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Fine Structure and Host-Virus Relationship of a Marine Bacterium and Its Bacteriophage

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ABSTRACT

VALENTINE, ARTRICE F. (Georgetown University, Washington, D.C.), AND GEORGE B. CHAPMAN. Fine structure and host-virus relationship of a marine bacterium and its bacteriophage. J. Bacteriol. **92**:1535–1554. 1966.—The fine structure of a gram-negative marine bacterium, *Cytophaga marinoflava* sp. n., has been revealed by ultrathin sectioning and electron microscopy. Stages in the morphogenesis of the bacterial virus NCMB 385, which has been shown to be highly specific for this organism, were also demonstrated in bacterial cells fixed according to the Kellenberger technique. The bacterium possessed a cell wall, cytoplasmic membrane, and nuclear and cytoplasmic regions typical of bacterial cells. Both the cell wall and the cytoplasmic membrane showed a tripartite structure, i.e., each was composed of two dense layers separated by a low-density zone. Intracytoplasmic membrane systems were also observed, especially in dividing cells and in cells in which new viruses were being formed. As many as 18 hexagonally shaped, empty phage heads (membranes only) were observed in untreated, infected bacterial cells. Phage heads, intermediate in density to empty heads and fully condensed ones, possibly representing stages in the morphological development of the virus, were also seen.

There are few reports in the literature of morphological studies of the lytic cycle of a bacteriophage within its host bacterium. Most of these reports are devoted to a single organism, viz., *Escherichia coli*. Definitive studies by Kellenberger et al. (19, 20), and more recent studies by Margaretten et al. (24), have shown the morphological changes occurring in *E. coli* cells after infection with bacteriophage. Both groups of investigators have described the morphological disintegration of the bacterial nucleus and its migration to the periphery of the cell. Kellenberger et al. (20) described the subsequent formation of a morphological pool of phage deoxyribonucleic acid (DNA) which becomes axially located and which represents the site of formation of new bacteriophages.

A study of the cytological changes of a bacteriophage-infected bacterium other than *E. coli* was undertaken in an attempt to increase the scope of our knowledge of the morphological events associated with the lytic cycle of a bacteriophage within its host bacterium. A marine bacterium-phage system was selected for the study because such a system had not yet been subjected to such investigation. Both the host organism and the virus employed were isolated by Spencer (32). The host organism was originally designated as an "unidentified flavobacterium" and was classified in the National Collection of Marine Bacteria (Scotland) as NCMB 397. The virus has the National Collection of Marine Bacteria number NCMB 385. Both the organism and the virus were obtained from the Torry Research Station, Aberdeen, Scotland.

From the phenetic data obtained in studies of NCMB 397 and previously isolated strains of similar morphology and physiology and from the results of studies of the DNA base composition of the organism, R. R. Colwell, R. V. Citarella, and P. K. Chen (Can. J. Microbiol., *in press*) have shown the organism to be a *Cytophaga* species and have designated it *Cytophaga marinoflava* sp. n. The general description of *C. marinoflava* represents it as a gram-negative, nonmotile, asporogenous, rod-shaped organism. No previous studies of the fine structure of this organism have been reported.

The morphology of the virus NCMB 385, as revealed by the negative-staining technique, was

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described in an earlier paper (35). Other properties of the virus, including its DNA base composition, were reported by Chen et al. (4).

This report then presents the morphological characterization of the marine oeganism, *C. marinoflava*, and the bacterial virus-host cell relationship, i.e., the infectious cycle of the virus from the adsorption stage to lysis of the host cell.

MATERIALS AND METHODS

Cells of *C. marinoflava* were grown in seawater-yeast extract medium, SWYE (6), consisting of 3.0 g of yeast extract (Difco) and 10.0 g of Proteose Peptone (Difco) in 1,000 ml of seawater prepared from a four basic salt solution. The four basic salt solution for marine bacteria (6, 34) consists of 24.0 g of NaCl, 0.7 g of KCl, 5.3 g of $MgCl_2 \cdot 6H_2O$), and 7.0 g of $MgSO_4 \cdot 7H_2O$ made on the basis of grams per 1,000 ml of distilled water. The medium was adjusted to *p*H 7.2 to 7.4 with 0.1 N NaOH solution. When a semisolid medium was used, 15 g per liter of agar (Difco) was added. Bacterial cells were grown at 17 C in 250-ml Erlenmyer flasks with aeration. Cells in the early logarithmic phase of growth, as determined by optical density readings (OD), were used for the morphological studies.

Bacteriophage stock. Routine stock preparations of NCMB 385 were made by plating the virus, with sensitive host cells, by the double-layer technique (1). For both the plates and the overlay, 1.5% SWYE agar was employed. The plates contained 1 ml of bacterial cells, 1 ml of appropriate virus dilution, and 1 ml of agar. The plates were swirled gently and then allowed to dry before being inverted for the final incubation. The phage was extracted from the completely lysed agar plates in SWYE broth (*p*H 7.2 to 7.4). Bacterial debris and agar were removed by two low-speed centrifugations. The supernatant fraction was then filtered (1) through a sterile membrane filter (pore size, 0.45 μ; Seitz or Millipore). Samples of approximately 9 ml each were removed and stored, as phage stock, in autoclaved vials, at 4 to 5 C. The phage filtrate was titrated initially by serial dilutions, and samples of 10^{-6} and 10^{-8} dilutions were plated with bacterial cells in the logarithmic phase of growth. After overnight incubation at either room temperature or 17 C, the plaques were counted, and the virus titer was calculated. Stock phage was routinely titrated before use. Virus titers ranged from about 6.0×10^9 to 1.2×10^{10}.

Infectious cycle. High-multiplicity ratios were used for the infection, i.e., a phage-to-bacterium (P/B) ratio of approximately 33:1 or 100:1. Bacterial cells were grown in SWYE broth at 17 C with aeration for times varying from 13 to 15 hr. Optical-density readings ranging from 0.092 to 0.150 indicated an early logarithmic stage. In some instances, plaque counts were conducted simultaneously with the electron microscope studies to serve as a virus assay. For such purposes, a second flask was inoculated with bacterial cells and grown under the same conditions as the cells inoculated with virus. These cells were used as sensitive cells for the plating experiments. The flasks remained at 17 C throughout the entire experiment, except when samples were removed. The aeration was discontinued after the addition of the phage. Samples for virus assay were taken simultaneously with the portions for electron microscopy, and 0.1 ml of sample was used to make virus dilutions of 10^{-4}, 10^{-6}, and 10^{-8}. A 0.05-ml amount of the 10^{-6} and 10^{-8} dilutions was added to agar plates followed by 0.5 ml of cells from the second flask. A 2-ml amount of 0.7% SWYE agar maintained at 45 C in a temperature-regulated water bath was used for the agar overlay. After drying, plates were inverted and incubated overnight at 17 C. Plaque counts were made the next day and were plotted against the time of the sample.

Preparation of specimen for electron microscopy. Bacterial cells were fixed by the standard technique of Kellenberger (17, 19) with slight modifications. Magnesium chloride was added to the buffer in the same amount as the calcium chloride solution, i.e., 0.25 ml of 1 M $MgCl_2$. The amount of tryptone was lowered from 0.1 ml per 10 ml of cells to amounts ranging between 0.05 and 0.01 ml. Instead of the prefixation step of Kellenberger et al. (17, 19), i.e., adding fixative to the sample before the initial centrifugation, 10-ml samples were removed from the flasks and immediately centrifuged in an International clinical model centrifuge (approximately 4,100 rev/min) for 5 min, the supernatant fraction was discarded, and 1 ml of fixative was added. Samples of cells were removed at time intervals of 5, 30, 60, 90, 120, 180, 190, 210, and 240 min. The fixative was gently pipetted off, after 4 to 5 hr, and fresh fixative and tryptone were added. Sampling times of the experiments resulted in a range of fixative times from 16 to 20 hr. Fixation was followed by washing the cells in buffer with 0.5% uranyl acetate. Blocking the cells in 2% agar was eliminated, and the cells were maintained as pellets throughout the procedures. Gentle removal of the fluids with a pipette was utilized in an effort to avoid disturbance of the pellet and to eliminate a second centrifugation prior to the washing period. The pellets of bacterial cells were dehydrated in a graded ethyl alcohol series: 50, 75, 95, and 100%. The cells were placed into propylene oxide and then into a mixture of 1:1 propylene oxide to Epon with accelerator. Epon embedding procedures according to Luft (22) were employed. Polymerization was carried out for 24 hr at 60 C.

Grids lightly coated with carbonized collodion (0.5%) or Formvar (0.25%) were used as support films (29). Sections were cut with either glass knives or a diamond knife on a Porter-Blum MT-1 ultramicrotome. Sections were either stained with alcoholic uranyl acetate (12) or doubly stained with alcoholic uranyl acetate and lead citrate (30, 37). Both an RCA EMU-2D and a Siemens Elmiskop I electron microscope, equipped with 50-μ objective apertures, were used.

RESULTS AND DISCUSSION

Initially, the cells were fixed according to the Kellenberger standard technique, without modi-

fication. However, large amounts of what appeared to be accumulations of precipitated osmium were found free in the sections and in many instances adhering to the bacteria. The elimination of the prefixation stage, the addition of fresh fixative after 4 to 5 hr, and the reduction of the amount of tryptone broth added to the tubes eliminated this problem. A range of fixation times of from 16 to 20 hr was used because of the 4-hr period during which samples were collected. This sampling time was necessary because of the 210-min latent period of the virus.

Adequate staining was obtained with either saturated aqueous or alcoholic uranyl acetate (12, 38), but the best staining results were obtained by use of the double staining technique with saturated alcoholic uranyl acetate and lead citrate (30, 37). The method of Venable and Coggeshall (37) was preferred, as it involved a shorter staining time and a greater ease of stain preparation.

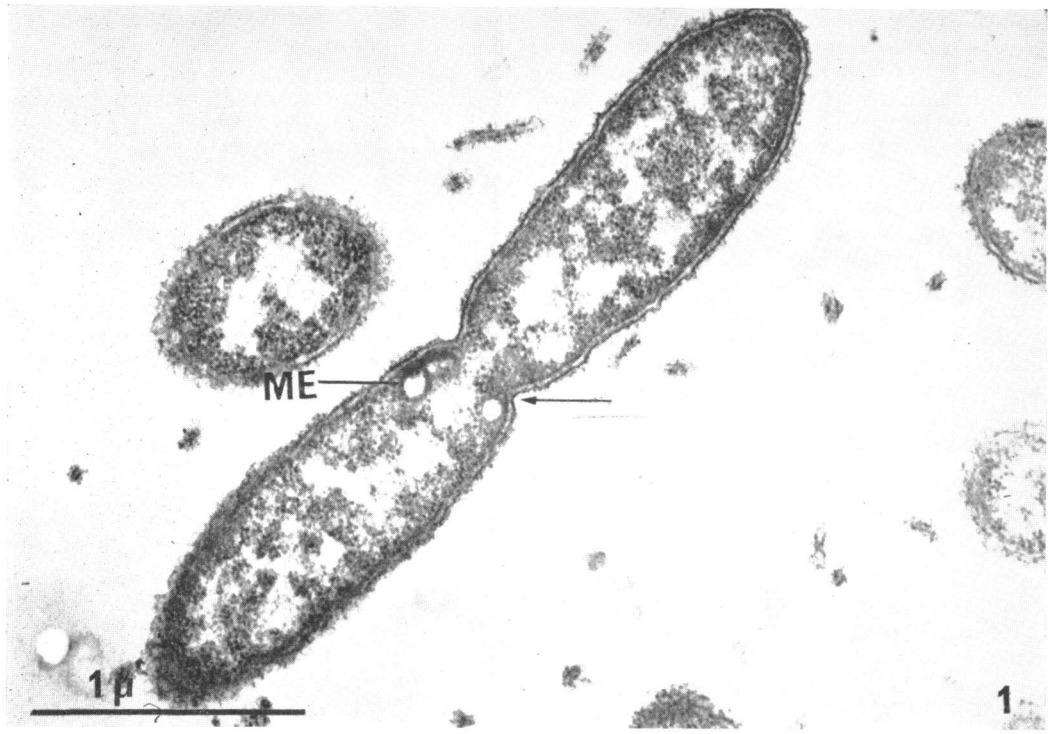
Control cells of *C. marinoflava* (Fig. 1 and 2) show a cell membrane, cell wall, and a granular type of cytoplasm, which in general is peripherally located but which also may be dispersed throughout the more axially situated nuclear region. In these cells, a finely arranged network of DNA is not apparent or is only faintly discernible within the matrix of the nuclear region. The filaments of DNA (DF) are indicated in the obliquely sectioned bacterium in Fig. 2. The network of DNA filaments is more clearly evidenced in other figures (Fig. 9, 10, 12, 14, and 15). The cell membrane is typical in appearance of the unit membrane described by Robertson (31), and the cell wall also has a tripartite structure. Both the cell wall and the cell membrane measured approximately 70 to 90 A thick. None of the bacterial cells revealed an extra 20- to 30-A component of the cell wall, as has been described by Murray et al. (28), but rather the cell wall was always seen as a structure composed of alternate dark, light, and dark layers as described by Kellenberger and Ryter (18). Their measurements of this structure in *E. coli* showed it to vary in thickness from 60 to 90 A. It is of interest that the cell wall of this marine organism, classified in the genus *Cytophaga* and order *Myxobacteriales*, has the same general morphology as the cell wall of gram-negative organisms of the order *Eubacteriales* [*E. coli* (18)] and the order *Pseudomonadales* [*Spirillum serpens* (28)]. This apparent structural similarity becomes of considerable interest when one recalls that the cell wall of *Cytophaga* is flexible whereas that of the other organisms mentioned is quite rigid. It may be that this difference in flexibility resides in a difference in macromolecular arrangement not detectable by our methods.

In dividing cells, there was no evidence of the formation of a cross wall or membrane septum. Rather, a constriction at the point of division was seen (Fig. 1 and 15). This is a type of cellular division different from that described for such gram-positive organisms as *Bacillus cereus* (3), *B. subtilis* (36), and an unidentified bacterium (2), where the centripetal growth of the wall or membrane to form the cross wall or membrane septum has been demonstrated. In general, septation characterizes the cellular process of gram-positive organisms. In contrast, cellular division of the gram-negative enteric organisms appears to be accomplished ordinarily by a mechanism of wall and membrane constriction unaccompanied by any architecturally differentiated deposition of membrane and wall at the plane of division.

Conti and Gettner (7) do describe a configuration which may represent a stage in the formation of a cross wall in a dividing cell of *E. coli* strain 15T⁻. However, they admit in their discussion that the identification of the partitioning structure from their electron micrographs is difficult. Their micrographs reveal a marked central constriction of the cell, but no developing membrane or wall septum is apparent. However, two of their figures (Fig. 5 and 5A of reference 8) show cells which possess clear but diminutive cross walls. "Peripheral bodies," described by Chapman and Hillier (3), which appear to be identical to the mesosome structures of Fitz-James (11) and which were seen to be associated with the sites of cross wall formation during the process of cellular division, were not observed by Conti and Gettner (7) in *E. coli*.

More recently, Steed and Murray (33) reported septa formation in the enteric, gram-negative organism, *E. coli*, and the gram-negative organism, *S. serpens*, when they were grown under certain unusual conditions. If the organisms were grown at 45 C, septa formation was observed. Septa occurred also in cells grown at 30 C when the buffer used in the Kellenberger fixative was diluted 1:6. These authors also described supernumerary asymmetrical septa and mesosomes in these organisms.

Initially, it was believed that gram-negative organisms were devoid of mesosome structures. However, reports of these structures in *E. coli* and *S. serpens* (16, 27), among other gram-negative organisms, show that they are not restricted to the gram-positive organisms. Kaye and Chapman (16) showed the presence of structures presumed to be mesosomes in *E. coli* after treat-



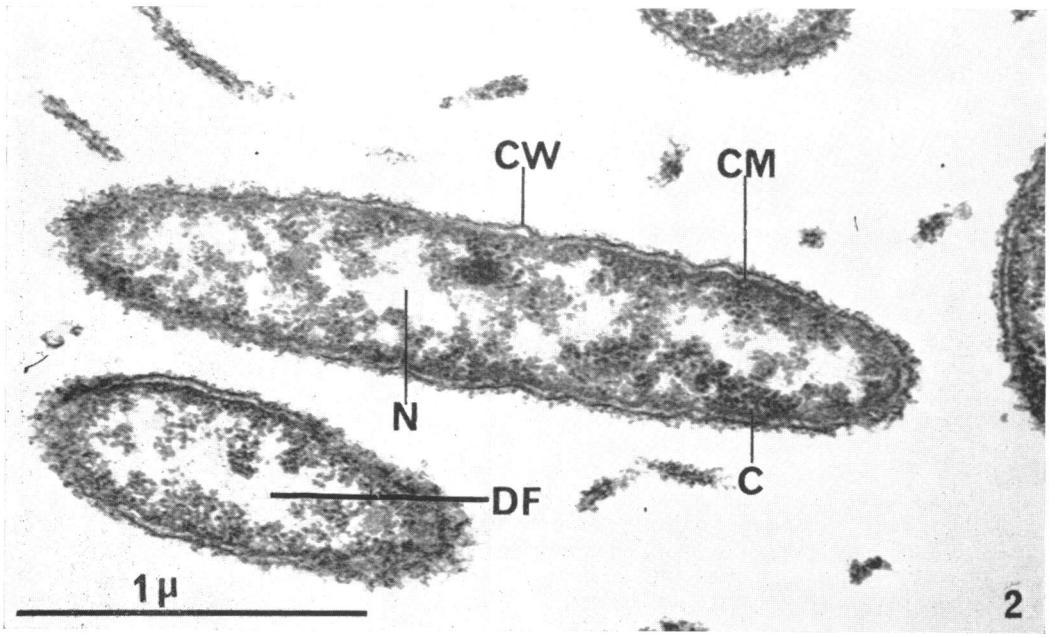


FIG. 1. *Control cell in the process of division. A constriction is seen at the point of division (arrow). There is no evidence of septum formation in this cell. Two intracellular membrane systems of the mesosome type (ME) are also seen in this region. Uranyl acetate stained. × 39,000. The magnification mark in each figure represents 0.1 μ unless otherwise stated.*

FIG. 2. *A second control cell representative of the vegetative state of the organism. Doubly stained with alcoholic uranyl acetate and lead citrate. Cell wall (CW), cytoplasmic membrane (CM), cytoplasm (C), and nucleus (N) are indicated. DNA filaments (DF) appear in the nuclear area. × 49,000.*

ment with the antibiotic, colistin sulfate. Also Murray and Birch-Andersen (27) have observed mesosomes of membranous intrusions in *S. serpens*. Kushnarev and Pereverzev (21), in a study of the isolated membranes of *E. coli*, resulting from autolysis, showed membranes, typically "unit membrane" in structure, which are supposed to represent intracytoplasmic membranes; they discussed whether or not these membranes are mesosomal, but no conclusion was given. Their results give no definite indication of how these membranes would position themselves in intact cells. There were no isolated structures resembling the mesosomes described by Fitz-James (11).

Intracytoplasmic membrane structures of four types were found in thin sections of *C. marinoflava*. One of the types was strongly reminiscent of the peripheral body of Chapman and Hillier (3) in that it was nearly empty in appearance and was peripherally located. Figure 1 shows the presence of this type of membrane structure (ME) in the region of constriction during cell division. The actual occurrence of the membrane lamellae in one of these bodies is seen in the somewhat more typical mesosome-type structure (ME) observed in Fig. 15. This body is obviously not so closely located to the actual region of division as is the system in Fig. 1. In both cases, although the structures are in close proximity to the cell membrane, a definite point of attachment is not seen. The structures have been described as "intracytoplasmic membrane structures" since they are not identical in appearance to the mesosomes of Fitz-James, but rather appear to be intermediate between them and a somewhat more myelin-type arrangement which Kaye and Chapman (16) observed in *E. coli* after treatment with colistin sulfate. An example of the more myelin-type configuration observed in *C. marinoflava* is shown in Fig. 5 (MY). The intracytoplasmic membrane structure found in Fig. 3 (ME) near the center of the cell, appears to be a part of the cytoplasmic membrane and is typical of many of the mesosomes of Fitz-James. This arrangement is also suggested in the system in the upper polar region of the cell (ME). Both of these arrangements of intracellular membranes resemble that in the intracytoplasmic membrane system described for *Streptomyces coelicolor* (13, 14). Although some time has been spent pointing out the variations in the appearance of these intracytoplasmic membrane systems, it should be noted that they are here considered to be morphological variations quite possibly reflecting differences in cell physiology.

Weibull (39) and Mitchell and Moyle (26) have reported the cytoplasmic membrane to be the carrier of respiratory enzymes in the bacterial cell. Therefore, if the intracytoplasmic membrane systems are derived from the cell membrane, as has been demonstrated morphologically (11, 13–15), they, too, would be presumed to be sites of certain enzyme systems. It would follow, then, that, in the case of a dividing cell, or a cell requiring more energy than that required by the vegetative cell, mesosomes or other types of intracellular membrane systems might be expected to appear or to become enlarged or more numerous. This would account for their frequently being observed in dividing cells of various species, including *C. marinoflava*, and in this particular instance would explain their being present in cells in the process of replicating bacteriophages. Another possible function for the intracytoplasmic membrane systems is that of increasing cell surface for the purpose of aiding in the transport of substances into and out of the cell. Such a mechanism might be anticipated in cells which were in the process of replicating phages. Mesosomes or mesosome-like structures have not been described in *E. coli* cells in direct association with sites of division or as a feature of the cytological changes occurring after infection with phage.

The cells in this study were not put into a synchronous growth phase. Therefore, cells at different stages of infection were observed in the various samples taken.

Attached phages were observed on bacteria, in the samples taken as early as 5 min after infection. Figures 4 to 6 show attached phages from samples taken 30 min after infection. The phage attaches to the outer surface of the bacterium by its tail (Fig. 4–6), but there was no evidence of rupture or infolding of the cell wall. There was also no indication of alteration of the phage tail or base plate structure as has been reported for T2 phages by Cota-Robles and Coffman (8). Empty heads (Fig. 4 and 5), empty heads with a core (Fig. 4), and heads in which the nucleic acid was still present (Fig. 6) were observed on the attached phages. The point of attachment, i.e., the terminal portion of the tail, to the cell wall seemed to be the same for all these types. No evidence of the mechanism of release or of penetration into the bacterium of the phage nucleic acid was obtained. However, as subsequent figures will show, it is obvious that release and infection did occur. It seems reasonable that there may be an enzyme associated with the tail structure of the phage that acts at the bacterial surface in a way that is not detectable in ultrathin sections at this level of resolution and with these preparative methods. The core seen here in the empty head (Fig. 4) may represent a special structure in the DNA-free region observed in sectioned T2 L bacterio-

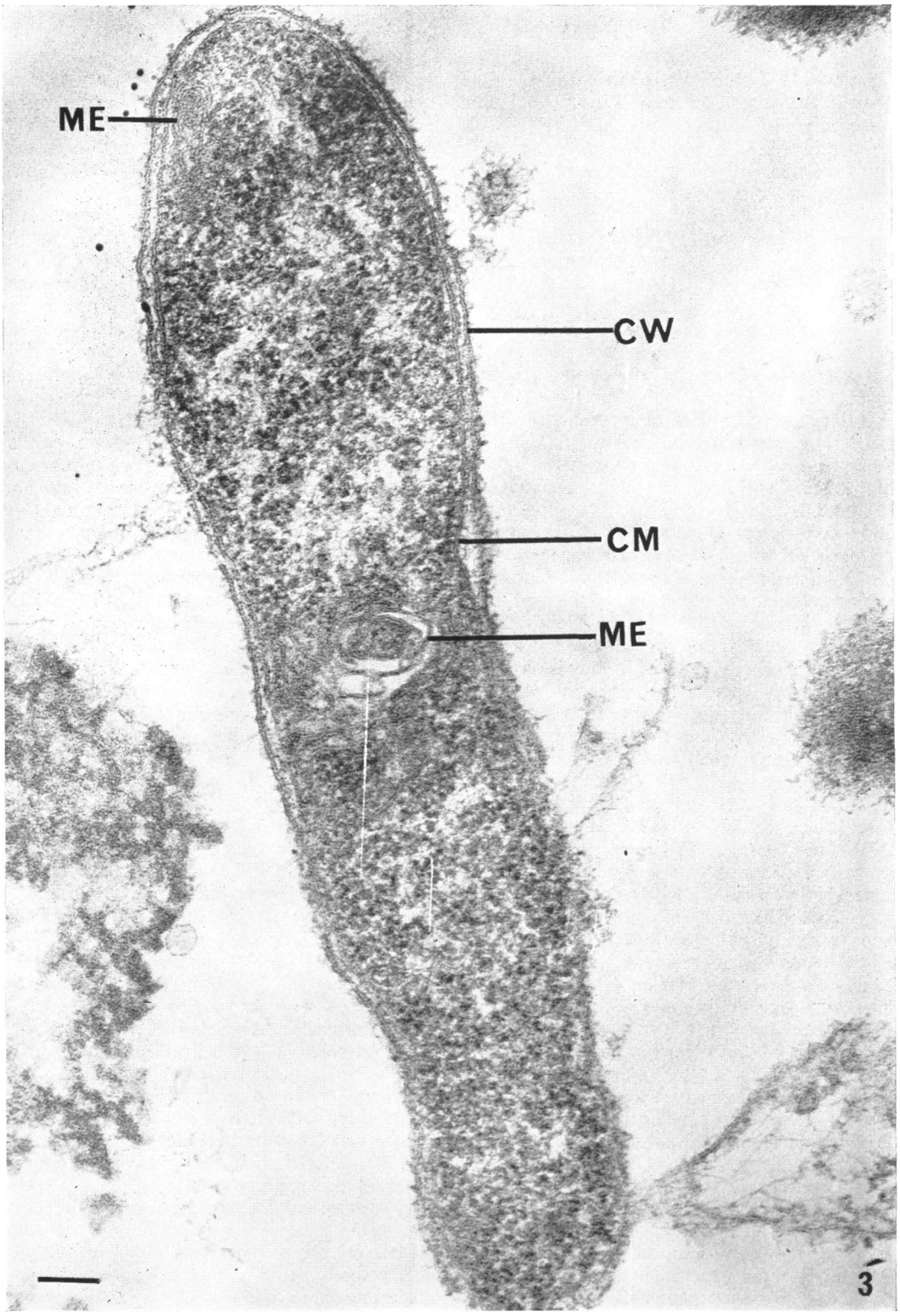


FIG. 3. *Intracellular membrane systems (ME) appear at the center and upper polar end of this cell taken 5 min after infection. The tripartite structure of the cell membrane (CM) and cell wall (CW) is very distinct. Doubly stained with alcoholic uranyl acetate and lead citrate.* × *100,000.*

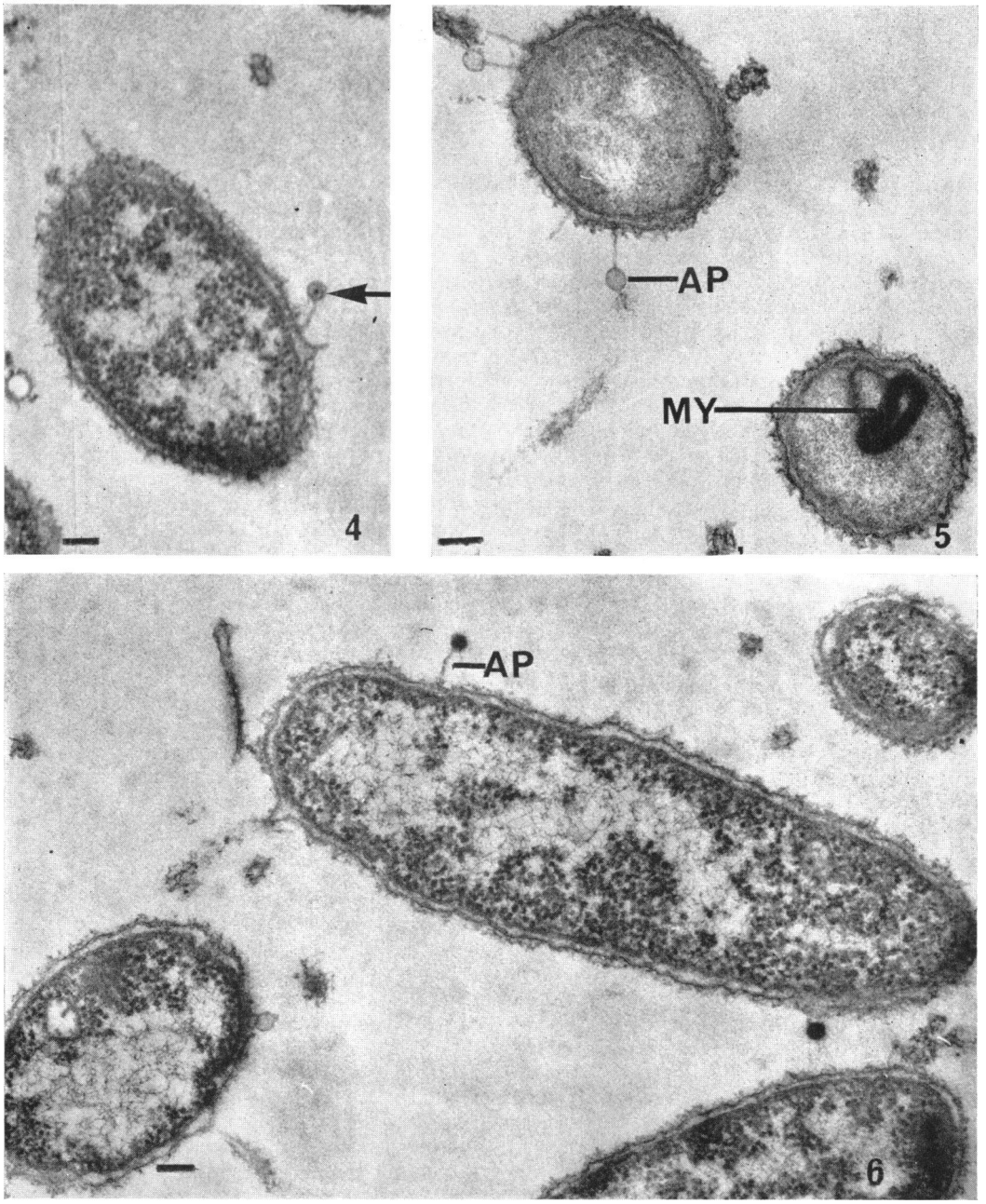


FIG. 4. *Transverse section of a cell 30 min after infection, showing an attached phage with empty head in which a centrally located dense body or core (arrow) is observed. Alcoholic uranyl acetate stained.* × *56,000.*

FIG. 5. *Attached phages (AP) with empty heads are observed in a transverse section of a bacterium 30 min after infection. An intracellular membrane system resembling a myelin figure (MY) is seen in a second cell. Doubly stained with alcoholic uranyl acetate and lead citrate.* × *65,000.*

FIG. 6. *Attached phage (AP) observed on a sagittal section of a bacterium 30 min after infection. The electron-dense head indicates the DNA within the phage. Alcoholic uranyl acetate stained.* × *56,000.*

phage by Cummings and Wanko (9). In their thin sections of bacteriophages, this area appeared as a less opaque zone in the opaque head region. This core structure does not resemble the structure described by Margaretten et al. (24), which appears as a hollow disc and has been observed both in empty heads found on attached bacteriophages and intracellularly. These authors suggest, that this is probably a protein component of the head. The structure described here and the one described by Margaretten et al. (24), even though morphologically dissimilar, may indeed be representative of a protein region of the head, and could function in a similar capacity.

No distinct cytoplasmic changes were observed during the earlier part of the infectious cycle, i.e., from 0 to approximately 90 min after infection. There was no apparent disintegration of the nucleus followed by migration of its former constituents to the cell periphery during this initial period of infection, as might have been anticipated from previous studies. In the light microscope studies of Luria and Palmer (23), cytological changes were detected in *E. coli* cells infected with T2 bacteriophage, but no migration was observed after infection with T1 bacteriophage. With T1 phage, a large number of the cells appeared normal until lysis occurred. The situation here may be comparable, in this respect, to that of T1 phage infection.

In samples taken at 120 min after infection, there was definite evidence of intracellular replication of phage (Fig. 7 and 8), and approximately 40 to 50% of the bacteria contained intracellular viruses. Empty heads and heads in which the DNA had condensed completely were observed. In the majority of sections, the membrane coat surrounding the nucleic acid was not seen, but, in some instances, the plane of section was such that the protein membrane surrounding the DNA was seen intracellularly (Fig. 11). The appearance of empty heads in relatively large numbers is particularly interesting, since intracellular, empty phage heads have only been observed in two instances: in cells fixed by the Kellenberger technique, after treatment with proflavine (20), and in the glutaraldehyde-fixed cells described by Margaretten et al. (24). In neither case were these empty heads seen in as large a number as those observed in this study. In Fig. 14, approximately 15 to 18 empty hexagonally shaped membranes were found in close proximity to one another. These empty heads do not seem to form any specific pattern, but appear to be a group of closely packed heads which are overlapping because of the plane of sectioning through the packets. They do not seem to be suggestive of the intracellular crystalline array of "uncondensed" phages described by Cole (5) for replicating phages in a group C *Streptococcus* infected with C1 bacteriophage. Because of the evenness of the edges of the head membranes seen in Fig. 7, 8, and 14, it is felt that they are not images resulting from a sectioning plane through incomplete phages, as is suggested by Kellenberger et al. (20). Since empty heads were seen by Kellenberger et al. (20) only in cells treated with proflavine, they considered them as products of the breakdown of defective or labile viruses in which the DNA had condensed. They would, therefore, be respresentative of aberrant forms rather than developmental forms. Margaretten et al. (24) also believed them to be abnormal rather than developmental forms, one of their reasons being the infrequency with which they are observed.

The empty heads observed in this study are in cells which have received no treatment as opposed to proflavine-treated cells in the Kellenberger study. However, since proflavine (10) is known to prevent the assembly of complete infective particles, even though both phage protein and nucleic acid continue to be synthesized, it might be speculated that the empty heads present here may have resulted from an inability of complete phage particles to be assembled, even though individual components are being produced. Another possibility would be that the empty heads are products of excess or aberrant protein synthesis or of a deficiency in DNA synthesis by the host organism, resulting in the production of "immature" phage or an excess of head membranes.

Admittedly, it is difficult to imagine a membrane being formed first and then the DNA being packaged into it, as this would defeat the concept of "cell economy," for a tremendous amount of energy would be required to pack the DNA of the bacteriophage into a membrane approximately 25 to 30 A thick and measuring 600 A in diameter. However, the large number of these empty heads found within the DNA region of the bacterial cell tempts one to consider them in the light of developmental rather than abnormal forms. It is also interesting that they occur in the 120-min sample, where morphologically complete, newly synthesized, intracellular viruses are first observed, in larger number than the completely condensed heads. However, this possibility, i.e., that they are developmental forms, seems very unlikely because of the previously discussed reasons and the fact that these cells are not growing synchronously. Furthermore, the fact that the phage-producing cells have been unaerated from the time of infection may in itself be a factor causing abnormal synthesis.

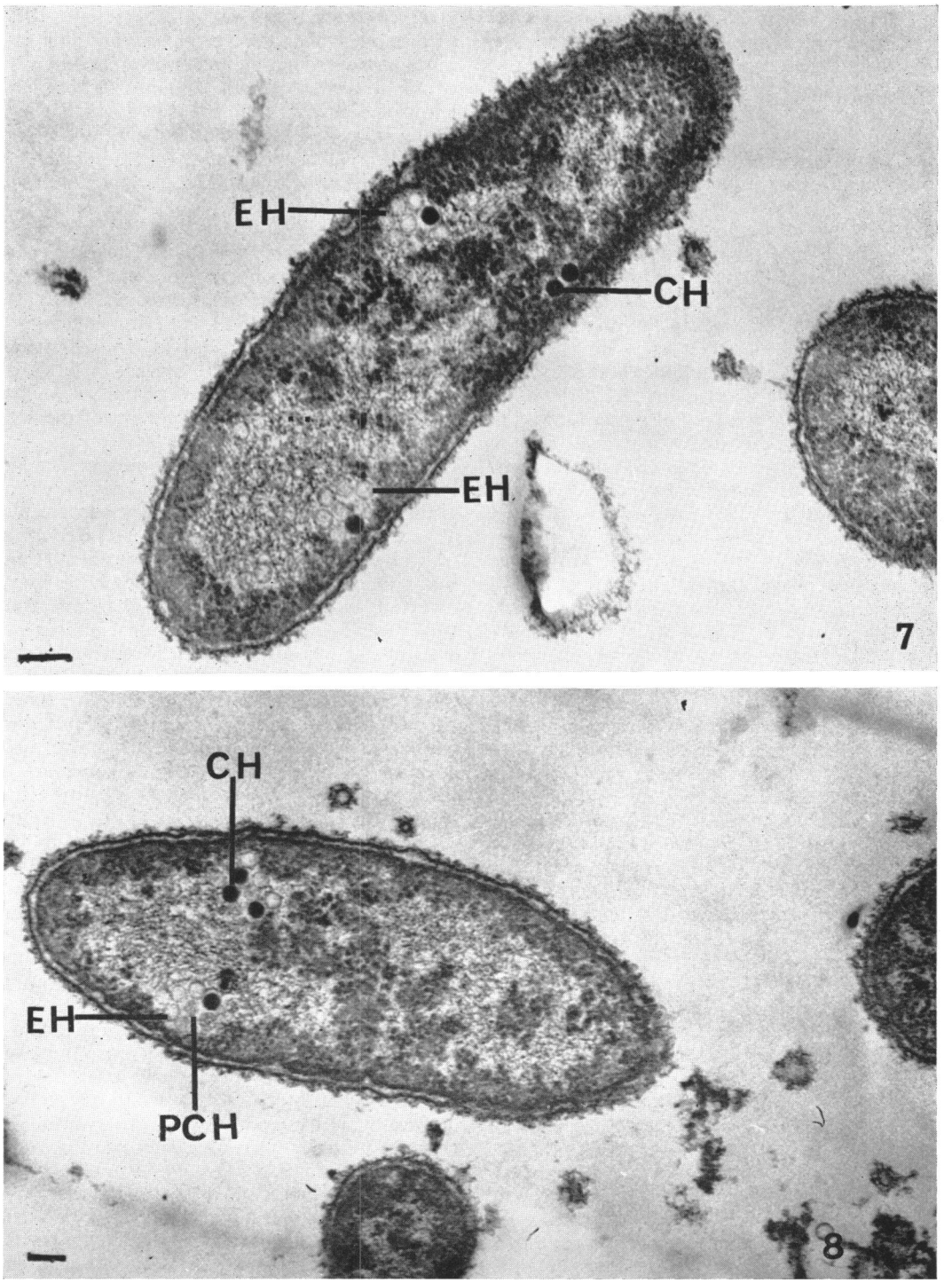


FIG. 7 and 8. *Definite intracellular phages are seen in these bacterial cells, 120 min after infection. Groups of hexagonally shaped phage head membranes (EH) are observed as well as partially condensed (PCH) and fully condensed heads (CH). Doubly stained with alcoholic uranyl acetate and lead citrate.* × *75,000 and 56,000, respectively.*

Cells at different stages of development are seen in samples taken at any given time after infection, as has been previously discussed, and as is exemplified by the large number of empty heads observed in the cell seen at 210 min after infection (Fig. 14). It would seem desirable in the future to remove free phage after infection to eliminate further infection of the remaining bacteria and to observe the developmental process of NCMB 385 in a synchronized culture.

The viruses are formed and remain situated in the nuclear region, which is generally axially located, and it appears that they have no affinity for any specific areas within the nuclear matrix (Fig. 7–17). The fact that they are always in contact with the nuclear regions seems to favor the concept of Kellenberger et al. (20) that the viruses develop from a pool of phage DNA. The identification of the nuclear material in this organism as being host or phage DNA was not attempted in this study. Margaretten et al. (24) suggested that the DNA, at the time of phage development, be designated nuclear material rather than a pool of phage DNA, as they did not observe phage particles in close proximity to the low-density nuclear regions. It was observed in this study (Fig. 7, 8, and 14) that the empty heads appear to occur in larger quantities in the peripheral areas of the DNA-containing regions.

At 180 min after infection, about 85 to 90% of the cells observed contained virus particles. In some instances, empty heads were observed, but, in general, the majority of the viruses had either partially condensed or fully condensed heads (Fig. 10–12). Figure 9 shows both an oblique and a longitudinal section of bacteria 180 min after infection. The longitudinal section serves to summarize the events involved in the intracellular development of the bacteriophages, in that viruses with empty heads (EH), partially condensed heads (PCH), and fully condensed heads (CH) are observed. If one examines some of the partially condensed heads, a corelike structure previously described in an attached phage is seen (small arrow). In a few instances, structures which appear greater in diameter than the DNA filaments forming the fibrillar network of the nuclear region are attached to the heads of bacteriophages. These structures have been interpreted as tails (arrows labeled with T, Fig. 9 and 10). However, the difficulty in resolving tail structures in sectioned material because of inadequate contrast or the masking of these structures by the cytoplasm or fibrillar content of the nucleoplasm, makes it possible that the structures labeled T are strands of nuclear DNA.

Kellenberger et al. (19, 20) were not able to observe tail structures within the bacterial cells, and reported the failure of preliminary attempts to stain or surround the tail structure with phosphotungstic acid. Mercer (25) employed osmium tetroxide fixation, followed by a postfixation treatment with either phosphotungstic acid or uranyl acetate, and showed intracellular T2 bacteriophages with tails. Margaretten et al. (24) showed elegantly preserved phages with tail structures in their glutaraldehyde-fixed material.

In addition to the developing phages, Fig. 9 also reveals an unidentified electron-dense structure (DS) and a membrane structure (ME). A protrusion of the cell wall and cell membrane is observed at different regions of the cell (large arrow). The cell in Fig. 12 appears to be close to the stage of lysis. A rupture of the cell wall is apparent (arrow), and disintegration of the cell membrane seems to be in progress.

Unfortunately, it was not possible to obtain, in this study, a cell at the point of lysis, in which viruses were being released into the environment. The determination of the range of time during which lysis occurred was based on the observation that a large number of intracellular phages were observed at 180 and 190 min after infection (Fig. 9 and 13), and that a large number of extracellular phages and much cellular debris were observed at 210 min (Fig. 14–17). It may well be that cells on the verge of lysis are so delicate as to be destroyed in the preparative treatments. This morphological evidence was substantiated by results of the plaque count assay taken simultaneously with the samples for electron microscopy.

This study of the sequence of events in the morphological development of NCMB 385 has contributed additional information on the cytological changes occurring in the host organism and the morphological development of the bacteriophage. The nucleus of the host organism does not show an initial disintegration followed by a peripheral migration. These studies give no indication of the existence of two morphologically distinguishable pools of DNA (19, 20). Adequate numbers of intracellular phages were obtained without the superinfection procedure utilized by Kellenberger et al. (20) to produce lysis inhibition. Interesting information has also been provided by the presence of a relatively large number of empty heads (as many as 15 to 18 per cell section) in untreated, infected bacterial cells. Such numbers of empty heads have not been reported previously in untreated bacterial cells studied in any of the other investigations of intracellular phage replication which have come to our attention (20, 21, 25, 26). The significance of these empty heads is open to

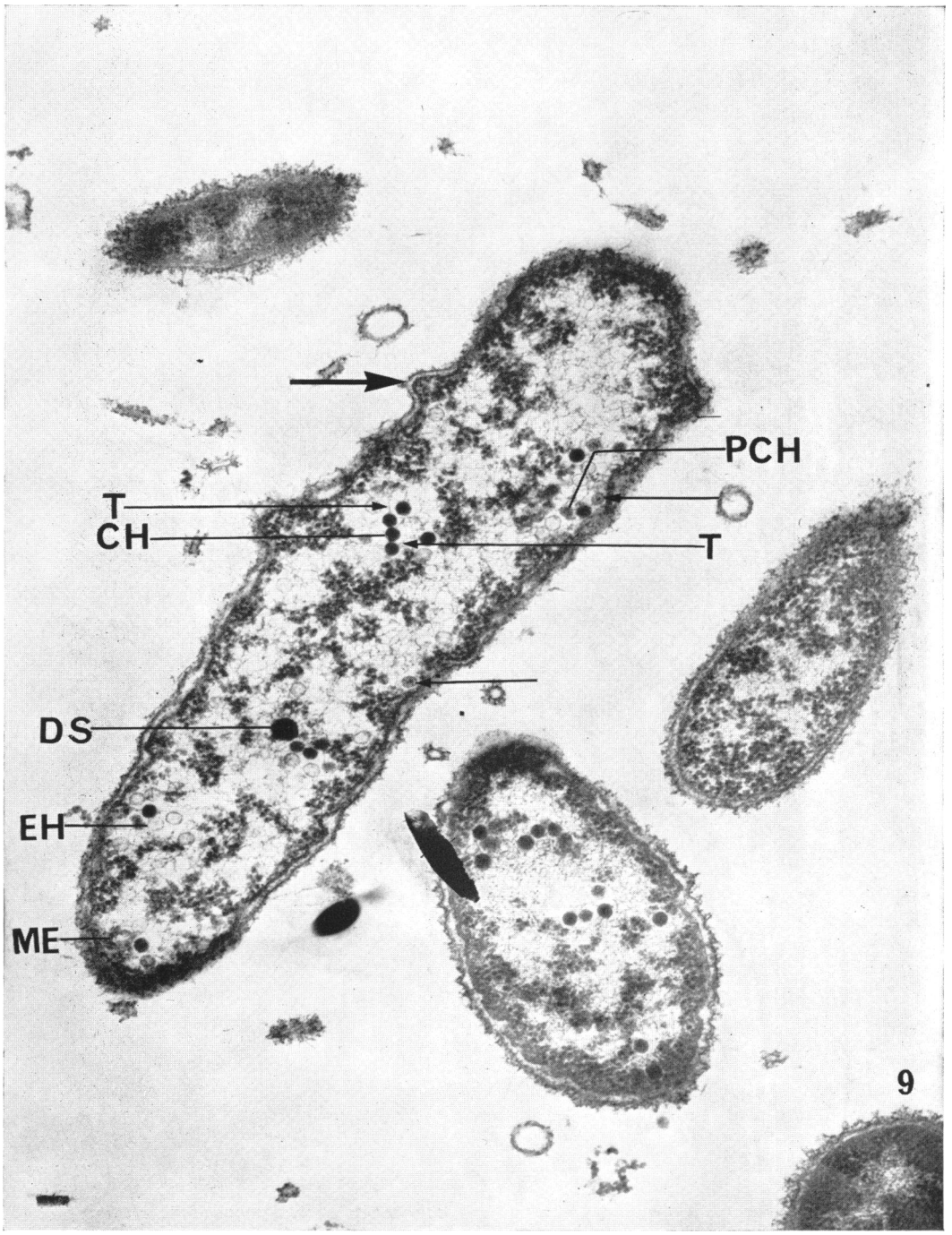


FIG. 9. *Intracellular phages in all stages of development are observed in this cell at 180 min after infection. Structures labeled T (see points of small arrows) may be interpreted as tail structures. However, it is equally possible that they may be strands of DNA. A dense structure (DS) and mesosome-type membrane system (ME) are seen. Protrusions of the outer surfaces of the cell are observed as indicated by large arrow. Doubly stained with alcoholic uranyl acetate and lead citrate.* × *49,000.*

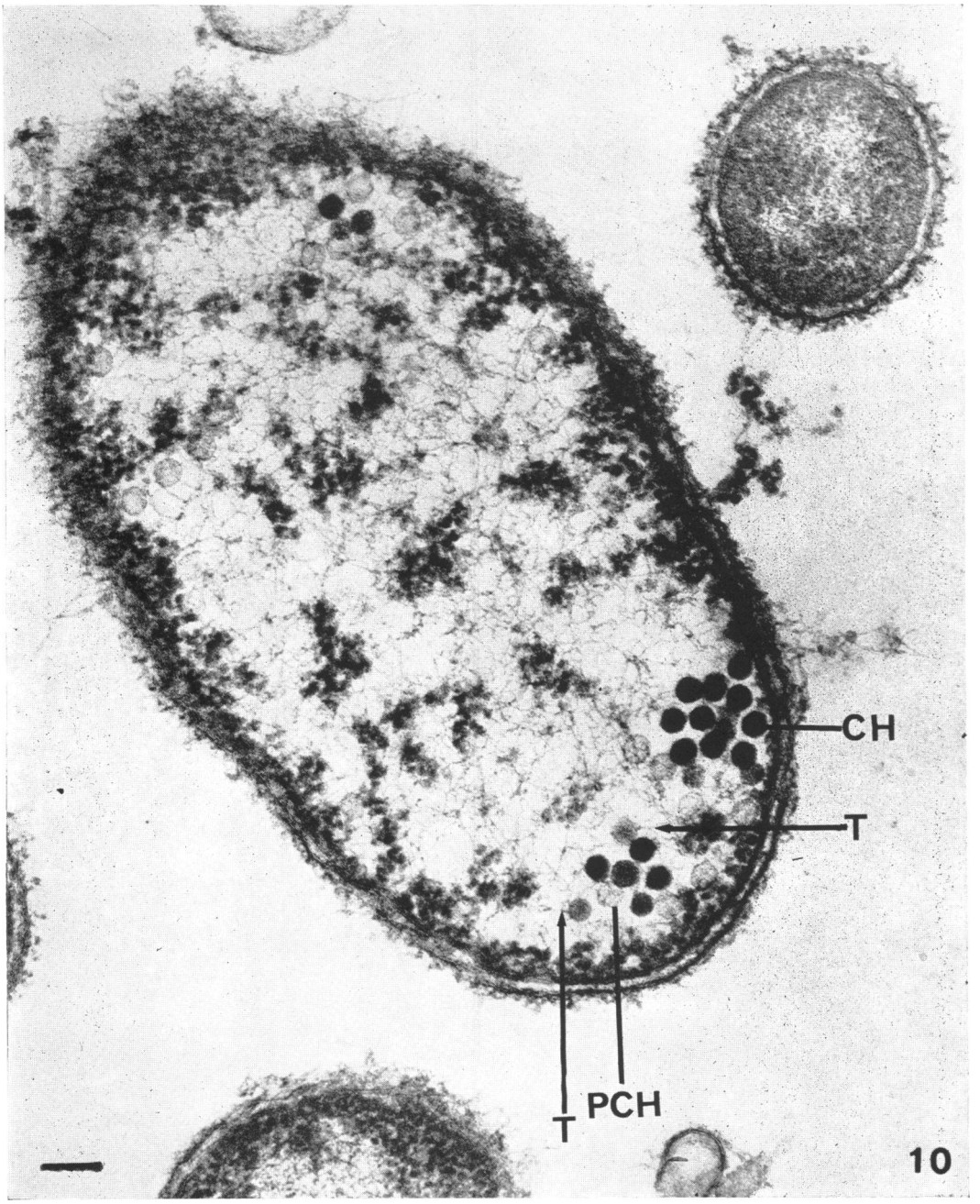


FIG. 10. *At higher magnification, intracellular particles with structures which may be interpreted as tails (T) are again observed. Also, partially condensed (PCH) and fully condensed (CH) heads are indicated. Doubly stained with alcoholic uranyl acetate and lead citrate.* × *90,000.*

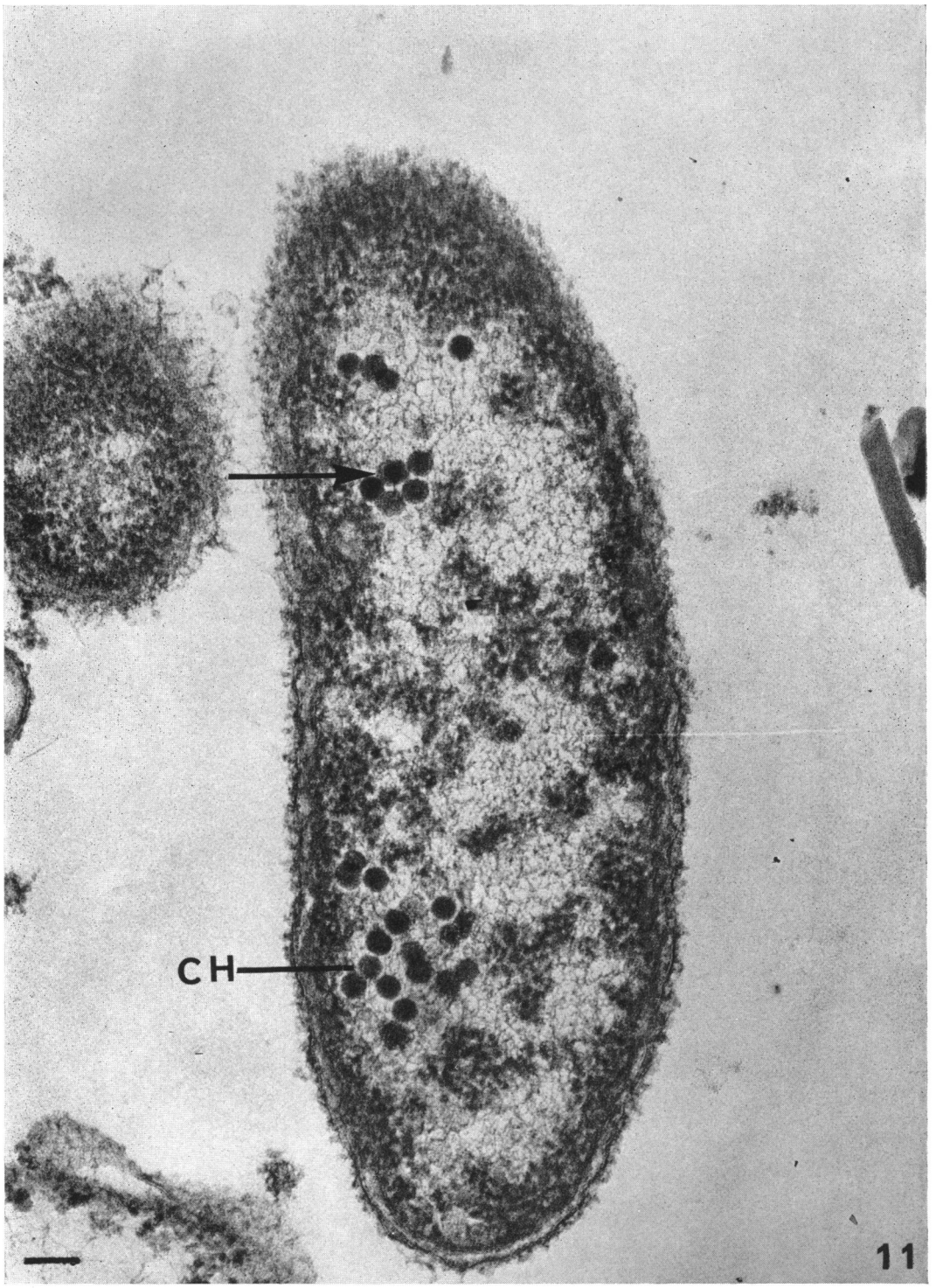


FIG. 11. *Phage particle sectioned so that the membrane surrounding the nucleic acid is evident, as indicated by arrow. In the majority of the condensed heads (CH), the membrane is not apparent. This cell is representative of a sample taken at 180 min after infection. Doubly stained with alcoholic uranyl acetate and lead citrate.* × *83,000.*

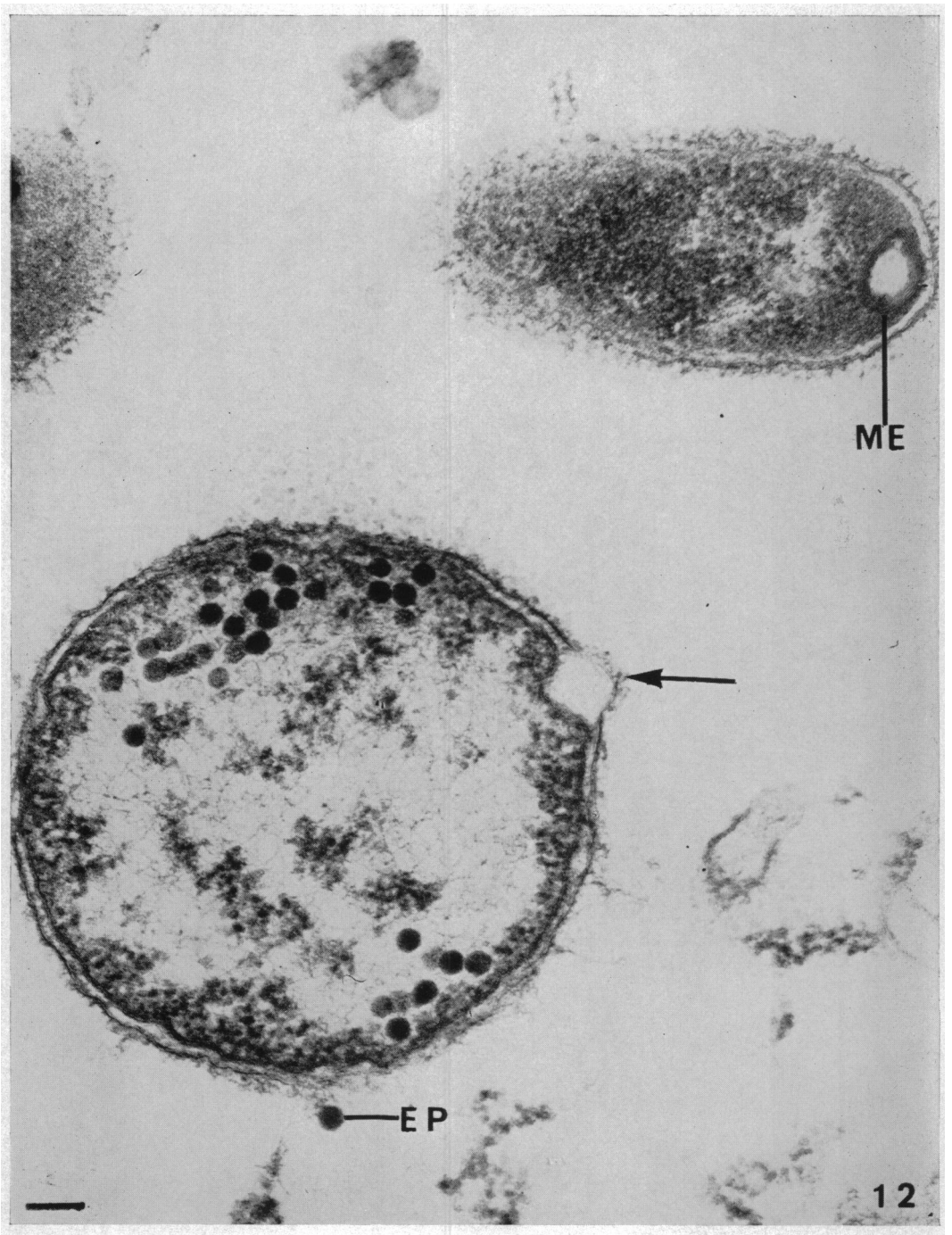


FIG. 12. *A bacterium considered to be at the point of lysis is observed 180 min after infection. A rupture of the cell wall is apparent (arrow). Extracellular phage is observed (EP) and also an intracytoplasmic membrane system (ME) in the cell at upper right. Doubly stained with alcoholic uranyl acetate and lead citrate.* × *90,000.*

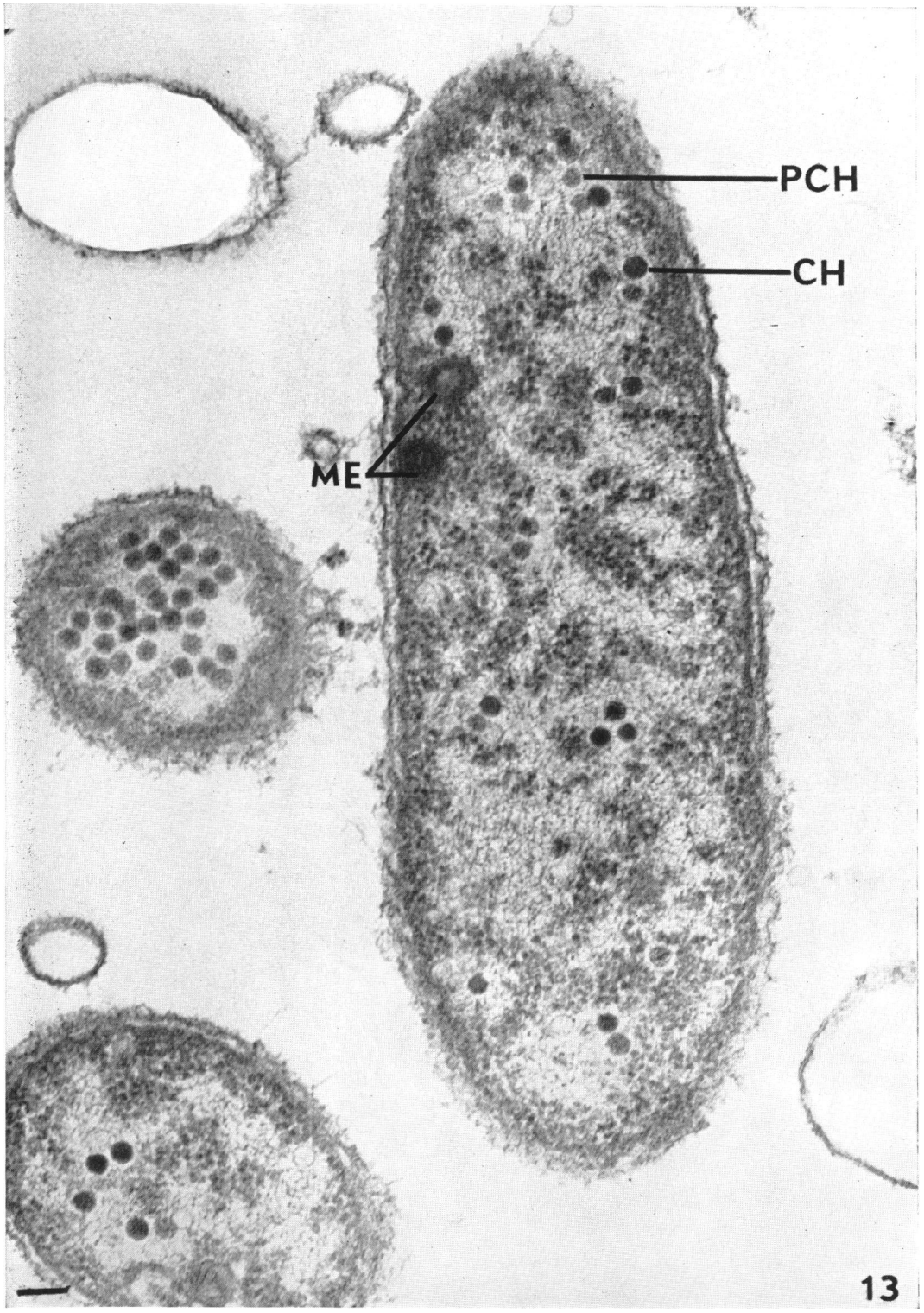


FIG. 13. *A cell at 190 min after infection showing two intracellular membrane systems, mesosome-like in appearance (ME), within a cell in the process of replicating phages. Phage heads in the partially (PCH) and completely condensed (CH) stages of development are seen. A transverse section of a second bacterium also shows many replicating phages. Doubly stained with alcoholic uranyl acetate and lead citrate.* × *75,000.*

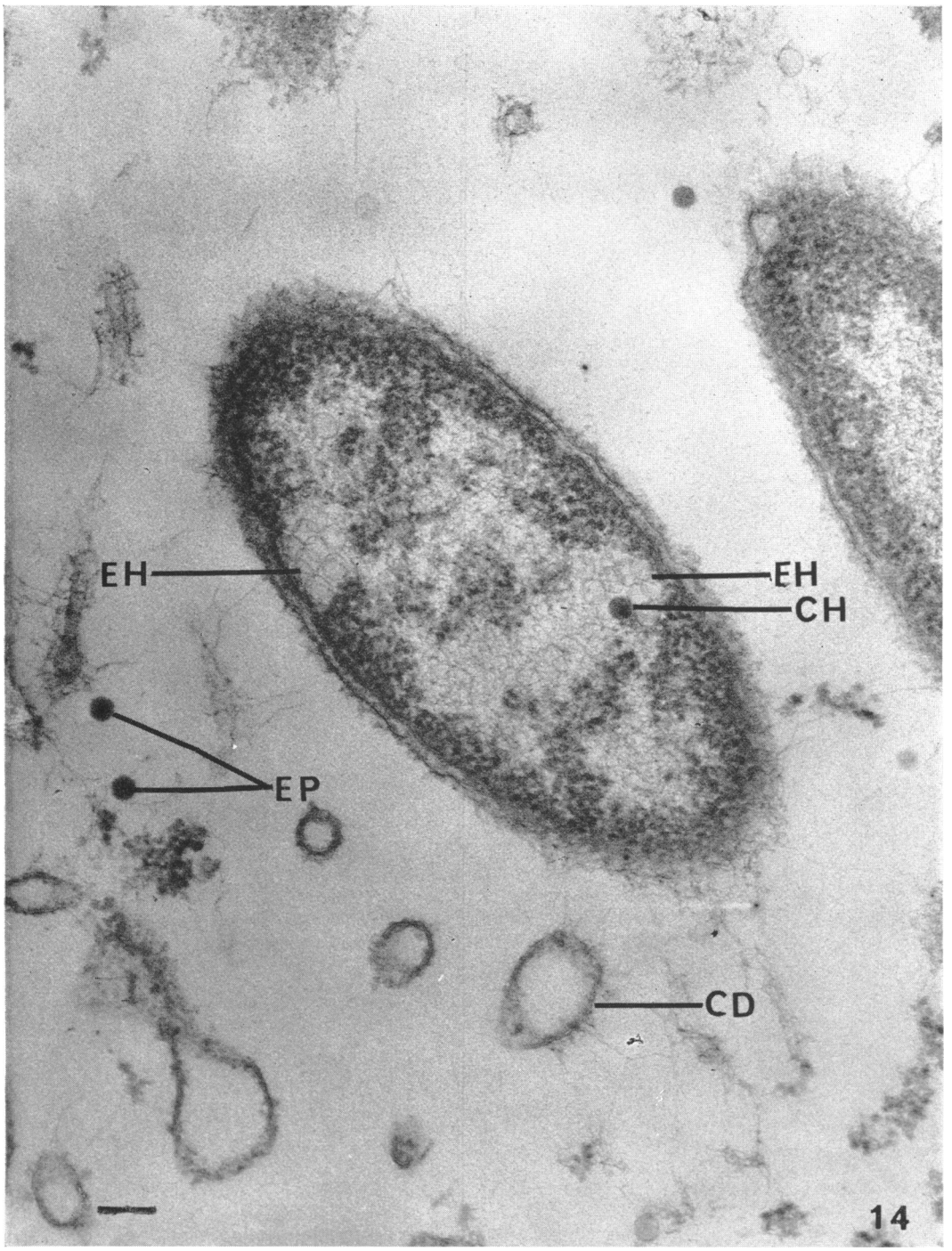


FIG. 14. *Packets of hexagonally shaped membranes, i.e., empty heads (EH), are observed in a cell taken at 210 min after infection. A completely condensed head (CH) is seen. Cellular debris (CD) and extracellular phages (EP) presumably resulting from the first lysis, are indicated.* × *90,000.*

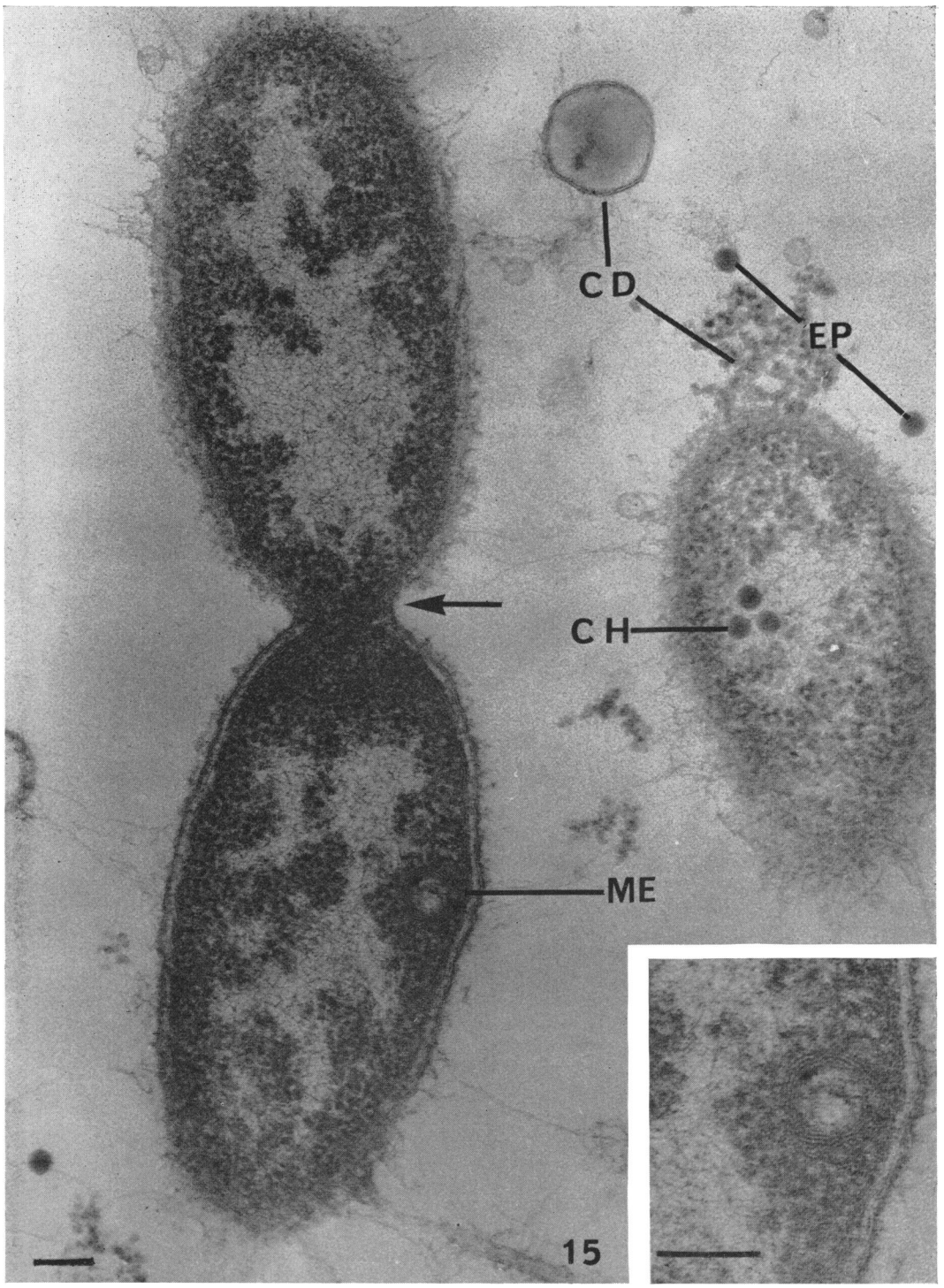
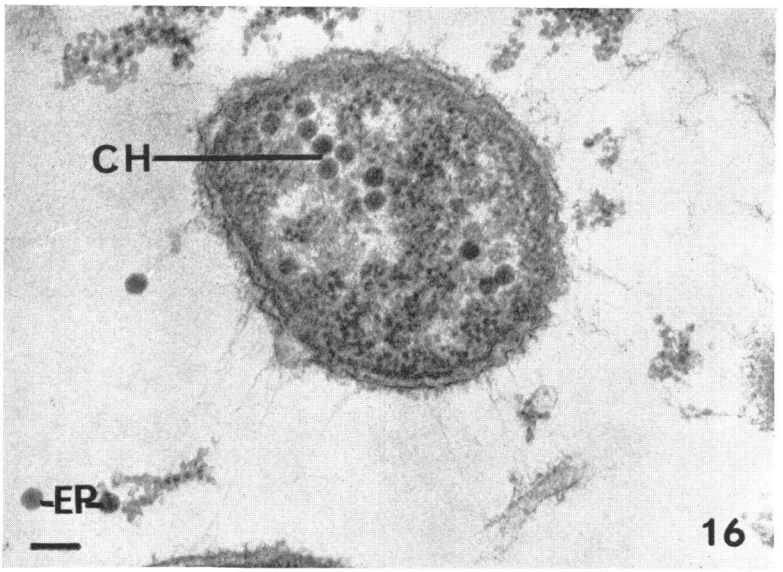


FIG. 15. *At 210 min after infection, extracellular phages (EP), presumably resulting from lysis, intracellular phages (transverse section) and a dividing cell with mesosome type structure (ME) are observed. The arrow indicates the constriction at the plane of division. The mesosome-like structure is seen in close proximity to the cytoplasmic membrane, but no point of attachment is observed. Alcoholic uranyl acetate-stained.* × *90,000. A higher magnification of the mesosome is seen in inset.* × *160,000.*



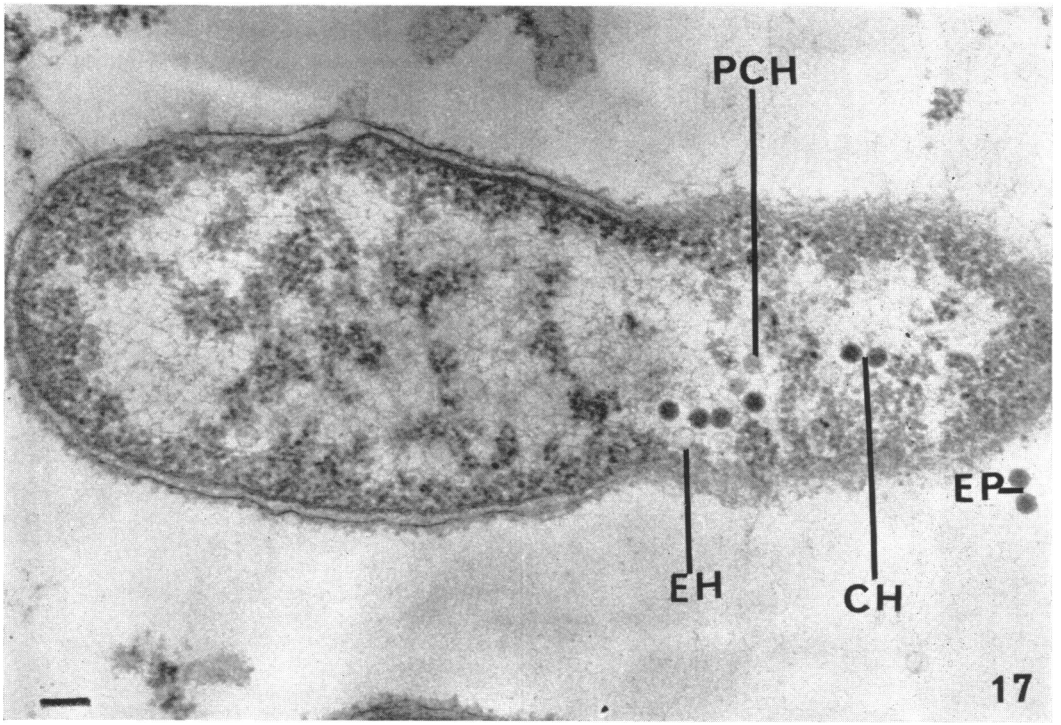


FIG. 16. *Transverse section of a bacterium 210 min after infection in which only completely condensed heads (CH) are observed. Extracellular phages (EP) are seen free in the section. Alcoholic uranyl acetate-stained.* × *68,000.*

FIG. 17. *Longitudinal section taken at the same time as the bacterium in Fig. 16 in which empty (EH), partially condensed (PCH), and completely condensed (CH) heads are observed. Extracellular phages are also indicated. Alcoholic uranyl acetate-stained.* × *68,000.*

speculation, and they present several intriguing possibilities for studies of intracellular phage development.

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