LIGHT AND ELECTRON MICROSCOPE STUDIES OF MYCOBACTERIUM— MYCOBACTERIOPHAGE INTERACTIONS

III. Further Studies on the Ultrathin Sections

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ABSTRACT

The process of multiplication of mycobacteriophage B-1 in its host cell was studied by means of an improved technic of ultrathin sectioning. The appearance of the nuclear apparatus was not altered throughout the latent period. Phage-shaped dense particles appeared about 30 minutes after infection in less dense areas neighboring the nuclear apparatus and occasionally at the margin of the nuclear apparatus. The less dense areas, which may correspond to the phage multiplication foci according to the authors' interpretation, were not filled with such arrays of fine-stranded fibrils as are seen in the nuclear apparatus. Empty phage heads could frequently be seen within and outside the lysed cells, along with the mature phage particles, at the end of the latent period. Moreover, it was indicated that empty head membranes may possibly exist within the cells during the latent period

INTRODUCTION

In the previous papers of this series (9, 10) the multiplication process of mycobacteriophage in its host cell was studied by light and electron microscopy. Ultrathin sections of infected cells were examined in a previously reported study in this laboratory (9) and also in that of Fukai and Sellers (1). However, the technic used in fixation and embedding appears to have been unsatisfactory (5). The purpose of the present paper is to report the process of phage multiplication as revealed by the electron microscope using an improved technic of ultrathin sectioning.

MATERIALS AND METHODS

Mycobacterium Jucho strain[1] and mycobacteriophage B-1, reported in previous studies (9, 10), were used throughout this investigation.

The cells, grown at 37°C in aerated 4 per cent glycerol broth for 16 hours, were collected by centrifugation. A homogeneous thick suspension of the cells (10^7 to 10^8 cells per ml) was prepared by grinding with glass beads. After incubation at 37°C for 2 hours, the actively growing cells were infected with a multiplicity of approximately 5 to 10 B-1 phage particles per cell. 5 minutes later, antiserum for B-1 was added at a concentration adequate to inactivate 99 per cent of the phage in 5 minutes. 9-ml samples were added to centrifuge tubes containing 1 ml of 1 per cent OsO_4 in acetate-veronal buffer and were immediately centrifuged at 3000 RPM for 30 minutes. The fixation and uranyl acetate treatment were

[1] This strain was originally isolated from fowl tuberculosis and designated as *Mycobacterium avium*, Jucho strain. However, recent studies of biological properties of this strain have disclosed that it has lost most of the properties characteristic of *Mycobacterium avium* and resembles saprophytic mycobacteria in many respects (11–13). Therefore, it is preferable to call this strain merely *Mycobacterium* Jucho strain.

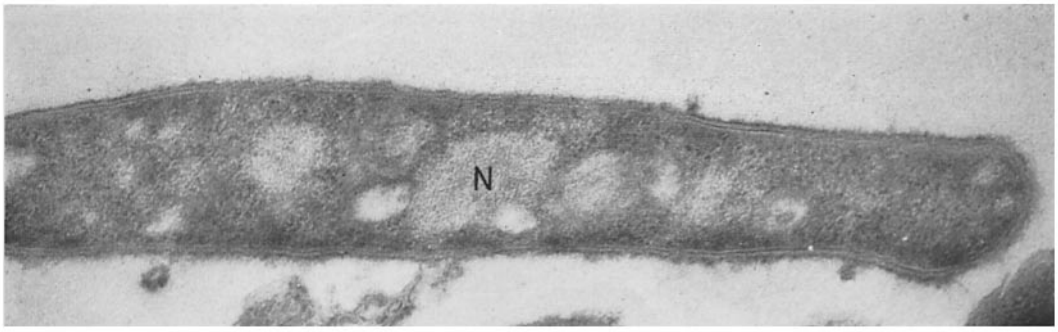

FIGURE 1

An example of the cells 20 minutes after infection with B-1 phages. The appearance of the nuclear apparatus (*N*) and other cellular components seems to be unaltered up to this time. × 52,000. For cellular components of healthy uninfected cells, see Koike and Takeya (5).

performed according to the method originally described by Ryter and Kellenberger (7). The cells were collected and fixed overnight at room temperature in 1 per cent OsO_4 solution in acetate-veronal buffer, pH 6.1, containing 0.01 M $CaCl_2$ and one-tenth volume of tryptone medium. After fixation the cells were collected by centrifugation, resuspended in melted 2 per cent agar, and deposited on a glass slide as a drop. Small agar blocks containing the fixed cells were treated with 0.5 per cent uranyl acetate solution in acetate-veronal buffer for 2 hours. The cells were then dehydrated in a series of acetone solutions and embedded in Araldite (2, 5). The dehydrated cells were immersed in a 1:3 mixture of freshly prepared embedding material (Araldite M, 10 ml; hardener 964, 10 ml; accelerator 964, 0.5 ml; dibutylphthalate, 0.7 ml) and absolute acetone for 30 minutes, followed by immersion for 30 minutes each in a 1:1 mixture and in a 3:1 mixture at room temperature. The cells were then put into a gelatin capsule filled with the embedding material and incubated at 30–35°C overnight, at 45°C for 1 day, and finally at 60°C for 1 or 2 days until polymerization of the resin was completed. Sections were cut with glass knives using a Porter-Blum microtome and were picked up on carbon-celloidin-coated copper mesh screens for electron microscopy. The specimens were examined with a JEM 5C electron microscope equipped with a 50 μ objective aperture.

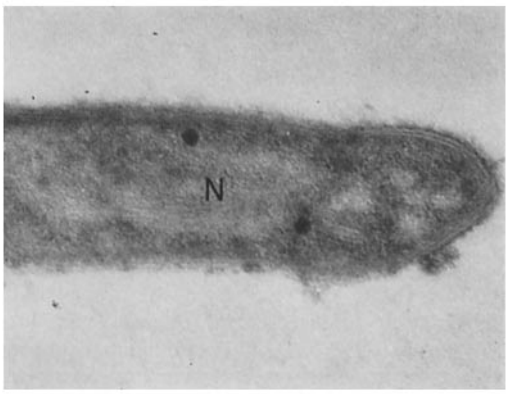

FIGURE 2

A cell 30 minutes after infection. Two dense phage-shaped particles are shown at the marginal part of the nuclear apparatus (*N*). × 53,000.

OBSERVATIONS

The cellular constituents appeared to remain unaltered until 20 minutes after infection (Fig. 1). After about 30 minutes a few dense phage-shaped particles appeared in the less dense areas of the cytoplasm neighboring the nuclear apparatus and/or at its margin (Figs. 2 and 4). These particles were found clustered in groups during the later stages of the latent period (Figs. 5 to 8). The parallel arrangement of fine-stranded fibrils seen in the nuclear apparatus was hardly visible in the less dense cytoplasmic areas. The two-dimensional crystal-like arrangement of phage-shaped particles, found by Kellenberger *et al.* (4) to occur with T2 phage infection, also seems to be relatively common here (Fig. 5). Besides the dense phage particles, doughnut-shaped lighter particles were also occasionally found (Figs. 3, 6 to 8, arrows). The nuclear apparatus appeared unchanged throughout the latent period.

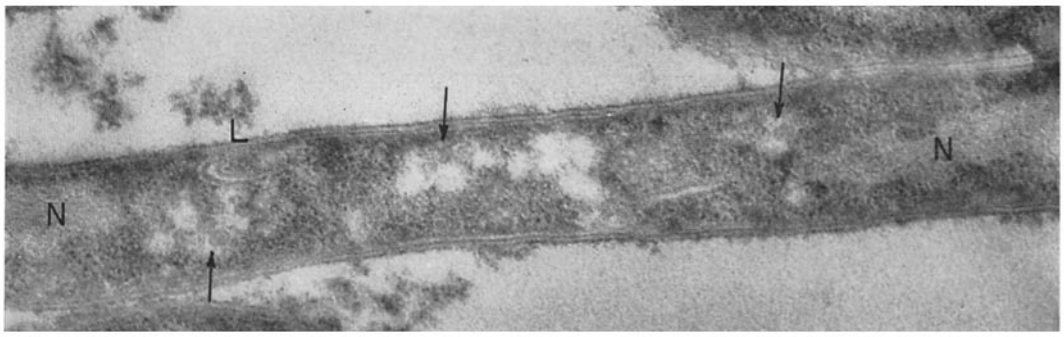

FIGURE 3

A cell 30 minutes after infection. Immature phage-shaped particles (arrows) are observed in less dense areas neighboring the nuclear apparatus (*N*). Most of the lighter particles appear to resemble the doughnut form. A lamellar structure (*L*), which has been supposed to be an extension of three-layered cytoplasmic membrane, is observable. See Koike and Takeya (5). × 55,000.

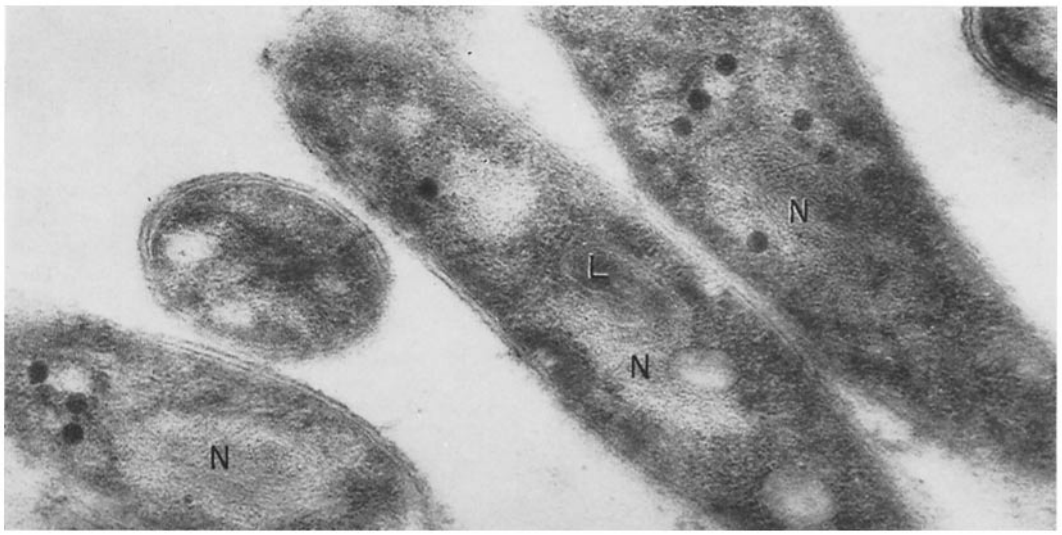

FIGURE 4

Cells 40 minutes after infection. Several dense phage-shaped particles are observed in less dense areas neighboring the nuclear apparatus (*N*). A few dense phage-shaped particles are seen at the marginal part of the nuclear apparatus. × 63,000.

At the end of the latent period, many lysed cells were observed. Inside and outside the lysed cells many doughnut-shaped particles were found along with mature, dense phage particles (Fig. 9).

DISCUSSION

The process of multiplication of T2 phage has been studied by Kellenberger *et al.* (4) using an excellent technic of ultrathin sectioning. The breakdown of the bacterial nuclear apparatus and the appearance of marginal vacuoles containing materials of nuclear origin are known to be the characteristic process at the initial stage of infection by T-even phages. On the other hand, a cytological examination of the process of infection by mycobacteriophage B-1 reported by Takeya *et al.* (10) has revealed that the bacterial nuclear apparatus was not morphologically modified in the same manner as it was in T-even phage infec-

tion. By the present electron microscope examination the appearance of the nuclear apparatus was proved to be unaltered throughout the latent period.

In the case of T2 phage infection, phage-shaped dense particles are reported by Kellenberger *et al.* (4) to appear in the morphological pool containing phage precursor DNA. According to the detailed studies of these authors, there seems to remain little doubt that condensation of phage DNA occurs in this pool and immature phage particles are surrounded by phage precursor DNA.

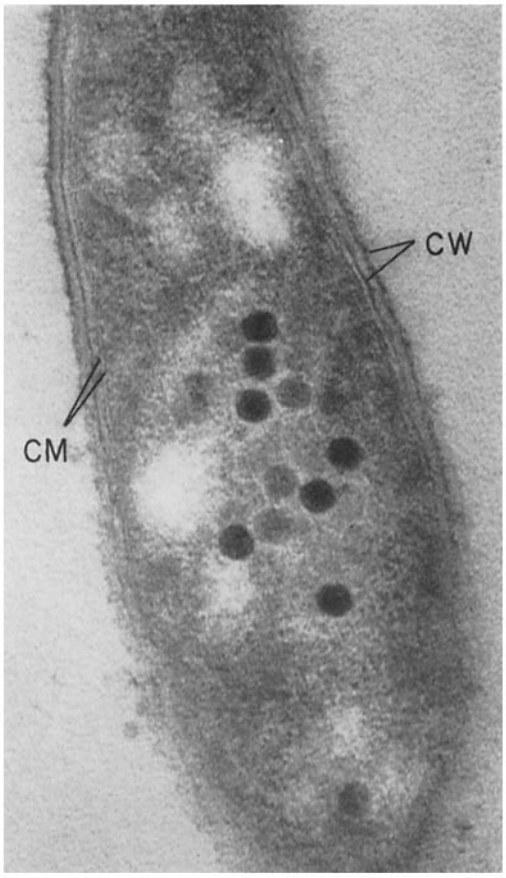

FIGURE 5

A cell 50 minutes after infection. A group of dense phage-shaped particles are seen in a less dense area of the cytoplasm. The density of the particles is not uniform. The least dense particles show about the same density as the ground substance of the area. The three-layered cell wall (*CW*) and the three-layered cytoplasmic membrane (*CM*) are clearly shown. × 100,000

The less dense areas, in which phage-shaped particles appeared and later clustered in a group, appear to represent the phage multiplication foci in the mycobacteria and may be interpreted analogously as the pool of the phage precursor DNA for mycobacteriophage B-1. On the other hand, fine-stranded material resembling the nucleoplasm has been reported to fill the phage precursor DNA pool for T2 phage (4), but such arrays of fine fibrils as are seen in the nuclear apparatus were not evident in the less dense cytoplasmic areas of the mycobacteria. A lower fibril concentration without parallel arrangement in these areas may account for this discrepancy. Formation of large chromatinic bodies in the course of the latent period, revealed by the previous cytological studies (9), suggests the fusion of the images of the nuclear apparatus with the neighboring less dense areas (possible phage precursor DNA pool). Because of the limitation of light microscope resolution, both would be stained as chromatinic substances by HCl-Giemsa stain. The above mentioned analogy is compatible with the result of this staining reaction but awaits verification.

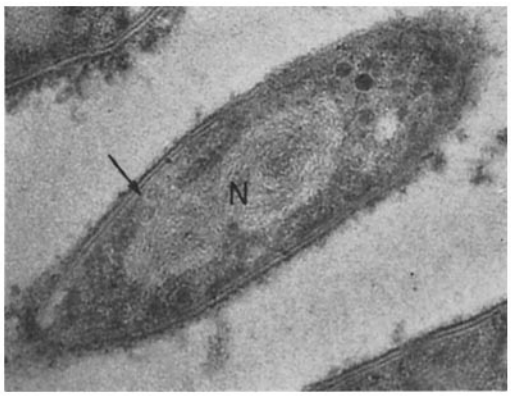

FIGURE 6

A cell 50 minutes after infection. Several phage-shaped particles are seen in an area neighboring the nuclear apparatus (*N*). A few doughnut-shaped particles (arrow) are found at the marginal part of the nuclear apparatus. × 53,000.

Careful examination of the sections has revealed that phage particles clustered in a group are all of almost the same size but are different in density (Fig. 5). The great difference in the density between the most and the least dense particles

may possibly represent steps in the condensation of phage DNA, but may also be due to a difference in the plane of sectioning, as indicated by the experiments of Kellenberger *et al.* (4) and Séchaud *et al.* (8) with T2 phage. These authors provided serial sections showing low density phages of unchanged diameter, originating from a normal phage, and drew their conclusion by statistical means. It appears, therefore, that only much more elaborate statistical means than those employed by the present authors and examinations of serial sections will allow a discussion of the former hypothesis for possible origin of low density sections of phage.

particles may arise as artifacts during preparation or may be images due to sectioning planes, but it seems possible that at least some of them represent empty head membranes. Empty heads seen in lysed cells (Fig. 9) are remarkably flattened in the direction of knife travel, which was ascertained by a knife mark found at another part of the same section, whereas mature phage particles remained in their characteristic polyhedral shapes. This probably occurs because the empty heads have insufficient content to endure the compression during the cutting process. The fact that the lighter particles resembling empty heads seen in Fig. 7 are also flattened by the compression may

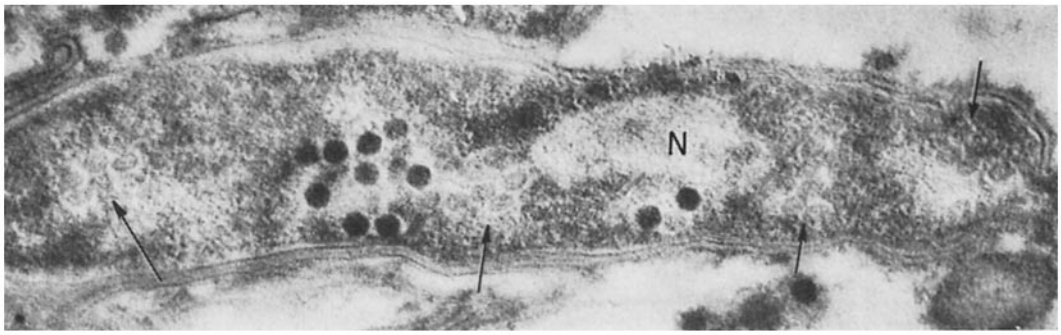

FIGURE 7

A cell 60 minutes after infection. A group of dense phage-shaped particles is seen in a less dense area neighboring the nuclear apparatus (*N*). Two dense particles are observable at the marginal part of the nuclear apparatus. Four groups of lighter particles (arrows), which resemble the doughnut form, are observed. × 80,000.

The empty phage heads or doughnut-shaped particles were not reported to be present within the cells infected with T2 (4). The empty heads found in the premature lysate or in the proflavin lysate were explained by Kellenberger *et al.* (4) as due to the breakdown products of immature fragile phages and/or the leakage products of abnormal proflavin phages. In the sections of proflavin-treated cells, the few completely empty phage-shaped vacuoles observed were considered to be another image, due to sectioning plane, of incompletely filled phages (4). The empty heads often found within and outside the lysed mycobacteria (Fig. 9) might also have been produced by the rupture of immature fragile phages after lysis. On the other hand, lighter particles resembling empty heads are also seen within the cells infected with B-1 (Figs. 3, 6 to 8). These support the interpretation that these particles are of a nature similar to that of the empty heads. However, more careful and quantitative investigations will be required to exclude artifacts completely and to confirm the presence of empty heads in infected bacterial cells. The same kind of empty protein shells were observed by Horne and Nagington (3) in HeLa cells infected with polio virus and were compared with the "top component" of turnip yellow mosaic virus preparations (6).

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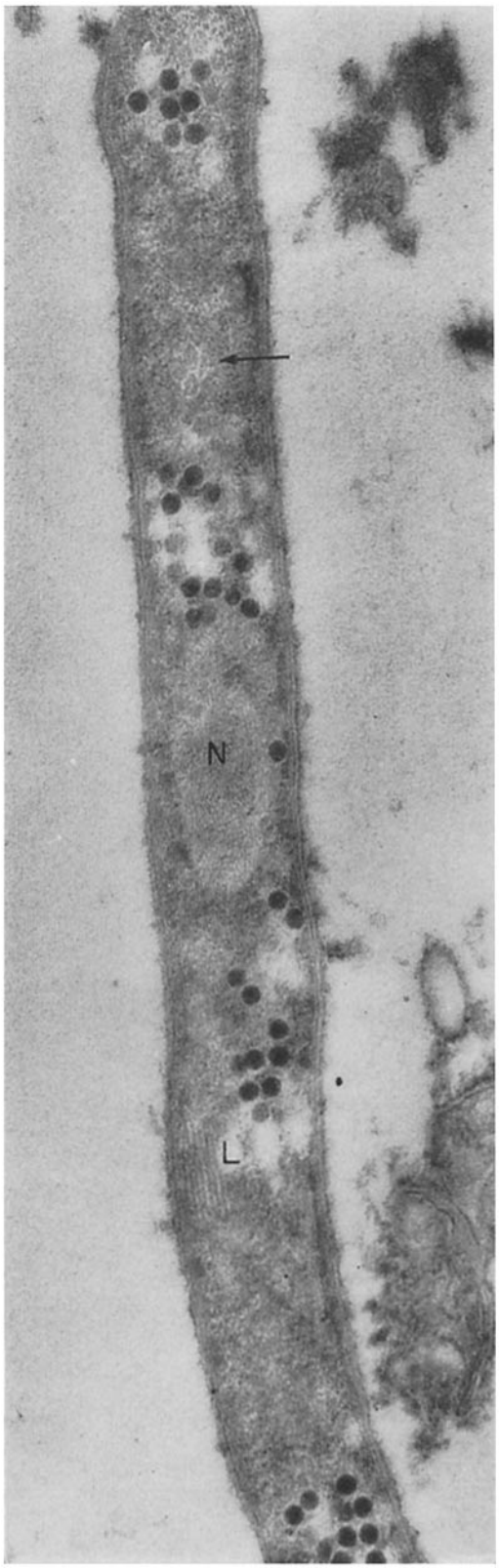

FIGURE 8

A cell 60 minutes after infection. Groups of phage-shaped particles are observed in several less dense areas. A group of lighter particles (arrow) is observable. *L*, lamellar structures. × 60,000.

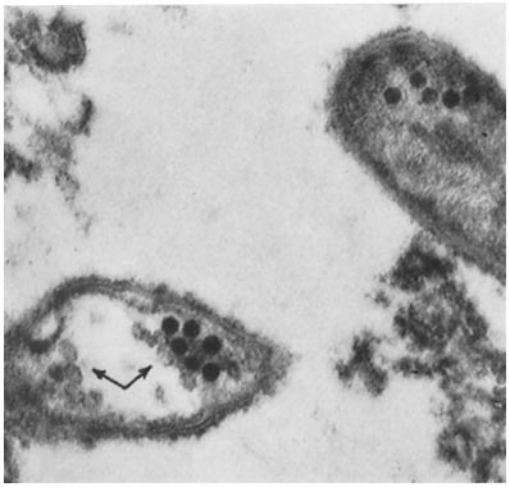

FIGURE 9

Cells 62 minutes after infection. Inside a lysed cell many doughnut-shaped particles (arrows) are observed along with mature phage particles. × 51,000.

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