Ultrastructure of *Escherichia coli* Cells Infected with Bacteriophage R17

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ABSTRACT

FRANKLIN, RICHARD M. (Institut de Recherches sur le Cancer, Villejuif, Seine, France), AND NICOLE GRANBOULAN. Ultrastructure of *Escherichia coli* cells infected with bacteriophage R17. *J. Bacteriol.* 91:834-848, 1966—Ultrastructural changes in *Escherichia coli* cells infected with ribonucleic acid (RNA) bacteriophage R17 were studied under conditions of one-step growth. No morphological alterations were seen during the latent period. During the period of rapid viral synthesis, a fibrillar lesion surrounded by ribonucleoprotein particles was observed in a polar region. Late in infection, paracrystalline arrays of virions were found in over 90% of the cells. When protein synthesis was blocked by chloramphenicol at 20 min postinfection, allowing continued viral RNA synthesis without production of coat protein, a dense fibrillar area appeared in a paranuclear region. Cytochemical studies were done on cells embedded in hydroxypropyl methacrylate, a water-miscible embedding agent. The paracrystalline arrays of virions were digested after extensive treatment with either pepsin or ribonuclease. Shorter digestion with pepsin resulted in better definition of the crystal regions. The fibrillar area found in chloramphenicol-treated cells was digested by ribonuclease but not by pepsin, and was also resistant to lead extraction. This region probably represents a pool of virus-specific RNA.

The following two papers deal with the morphological changes associated with the multiplication of the ribonucleic acid (RNA) bacteriophage R17 in its host cell *Escherichia coli*. Bacteriophage R17 multiplies rapidly in male strains of *E. coli* and reaches high intracellular phage titers (15). The presence of paracrystalline arrays of virions has been reported for several related RNA phages (6, 21). The present report includes a description of paracrystalline arrays of R17 in the later stages of infection but emphasizes the morphological alterations in the earlier stages.

Chloramphenicol, an inhibitor of protein synthesis, prevents viral RNA synthesis when added prior to 8 min postinfection (14). But, when chloramphenicol is added 15 to 20 min after infection, there is an accumulation (3, 14) and continued synthesis of viral RNA (3, 5). These facts are utilized in this study in a search for morphological evidence for a pool of accumulated viral RNA.

In morphological studies of this type, identification of structures in terms of their macromolecular components is tenuous. Therefore, use is made of differential enzyme digestions accord-

ing to principles enunciated by Bernhard and Tournier (2). This is possible through the use of a new hydrophilic embedding agent, hydroxypropyl methacrylate (11). Studies utilizing differential extraction or differential enzyme digestion are further corroborated by means of specific labeling of macromolecular components by tritiated precursors, followed by high-resolution autoradiography. This technique is discussed in the accompanying paper (8a).

MATERIALS AND METHODS

Media. MS broth was prepared according to the formula of Davis and Sinsheimer (4), without the addition of thiamine hydrochloride. MS broth was used for the growth of the host cell and in phage growth studies.

For plaque assays, both bottom and top agar contained the same ingredients as MS broth (no thiamine hydrochloride). Bottom agar contained 1% agar (Difco) and top agar contained 0.8% agar.

Sources of materials. Chloramphenicol was obtained from Parke, Davis & Co., Detroit, Mich. Pepsin (two times crystallized) and ribonuclease (pancreatic, fraction A, phosphate-free, lyophilized) were obtained from Worthington Biochemical Corp., Freehold, N.J.

2-Hydroxypropyl methacrylate (HPMA) was obtained from Rohm and Haas Co., Philadelphia, Pa.; α, α' -azobisisobutyronitrile, from Fluka A. G., Buchs, Switzerland; and Carbowax 1500, from Gurr Limited, London, England.

Antiserum. Antiserum to R17 phage was prepared by injecting rabbits subcutaneously with highly purified virus preparations (*see* Granboulan and Franklin, *manuscript in preparation*) mixed with an equal volume of a Freund-type adjuvant (9 parts of Drakeol 6VR to 1 part of Arlacel A). Each rabbit received between 45 and 90 mg of virus or 30 to 60 mg of virus protein. Only one injection was needed, and the antibody titer remained constant between 1 and 9 months after the initial injection.

Cells and virus. The original strain of *E. coli* K-12 strain Hfr₁ (λ) *meth*⁻ was kindly provided by E. B. Ellis and A. F. Graham. A nonlysogenic strain was derived from this by picking survivors of a heavy dose of ultraviolet radiation. This strain is designated as Hfr₁ (λ ⁻). The original strain and the derived strain grow, to a limited extent, in TPG (22) without Casamino Acids (Difco) but supplemented only with L-methionine at 100 μ g/ml. Phage λ was assayed on *E. coli* strain W1327 (nonlysogenic) kindly provided by L. M. Morse. No λ phage could be detected in the supernatant fluid of a culture of Hfr₁ (λ ⁻) or by plating Hfr₁ directly on W1327.

The original strain of bacteriophage R17 was kindly provided by E. B. Ellis and A. F. Graham. Grown on Hfr₁ (λ), the stock of R17 contained 1 λ plaque-forming unit (PFU) per 4.8×10^{11} R17 PFU (assayed on the F⁻ strain W1321). Phage λ -free R17 was isolated by four successive plaque passages on *E. coli* Hfr₁ (λ ⁻). Neither growth of the RNA phage nor metabolism of the infected cells was influenced by the presence or absence of phage λ (Franklin, *unpublished data*).

Growth of virus. For virus growth curves, the cells were grown in the appropriate medium (MS or TCG, *see* 7) and harvested during logarithmic growth (2×10^7 to 5×10^8 cells per milliliter). The centrifuged pellet of cells was mixed with a small volume of virus at an appropriate multiplicity. After allowing virus adsorption to take place (5 min at room temperature), the complexes were diluted in cold TPG salts and centrifuged. In some experiments, the pellet of infected cells was incubated for 5 min at room temperature with a 1:100 dilution of R17 antibody ($K = 8,900$ min⁻¹) and then washed three times in cold TPG salts. Dilution of the cells into the appropriate warm medium was considered the "zero time" of virus multiplication. At given time intervals, samples were diluted 1:10 into MS broth containing 10% (v/v) chloroform. Phage was titered by the usual plaque technique.

For electron microscopy, cells were infected at a multiplicity of approximately 10 to 20 PFU per cell, again in a small volume of medium at room temperature. After allowing 5 min of adsorption, the cells were diluted into MS broth at 37 C to start viral multiplication. Samples were collected and fixed at appropriate times.

Preparation of specimens for electron microscopy. For standard ultrastructural studies, bacteria were fixed according to the procedure of Ryter and Kellen-

berger (18). A 15-ml amount of culture was mixed with 1.5 ml of fixative (1% OsO₄) in Veronal-acetate buffer and was immediately centrifuged for 8 min at 5,000 rev/min. The pellet was suspended in 1 ml of fixative with 0.1 ml of tryptone broth added and was left at room temperature overnight. This suspension was then diluted in 8 ml of Veronal-acetate buffer and centrifuged, and the pellet was suspended in 3 drops of Agarose (1.5%) at 45 C. Upon solidification, the Agarose was cut into approximately 1-mm cubes, which were treated for 2 hr at room temperature in uranyl acetate solution (0.5% in Veronal-acetate buffer).

For ultrastructural cytochemistry, bacteria were fixed as just described, but were embedded in hydroxypropyl methacrylate (11) without prior uranyl acetate treatment.

For embedding in Epon, the blocks were dehydrated in acetone (25, 50, 75% for 15 min; 90 and 100% for 30 min) and embedded as follows: 20% Epon-80% acetone for 30 min, 50% Epon-50% acetone for 30 min, 70% Epon-30% acetone for 30 min, Epon overnight, and final embedding in Epon the next day with polymerization at 60 C.

For HPMA embedding, the blocks, stained or unstained with uranyl acetate, were partially dehydrated in aqueous solutions of Carbowax 1500 (1) according to the following schedule: 70 and 85% for 15 min at 37 C, embedding in the final embedding solution of HPMA prepolymerized with the catalyst α, α' -bisobutyronitrile (0.4%) followed by ultraviolet polymerization at 4 C.

Enzymatic digestions were done with sections of unstained specimens embedded in HPMA. Reduced OsO₄ was removed by floating the sections on 6% H₂O₂ in phosphate buffer for 10 min at room temperature (12, 13). Enzymatic digestions (10, 12) consisted of treatment with pepsin, 0.01% in 0.1 N HCl, pH 1, for 30 min at 37 C and with ribonuclease A, 0.1% in distilled water, pH 6.8, for 30, 45, or 120 min at 37 C. Control sections were floated on H₂O₂ as described above, followed by floating on distilled water for the same time and at the same temperature as the sections treated with enzymes.

Unstained sections embedded in HPMA were stained by one of the following treatments: (i) 5% uranyl acetate in Veronal-acetate buffer (pH 4.8) for 5 or 10 min, (ii) lead citrate for 10 min (17), or (iii) uranyl acetate for 5 min followed by lead citrate for 5 to 10 sec. All specimens were examined with a Siemens Elmiskop I electron microscope operating at 80 kv with an objective aperture of 50 μ .

RESULTS

Growth of bacteriophage R17 in *E. coli* Hfr₁ (λ ⁻). Many factors influenced the growth of RNA bacteriophages (*see* 15). These included the nutrient medium, age of cells, multiplicity of infection, cell density, and state of cells (preirradiated with ultraviolet light, pretreated with actinomycin, etc.). Two growth curves, shown in Fig. 1, illustrate the conditions most applicable to this and the following study.

FIG. 1. Growth of bacteriophage R17 in *Escherichia coli* Hfr₁ (λ^-) under conditions of (A) low multiplicity, low cell density, in TCG synthetic medium (open circles) and of (B) high multiplicity, relatively high cell density, in MS nutrient broth (closed circles).

The first set of conditions (A) consisted of growth in TCG synthetic medium (see following paper) at low multiplicity and low cell density (open-circled curve of Fig. 1). Cells were grown in TCG to a titer of 2.95×10^8 per milliliter, centrifuged, and resuspended in TPG salts (the balanced salts of TCG) at one-tenth the original volume. Virus was added at an input multiplicity of 0.15 in the presence of 0.01 M KCN. After 10 min at room temperature, the cells were diluted 10-fold in TPG salts containing 0.01 M KCN, centrifuged, and diluted into warm TCG to initiate virus multiplication. The final cell concentration was 1,000 cells per milliliter. One portion was assayed for infective centers and samples were harvested at appropriate times; the cells were lysed with chloroform and assayed for total PFU. The actual multiplicity, determined from the infective center data, was 0.046. The data are plotted in Fig. 1 as total PFU per infective center.

The second set of conditions (B) consisted of growth in MS broth at high multiplicity and high cell concentration (closed-circle curve of Fig. 1). Cells were grown in MS broth to a titer of 2.40×10^8 per milliliter, centrifuged, resuspended in 0.9

ml of TPG salts, and mixed with a small volume of virus at an input multiplicity of 21.7. After 5 min at room temperature, the cells were diluted 10-fold in TPG salts, centrifuged, and resuspended in 10 ml of TPG salts containing a 1:100 dilution of anti-R17 rabbit serum to neutralize any unadsorbed virus. After 5 min at room temperature, the cells were washed three times with TPG salts and then diluted into warm MS broth to initiate virus growth. Samples were taken at appropriate time intervals, the cells were lysed by the addition of 10% (v/v) chloroform, and the virus was assayed by the plaque test. In this experiment, it was assumed that all cells were infected. The data in Fig. 1, therefore, are plotted in this case as total PFU per cell rather than per infective center. This procedure is justified, since paracrystalline arrays of virus can be seen in over 90% of cells infected as described here.

The latent period in experiment A was 29 min and in experiment B it was 35 min. The growth cycle was complete by 60 min in A, but even after 80 min in B there was a continued slow increase in phage titer. The yield was 675 PFU per cell in A and 4,500 PFU per cell (at 140 min) in B. On the average, there were about 5.5 physical particles per PFU (see Granboulan and Franklin, *in preparation*); therefore, the particle yield per cell is approximately 3,700 in A and 25,000 in B.

These two experiments compared with other growth experiments not presented in detail, and with the data of Paranchych and Graham (15), make possible the following generalizations. (i) The growth curve examined under conditions of low cell density was sharper and terminated earlier than that performed at high cell density. There was probably delayed lysis under conditions of high cell density, but the final yield was not greatly affected by cell density. (ii) Growth in synthetic media resulted in lower virus yields than did growth in broth. (iii) Multiplicity of infection did not affect the latent period, at least in the present study. (iv) Intracellular virus accumulated before there was release.

Ultrastructure of uninfected bacteria. The ultrastructure of the strain of *E. coli* used here and embedded in Epon was similar to that of *E. coli* K-12 as described by Kellenberger, Ryter, and Séchaud (9). Deoxyribonucleic acid (DNA) filaments about 30 Å in diameter formed a network in the central nucleoid (Fig. 2a). Although ribosomes were very densely packed in the cytoplasm, it was often possible to distinguish groups of ribosomes which seemed to be associated in topologically linear arrays (Fig. 2a, b). In some sections, the filamentous DNA of the nucleoid extended to the periphery and was in close contact with the bacterial membrane.

FIG. 2. (a) *Uninfected bacterium. Epon. N = Nucleus. $\times 80,000$.* (b) *Detail of (a) showing linear arrays of ribosomes. $\times 180,000$.*

Ultrastructure of infected bacteria. Prior to 45 min postinfection, there was no change in the bacterial ultrastructure. At 45 min postinfection, one or a few regions devoid of ribosomes, but packed with a dense fibrillar material, were found within the cytoplasm, usually at one pole of the cell (Fig. 3; 4a, b). These areas were surrounded by ribosomes (Fig. 4a, b). Similar structures have been observed in *E. coli* infected with bacteriophage f2 (F. M. Schwartz, *personal communication*).

Paracrystalline arrays of virions were first found at 70 min postinfection. Prior to this time, it was not possible to distinguish virions, which are about as large as ribosomes. The paracrystals were generally located at one pole of the bacteria (Fig. 5). At this stage, the paracrystals were rare but they were more frequently found at 90 min postinfection and were present in almost every bacterium at 120 min postinfection. These crystalline structures were easily recognizable, but it was not possible to study the morphology of the individual viral particles within them (Fig. 6).

Ultrastructure of bacteria treated with chloramphenicol. The chloramphenicol experiments were designed to investigate the accumulated viral RNA synthesized when the drug was added to cells at 20 min postinfection (see introduction).

No ultrastructural change was found in uninfected bacteria treated with chloramphenicol (50 $\mu\text{g/ml}$) for 15 or 30 min. After 80 min, a vesicular alteration of the nucleoid was sometimes found, and was more frequently observed in cells treated for 100 min. This morphological alteration resulting from chloramphenicol treatment is well known (9).

Infected bacteria were treated with chloramphenicol (50 $\mu\text{g/ml}$) at 20 min postinfection, a time when there are no ultrastructural alterations (Fig. 7). The first ultrastructural changes were then seen at 50 min postinfection in the form of fibrils packed in a paranuclear area (Fig. 8). This area became larger and denser in fibril content by 120 min postinfection (after 100 min of chloramphenicol treatment). At this time, the area appeared as an "inclusion body," usually located between the vesiculated nucleoid and the cytoplasm (Fig. 9).

Ultrastructural cytochemistry of uninfected bacteria. Bacteria fixed according to Ryter and Kellenberger (18) and embedded in HPMa gave well-preserved ultrastructure (Fig. 13). The cytoplasm, however, was dense and homogeneous, and therefore it was difficult to distinguish ribosomes (Fig. 13). When bacteria were fixed only with osmium tetroxide, the filamentous structure (DNA) was not well preserved, but the cytoplasmic ultrastructure was satisfactory (Fig.

10a), although dense and homogeneous (Fig. 10a). Ribosomes could be observed, however, after pepsin digestion of ultrathin sections (Fig. 10b), but they disappeared after ribonuclease treatment (Fig. 10c). After ribonuclease digestion, the high electron density of DNA resulting from the complex of DNA, ribonuclease, and uranyl acetate (23) was not seen consistently, but was frequently observed (see Fig. 10c and 15c).

Ultrastructural cytochemistry of infected bacteria. No ultrastructural change was found in infected bacteria until 45 min postinfection. At this time, the same homogeneous areas found in infected bacteria embedded in Epon were seen at one pole. These areas were somewhat less evident in HPMa than in Epon. The results of enzymatic digestion with pepsin or ribonuclease were not clear enough to allow an interpretation.

The paracrystalline arrays of virions seen at 120 min postinfection were well preserved—better, indeed, than in Epon-embedded material (Fig. 11). The diameter of the virion in these crystals was about 200 Å, and the center-to-center distance was 230 Å. A short (15 min) treatment with pepsin improved the resolution of the individual virions. The virus particle appeared to be composed of a central dense core surrounded by a less dense, narrow zone, probably corresponding to the capsid (Fig. 12a). The structure of the crystal disappeared completely after 1 hr of ribonuclease treatment (Fig. 12b).

Ultrastructural cytochemistry of bacteria treated with chloramphenicol. The paranuclear fibrillar zone seen in Epon sections was also evident in HPMa sections (Fig. 13). Although the fibrillar area was less well preserved in bacteria only fixed with osmium tetroxide, it was easily recognizable (Fig. 14a). When sections were stained with lead citrate alone, the electron density of these fibrils increased, whereas the nuclear material was extracted. In tissues fixed only with OsO_4 , RNA and DNA were differentially affected by lead staining. The contrast of RNA was increased whereas DNA was partially or totally extracted (*see* 12). The paranuclear fibrillar area therefore reacted like RNA. Further evidence that the fibrils may be RNA came from enzymatic digestion studies. The fibrils were not attacked by pepsin, even after a prolonged treatment (Fig. 14b), but they disappeared after ribonuclease treatment (Fig. 14c).

DISCUSSION

The normal *E. coli* cell has been studied in detail by Kellenberger and his coworkers (9, 18). Although the techniques are different in the present study, the fine structure of the cytoplasm is well defined, and linear arrays of ribonucleopro-

FIG. 3. Cell 45 min after R17 infection. Epon. Note homogeneous zone (arrow). $\times 75,000$.

FIG. 4. (a) High magnification of the lower portion of Fig. 3a, showing the area containing closely intertwined fibrils but no ribosomes. Ribosomes surround the area. $\times 240,000$. (b) Ribosomes around and penetrating into a similar area (arrow). $\times 180,000$.

FIG. 5. Cell 70 min after R17 infection. Epon. A paracrystal region (c) may be seen at one pole of the bacterium, adjacent to the nucleus (N). $\times 75,000$.

FIG. 6. Cell 90 min after R17 infection. Epon. Section of a paracrystal. The individual viral particles cannot be distinguished. $\times 100,000$.

FIG. 7. Cell 20 min after R17 infection, just prior to addition of chloramphenicol. Normal nonvesicular appearance. $\times 80,000$.

FIG. 8. Cell 50 min after R17 infection. Chloramphenicol added at 20 min postinfection. Epon. Cross section as in Fig. 7. Fibrillar zone (F) close to the nucleus (N). $\times 80,000$.

FIG. 9. Cell 120 minutes after R17 infection. Chloramphenicol added at 20 min postinfection. Accumulation of fibrillar material (F). $\times 100,000$.

FIG. 10. Uninfected bacterium. Osmium tetroxide fixation. HPMA. The nucleus (N) is poorly preserved. (a) No enzymatic digestion. Staining with uranyl acetate and lead citrate. Cytoplasm appears rather homogeneous and very dense. $\times 60,000$. (b) Section treated with H_2O_2 followed by pepsin for 30 min. Ribosomes are now apparent. $\times 60,000$. (c) Section treated with H_2O_2 followed by ribonuclease for 45 min. Ribosomes are probably extracted. $\times 60,000$.

FIG. 11. Cell 120 min after R17 infection. OsO_4 fixation, HPMA. Section treated with H_2O_2 and stained with uranyl acetate and lead citrate. Crystalline arrays of virions. $\times 130,000$.

tein (RNP) particles (ribosomes) are seen. These arrays may represent polyribosomes. Configurations of RNP particles which might represent polyribosomes have also been observed in *Bacillus cereus* cells which were partially lysed by freezing and thawing (16). In this case, the RNP particle arrays were distributed throughout the cytoplasm as they are in the present study. These observations conflicted with the original report of Schlesinger (19), who reported that at least 25% of the ribosomes of *B. megaterium* were bound to protoplast membranes as polyribosomes capable of controlling protein synthesis. This apparent discrepancy has now been resolved by ultrastructural studies indicating a reticular matrix extending through the cytoplasm of *B. megaterium* to which the ribosomes are bound (20). Upon lysis of the cell, this reticulum may fractionate with protoplast membranes.

If we compare the virus growth curve (Fig. 1) with the ultrastructure of the infected cell (Fig. 3-6), the lack of morphological changes through

the latent period are most notable. Not until 45 min postinfection, when there is a maximal rate of virus synthesis, is it possible to observe any difference between uninfected and infected cells. The fibrillar areas seen at this time are surrounded by ribosomal arrays, suggesting that these might be localized foci of viral synthesis. Presumably, prior to 45 min postinfection, these foci are too small to be distinguished from normal cytoplasmic components. Although these foci can be seen in HPMA-embedded bacteria, it has not been possible to identify the contents of the foci by differential enzymatic digestion. This is possibly due to the small size of the foci and, therefore, the inability to unambiguously identify them in sections treated with hydrogen peroxide and enzymes. Crystals are first observed in some infected cells at 70 min postinfection. At this time, there is an average of 400 to 500 PFU or 2,000 to 2,500 virions per cell. Since only a few cells contain crystals at this time, it may be that crystal formation only occurs when the intracellular virion concentra-

FIG. 12. Same specimen as in Fig. 11. (a) Section treated with H_2O_2 followed by pepsin for 15 min. Same staining as in Fig. 11. $\times 150,000$. (b) Section treated with H_2O_2 followed by ribonuclease for 1 hr. Same staining as in Fig. 11. Viral particles no longer visible in the paracrystalline area (c). $\times 80,000$.

FIG. 13. Cell 120 min after R17 infection. Chloramphenicol added at 20 min postinfection. HPM. Accumulation of fibrillar material (F) at one pole of the bacterium and close to the nucleus (N). $\times 105,000$.

FIG. 14. Same specimen as in Fig. 13. OsO_4 fixation. HPMA. Nuclei poorly preserved (N). (a) No enzymatic digestion. Staining with uranyl acetate and lead citrate. Same fibrillar area (F) as in Fig. 13 but not so well preserved. $\times 70,000$. (b) Section treated with H_2O_2 followed by pepsin for 20 min. Same staining. Ribosomes clearly visible. No alteration in the fibrillar area (F). $\times 70,000$. (c) Section treated with H_2O_2 followed by ribonuclease for 45 min. Same staining. Positive staining of the nucleus (N); ribosomes no longer visible. Complete digestion of fibrillar area (F). $\times 70,000$.

tion is somewhat higher. Most cells contain crystals at 90 min when there are about 10,000 virions per cell. It was not possible to identify virions outside of the paracrystalline areas, since they are of approximately the same size and density as RNP particles. In the study of De Petris and Nava (6) on μ_2 , another RNA phage, the number of virions per cell was estimated at 10,000 from counts of virions in paracrystals in sectioned bacteria. Crystalline arrays of virions which filled over half of a bacterial section were reported by Schwartz and Zinder (21). Possibly all of the intracellular virions coalesce to form one or several intracellular crystals if the bacteria do not lyse before the virion density reaches some critical concentration of 2,500 to 10,000 per cell. Since the volume of an *E. coli* cell is approximately 2×10^{-12} cm³, this would be a concentration of 10^{15} to 5×10^{15} virions per cm³. It is possible to obtain such concentrations of purified virus and to obtain crystal formation from such solutions. Thus, in vivo crystal formation may be a physical process similar to in vitro crystallization.

The virus in the paracrystalline regions can be seen more clearly after "etching" with pepsin. More extensive pepsin or ribonuclease digestion removes these crystalline areas. This suggests that there may be a protein and RNA matrix by which the crystalline areas are firmly bound in the cytoplasm. In well-defined areas of "etched" crystals there is hexagonal close-packing of virions. The packing is not uniform throughout an entire section, however, thus emphasizing the paracrystallinity.

From the biological and biochemical studies on the synthesis and possible accumulation of bacteriophage RNA in the presence of chloramphenicol (3, 5, 14), it appears likely that the paranuclear fibrillar area found in infected cells treated with chloramphenicol at 20 min postinfection is a pool of viral RNA. To support this contention, we may note the absence of this morphological feature in uninfected cells treated with chloramphenicol, its digestion by ribonuclease but not by pepsin, and its resistance to prolonged extraction with lead salts. All of these properties suggest that the fibrillar structures in the paranuclear area represent RNA of a type found only in the bacteriophage-infected cell. Unfortunately, it was not possible to distinguish single- and double-stranded RNA or the replicative intermediate (7, 8) in this sectioned material.

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