCl buffer followed by uranyl-acetate treatment and embedding in araldite also revealed a well-preserved nuclear structure filled with fine fibrils (Fig. 11).

A membranous structure which was tentatively designated as "lamellar structure" (9, 10) was clearly observable in all the preparations examined (Figs. 12 to 21). This structure has no limiting membrane. The arrangement of the unit membrane found in this structure usually appears lamellar, like the cross-section of an onion (Figs. 12 to 19), and is entirely different from the arrangement of the cristae mitochondriales seen in cells of higher organisms. The thickness of each of the inner and outer dense layers was 25 A and the lighter interspace was 30 A in width. These dimensions are much smaller than those of the cristae mitochondriales and coincide well with those of the three-layered cytoplasmic membrane (9, 18). A parallel arrangement with irregular contour was also occasionally observed (Figs. 20, 21). This membranous structure was also clearly demonstrated in the specimens fixed with 1 per cent OsO_4 in potassium dichromate buffer as reported before (2). It is very interesting that the unit membranes appear to be continuous with the three-layered cytoplasmic membrane (10) (Figs. 12, 13, 16, 17).

Fig. 22 is a schematic representation of the fine structure of an ultrathin section of *Mycobacterium.*

DISCUSSION

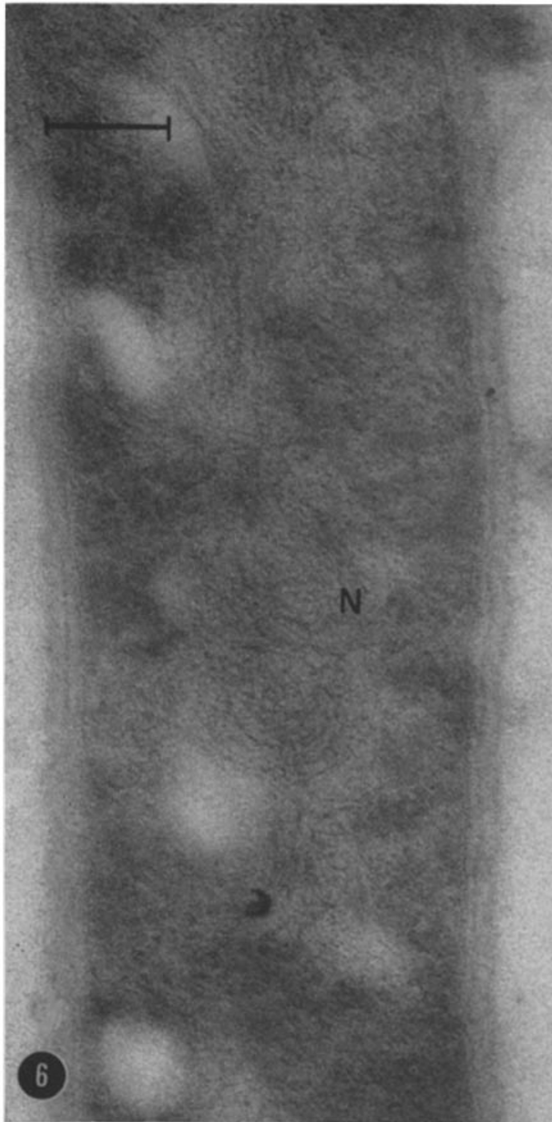
Despite extensive studies the real structure of the nuclear apparatus in ultrathin sections of bacteria is still controversial. The appearance of the nuclear apparatus has been reported to be easily affected by change of electrolyte concentration in the cell environment, by light microscopy (20), and by electron microscopy (21, 22). The preservation of the nuclear apparatus was also found by the present authors (2) to be greatly affected by the composition of the fixative solution. On the other hand, effort has been made by Maaløe and Birch-Andersen (23), Ryter and Kellenberger (24), and Glauert and Glauert (18) to find a better embedding material than methacrylate for preserving the cell constituents. In most of the micrographs hitherto reported the nuclear apparatus was shown as a vacuole-like region filled with

FIGURE 5

Cells fixed with 1 per cent OsO_4 in acetate-veronal buffer (pH 6.1) followed by uranyl-acetate treatment and araldite embedding. The nuclear apparatus is filled with fine fibrils in parallel and/or concentric arrangement. A "lamellar structure" composed of thin unit membranes is observable. The three-layered cell wall and also the three-layered cytoplasmic membrane are clearly seen. × 127,000.

FIGURE 6

Higher magnification of the same bacterium as in Fig. 5. Fine fibrils, about 30 A in diameter, fill the less dense nuclear region. × 170,000. Figs. 6 and 7 originally appeared in references 9 and 10 and are republished here.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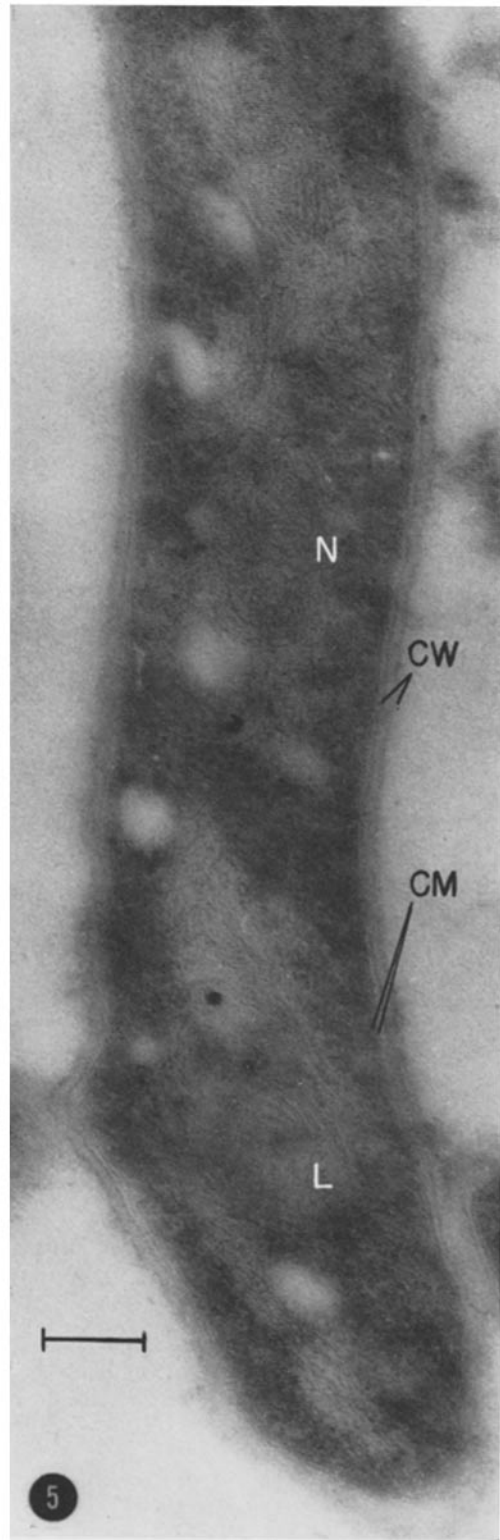
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threads, coarse fibrils, and/or fine granules, whereas a homogeneous, smooth structure, lacking a vacuole-like space, was reported as the nuclear structure by Tokuyasu and Yamada (22) using $KNnO_4$ fixation. Recently, Ryter and Kellenberger (16) have succeeded in obtaining a well-preserved nuclear apparatus filled with fine-stranded fibrils in several bacterial species. Kellenberger *et al.* (19) have discussed the nature of the bacterial nuclear apparatus based on the understanding that this represents the real nuclear structure, independently of the physiological state of bacteria. On the other hand, Chapman (25) inclines to believe the thread-like and the fibrillar structures are alternative forms of the chromatin material. This opinion is supported by the findings of Chapman and Kroll (26) and Preusser (27) that the size, shape, and content of the nuclear apparatus vary according to the age of the culture.

In this experiment the nuclear apparatus of mycobacteria, obtained by fixation with 1 per cent OsO_4 in acetate-veronal buffer followed by uranyl-acetate treatment and araldite embedding, showed a similar appearance to that observed by Ryter and Kellenberger (16) in other bacterial species. The uranyl-acetate treatment was found indispensable for preserving the nuclear structure, but the pH of the fixative solution could be changed to neutral. The buffer could also be replaced by collidine-HCl buffer. The nuclear structure presented by Ryter and Kellenberger (16) and in this experiment is considered to be preserved in a most significant way and appears to represent the real nuclear structure, at least of actively growing bacteria, though several investigators, including Chapman (25), have still reserved judgment as to the significance of this structure.

A mitochondrion-like structure was first reported by Shinohara *et al.* (5) and Zapf (7) in mycobacteria and was considered to be equivalent to mitochondria of higher organisms. Later, a similar structure was found in human-type tubercle bacilli by Yoshida *et al.* (28). Further detailed observation with improved technic for the preparation of specimens by Takeya *et al.* (2) has revealed that this structure is composed of extremely thin unit membranes, the thickness of which is much less than that of the cristae mitochondriales. This structure was described by Shinohara *et al.* (6) as round or elliptical in shape and as separated from the cytoplasm by a distinct membrane. However, the method of fixation and embedding used in their experiments seems to have been inadequate because coarse aggregation was seen everywhere in the cytoplasm and the nuclear region. In the present experiment, the technic used for preparation seems to be adequate to preserve and reveal fine details of the structure, because other cellular constituents were demonstrated with finer details than those so far reported. As the arrangement of these membranes was found to be usually lamellar, this structure was tentatively designated as "lamellar structure" (9, 10). The striking finding which was obtained in this experiment is that this unit membrane system appears to be continuous with the three-layered cytoplasmic membrane. The fact that the measurement of each layer of the membrane in the structure coincides well with that of the corresponding layers of the cytoplasmic membrane will support

FIGURES 7 and 8

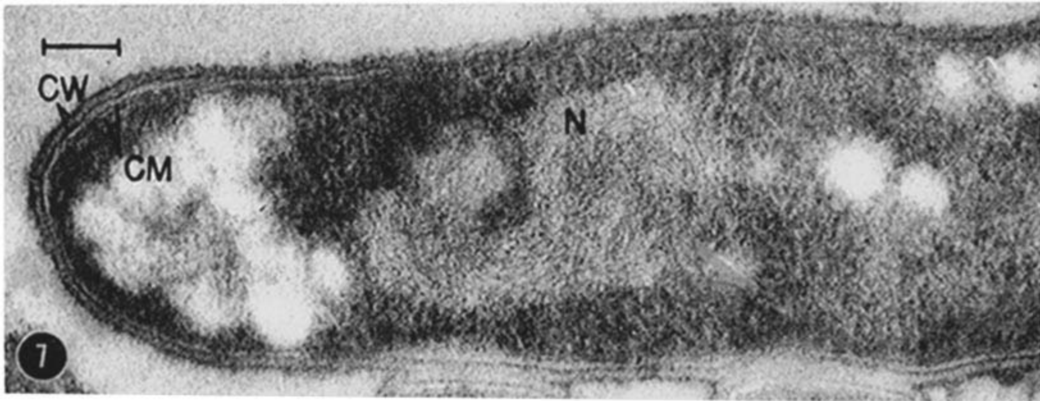
Cells fixed with 1 per cent OsO_4 in acetate-veronal buffer (pH 6.1) followed by uranyl-acetate treatment and araldite embedding. Fine fibrils in parallel and/or concentric arrangement fill the less dense nuclear region. A dense polyphosphate granule is observable in Fig. 8. Fig. 7, × 96,000; Fig. 8, × 80,000.

FIGURE 9

Cells fixed with 1 per cent OsO_4 in acetate-veronal buffer (pH 7.4) followed by uranyl-acetate treatment and araldite embedding. The nuclear apparatus is filled with fine fibrils but the contour of the nuclear region is somewhat vague. × 82,000.

FIGURE 10

Cells fixed with 1 per cent OsO_4 in acetate-veronal buffer (pH 6.1) followed by embedding in araldite. Coagulation of the nuclear material is seen. × 82,000.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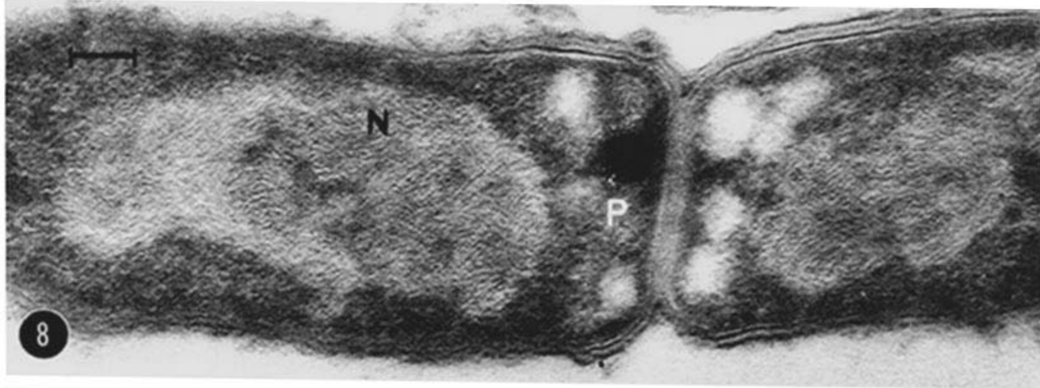
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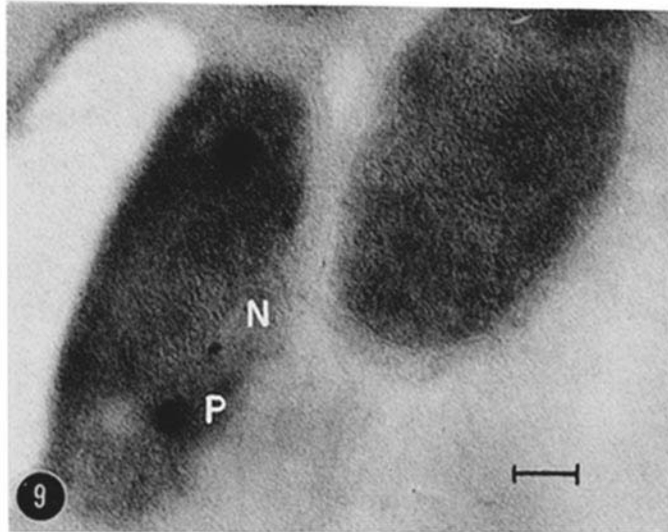
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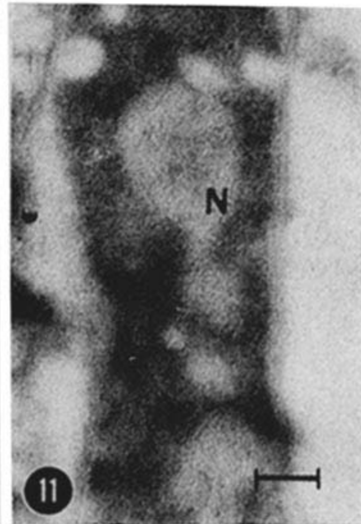
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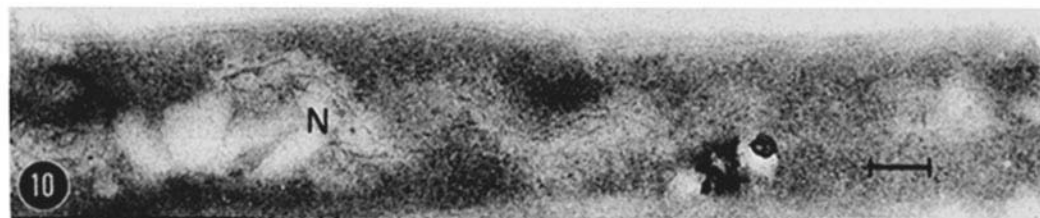
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this finding. This structure has no limiting membrane. The limiting membrane reported by Shinohara *et al.* (6) might have been an artifact. The continuity of the unit membrane with the cytoplasmic membrane, and the absence of a limiting membrane, indicate that the "lamellar structure" is a folded-up extension of the cytoplasmic membrane into the cytoplasm.

The enzymic activity of the cytoplasmic membrane has recently been extensively investigated by many biochemists. According to Mitchell (29), the cytoplasmic membrane of *Staphylococcus aureus* contains more than 90 per cent of the recoverable cytochrome, several dehydrogenases, and acid phosphatase. Moreover, the initial stages of protein synthesis in *Bacillus megaterium* are reported by Butler *et al.* (30) to take place at sites on or closely associated with the cytoplasmic membrane. These findings indicate that the cytoplasmic membrane plays an important part in the metabolism of bacteria. The extension and concentration of the cytoplasmic membrane into the "lamellar structure" may serve in intensifying the metabolic activity of the bacteria. Circumscribed sites of oxidation-reduction in mycobacteria were observed by mitochondrial stains in both light and electron microscopy (31, 32). Moreover, reduced tellurite was found localized as crystals (150 A × 200 to 1000 A) or fine particulate deposits of high density (30 to 100 A in diameter) in discrete regions of reducing sites by electron microscopy (2). The extension of the cytoplasmic membrane into the "lamellar structure" may account for these findings. The same kind of lamellar membranous structure was recently reported in *Bacillus subtilis* (16), in *Streptomyces* (33), and in *Mycobacterium lepraemurium* (34). In the latter two organisms essentially the same pictures as those obtained in this experiment, which show continuity of the membranous system with the cytoplasmic membrane, have been presented.

The cytoplasmic membrane of bacteria appears to correspond to the cell membrane of cells of higher organisms. Therefore, the extension and concentration of the cytoplasmic membrane into the "lamellar structure" may suggest the phylogenetic origin of the membranous systems, *i.e.*, the endoplasmic reticulum, the Golgi apparatus, and the mitochondria in the cells of higher organisms. It is conceivable that in the process of evolution the cell membrane originally infolds into the cytoplasm and differentiates into independent organelles according to their specialized functions. The continuity of the cell membrane with the endoplasmic reticulum reported by Palade (35) supports this speculation.

Addendum: Just after the completion of this report, a paper by Glauert and Hopwood on the fine structure of *Streptomyces* (37) appeared. Their considerations on the function of the cytoplasmic membrane system

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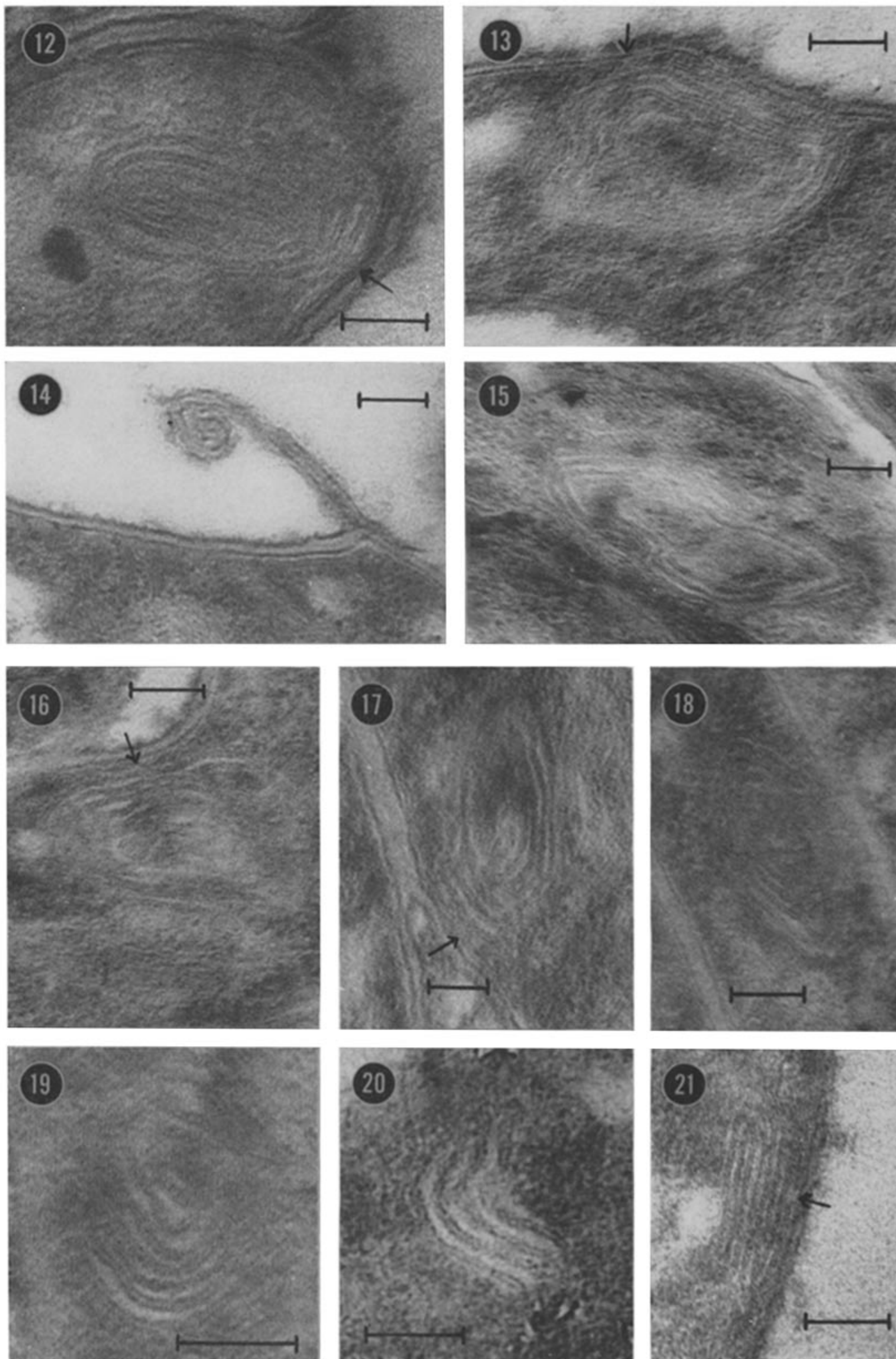
FIGURE 11

A cell fixed with 1 per cent OsO_4 in collidine-HCl buffer (pH 7.4) followed by uranyl-acetate treatment and araldite embedding. The nuclear apparatus is filled with fine fibrils. × 71,000.

FIGURES 12 through 21

Cells fixed with 1 per cent OsO_4 in acetate-veronal buffer (pH 6.1) followed by uranyl-acetate treatment and araldite embedding. All the figures represent the "lamellar structure" composed of extremely thin unit membranes. The dimensions of each layer of the membrane in the structure coincide well with those of the corresponding layers of the cytoplasmic membrane. The continuity between the unit membrane and the cytoplasmic membrane is shown in Figs. 12, 13, 16, 17, and 21 (arrows). The arrangement of the unit membrane looks like a cross-section of an onion, as shown in Figs. 12, 13, 15, 19, or is occasionally found to be parallel, as shown in Figs. 20 and 21. Two firmly adhered cells, the upper a ghost and the lower an intact cell, are shown in Fig. 14. A lamellar structure of the upper ghost cell still remains, showing the continuity with the cytoplasmic membrane. (*Cf.* with Fig. 19 in Takeya *et al.* (36).) Fig. 12, × 127,500; Fig. 13, × 121,000; Fig. 14, × 97,500; Fig. 15, × 101,000; Fig. 16, × 110,000; Fig. 17, × 90,000; Fig. 18, × 115,000; Fig. 19, × 180,000; Fig. 20, × 150,000; Fig. 21, × 125,000.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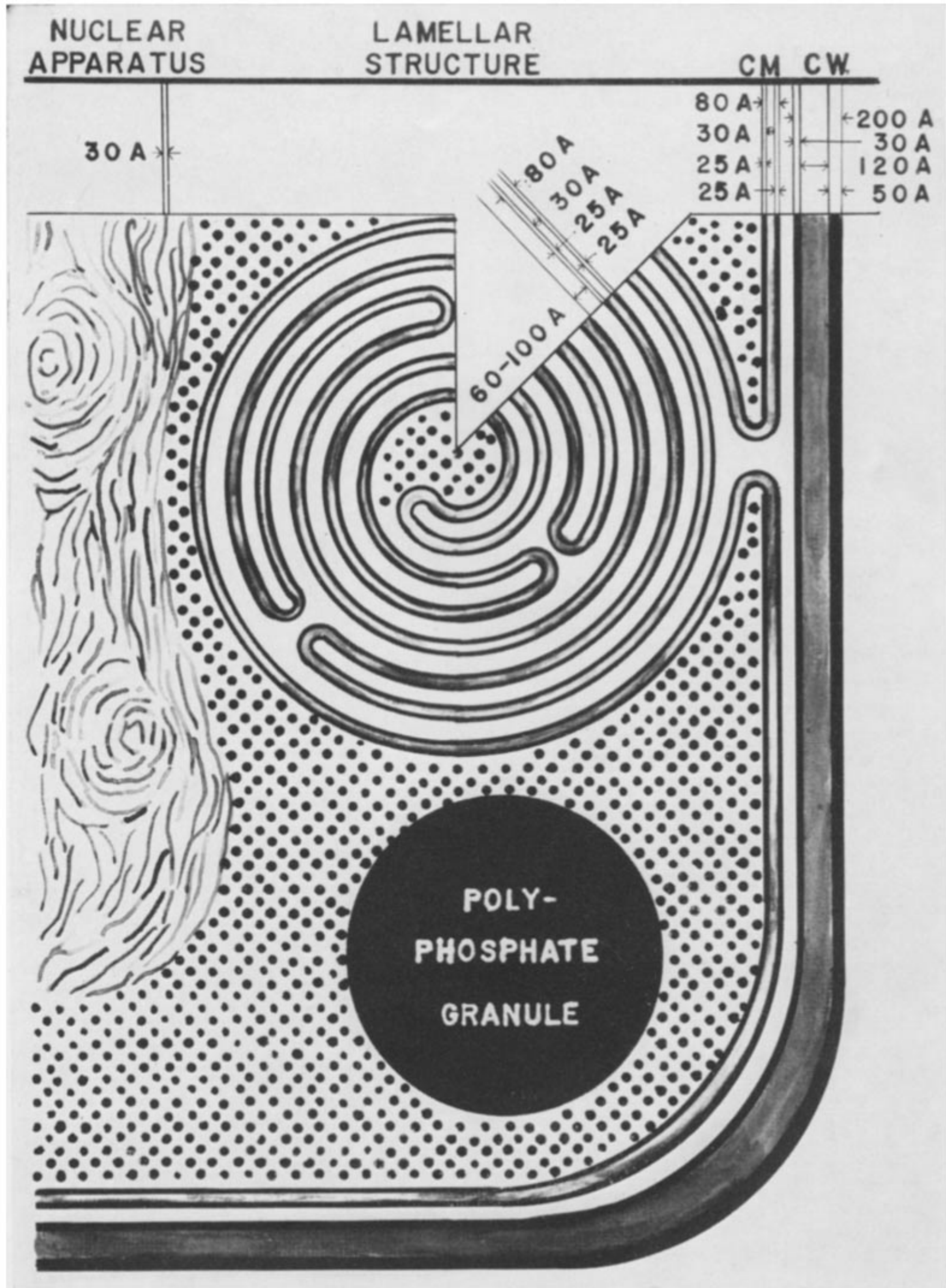

FIGURE 22

A schematic representation of the fine structure of an ultrathin section of *Mycobacterium*.

in *Streptomyces* essentially agree with those described for *Mycobacterium* in this paper. More recently, a paper by Giesbrecht (38) has appeared, in which he describes the same kind of lamellar structures in *Bacillus megaterium* in great detail and designates them as mitochondria with tubuli mitochondriales.

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