THE ELECTRON MICROSCOPY OF DEVELOPING BACTERIOPHAGE

III. TECHNIQUES TO VISUALIZE DEVELOPING FORMS

by

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In previous papers in this journal[1,2] methods have been described which we have been using to study the development of bacteriophage from infected bacteria. These procedures have led to much new information but it is apparent that by itself this information is not sufficient to give an adequate demonstration of all essential steps in the multiplication of the bacteriophages against *Escherichia coli* we have been investigating.

In one of these procedures the broth cultures of infected but not completely lysed bacteria have been inactivated and then repeatedly sedimented and resuspended first in saline and then in water before being spread on a substrate for examination. This has the advantage of giving especially "clean" preparations that show the fine details of bacterial structure as well as of permitting an accurate control of the period of incubation. Its obvious disadvantages are that the bacteria are not observed under the conditions under which growth and infection proceeds, that the infected bacteria may be altered by the manipulations involved in washing and that all light objects such as particles of bacteriophage are not retained in the final preparation. In the other procedure the preparation is a "replication" of the top surface of an agar plate on which plaques have fully developed. This technique shows the products of the lytic process undisturbed from their natural positions. It has the great disadvantage, however, of showing only the end product of this process and not any of the essential steps through which it has been reached.

We have since developed and are using techniques of specimen preparation which avoid these and certain other disadvantages of the two foregoing methods. For a full understanding of the mechanism of bacteriophage growth it is clear that detailed studies should be made of several types of bacteriophage using all these and perhaps other methods of specimen preparation. It will require a long time to complete such a study, but in the meantime it has seemed worthwhile to outline the new modifications in procedure and to illustrate the new knowledge their application brings out.

The essential step of catching for observation all the contents of a lysing bacterial culture after short periods of incubation can be taken by promptly killing such a broth culture, as by the addition of 4% formalin, pouring it over an agar plate and, after draining and incubating until the broth has just soaked into the agar, "replicating" in

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the manner previously described for examination of plaques[1]. When the drying is properly carried out the broth and salt will soak so thoroughly into the agar that very "clean" preparations are obtained (Fig. 1), while at the same time the infected bacteria and their products are picked up by the replicating collodion film after a minimum of mechanical treatment. Preparations made in this way have thus far been of most value in revealing the contents of bacterial residues after incubations about as long as the burst time, or in the case of infections with slow-lysing "wild" strains of bacteriophage, after still longer incubations. In such bacteria they have revealed many of the newly formed particles of bacteriophage and many forms which may be these particles in course of development. These probably immature forms are of various sorts. Among them are the more or less "empty" heads shown in previously published photographs[2,3] and illustrated and discussed by HERČÍK[4]. These have substantially the diameters of the recognized bacteriophage particles; they show all gradations between the electronically opaque thick particles of the mature bacteriophage and heads that seem to contain little more than a granule or two. Attached to many of the insubstantial heads are tails that have about the expected diameters but are not more than half the usual lengths. These abbreviated tails are seen at many points within and around the residue of Fig. 1. They occur in small clusters and frequently they radiate[5] from central masses that may be found both within protoplasmic masses and free in the bacterial debris (Fig. 2). Flattened heads are often attached to the short tails and it is very possible that they and the wheel-like objects are steps in bacteriophage formation. If this proves to be the case, then the thick tails of the even-numbered coli bacteriophages have an unexpectedly important role in the mechanism of their proliferation.

Many other interesting forms related to developing bacteriophage have already been observed in preparations made as just described. Thus after infection of slowly metabolizing *E. coli* (in an aging culture or in the cold) many ribbons or rods are found[6] that always have the diameters of bacteriophage heads. Similar forms have also been seen by HERČÍK[7] in cultures lysed by freshly isolated strains of coli bacteriophage. Many of these thick filaments are segmented (invariably into elements having the dimensions of bacteriophage heads) and they may represent a stage in an alternative process of multiplication. Bands like these have also been seen within the bodies of infected bacteria, especially when the bacteriophage is T5, and it would appear that their further study is important for a thorough understanding of how bacteriophages grow. Along with these thick bands are also many substantial, narrow rods that approximate the bacteriophage tails in diameter (Fig. 2). These occur separately on the substrate, in bacterial debris and protruding from seemingly intact cells. Their relation, if any, to the tails of bacteriophage is not yet evident. Whatever their significance they are found only in bacteriophage-diseased cultures and they cannot be confused with the numerous peritricate flagellar threads that sometimes develop from the borders of certain variants of the B strain of *E. coli* with which we have worked.

Infected bacterial residues in the preparations made as described above are flattened like those obtained by the techniques described in previous papers[1,2]. Since this undoubtedly was a consequence of drying from an aqueous medium of high surface tension, some experiments to avoid this have been made by drying from a liquid of low surface tension. This was done by separating the killed infected bacterial masses from a broth culture, sedimenting them from alcohol-water mixtures of increasing alcohol content and finally suspending them in amyl acetate for deposition on the supporting formvar film. As Fig. 3

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Fig. 1. The residue of a bacterium incubated in broth for 45 minutes after infection with T2r+ and prepared as a pseudo replica. Many objects with abbreviated tails and flattened heads appear above and below the still intact residue, as well as within its mass. Note also the "empty heads" distributed over this mass. 30,000 ×

Fig. 2. Debris of bacteria lysed by prolonged incubation after infection with T2r+. Part of a "wheel-like" aggregate with radiating short tails is just right of center. Note the lengths of filament of a diameter approximating these tails which are distributed over the lower right quadrant of this photograph. 20,000 ×

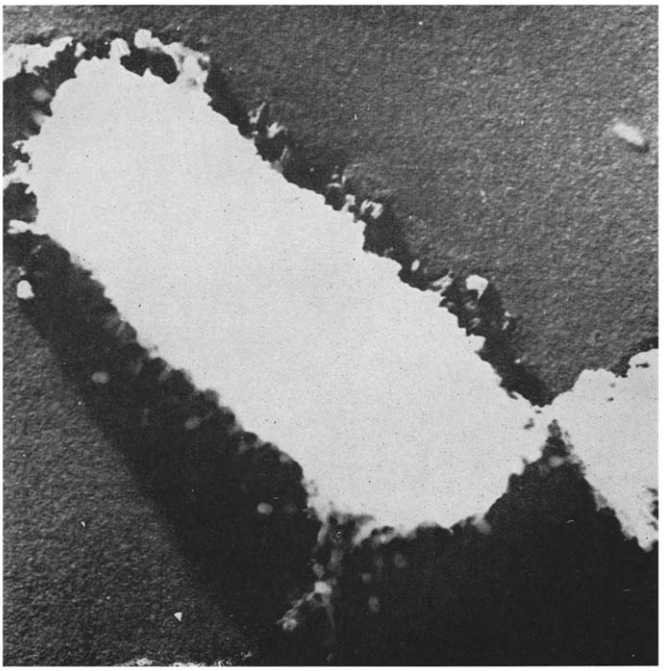

Fig. 3. A bacterium after 40 minutes incubation with T2r+ prepared by dehydration and final deposition from amyl acetate. It retains its original shape without the serious flattening that results from drying down from aqueous suspension. Its irregular outlines suggest that the bacterial membrane has been lost. 40,000 ×

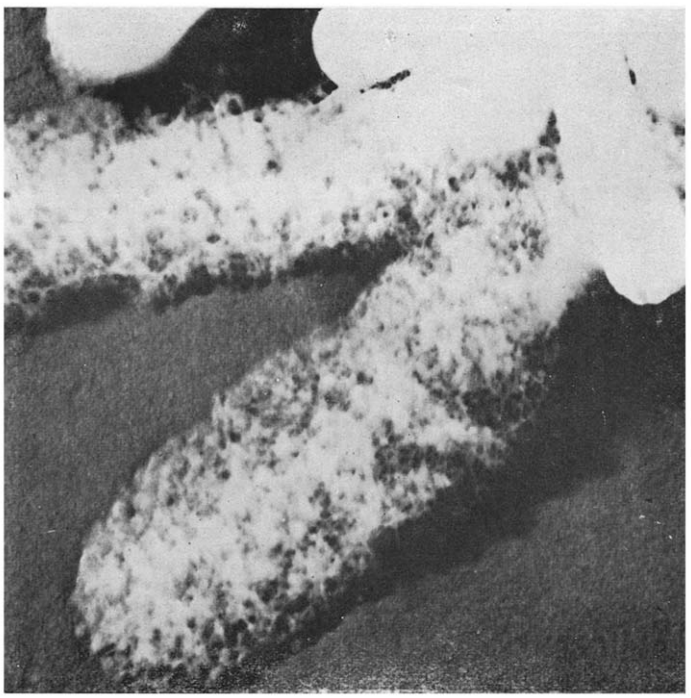

Fig. 4. An infected bacterium like that of Fig. 3 from a mass that was repeatedly sedimented from water before dehydration. In spite of the loss of newly formed bacteriophage particles, the residual spongy framework retains its original shape. Some bacteriophage particles can be seen still held by its meshes. 40,000 ×

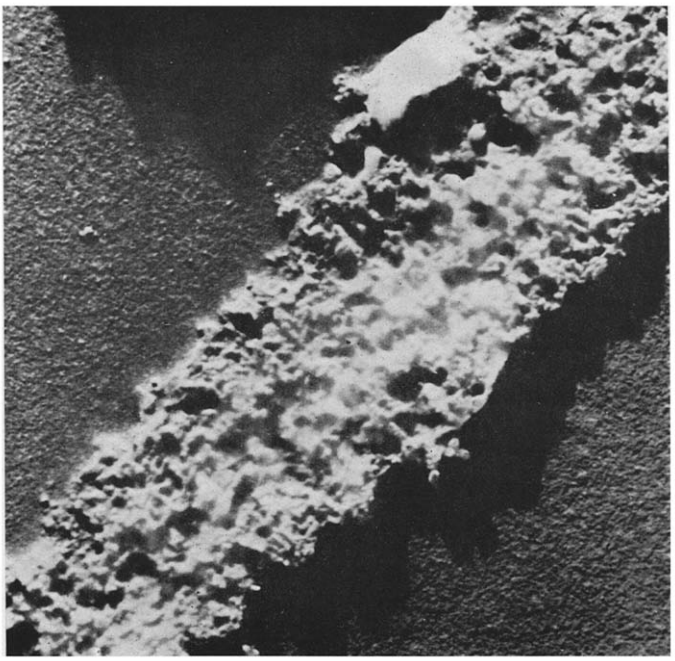

Fig. 5. A sectioned bacterium incubated 20 minutes after infection with T2r. A few particles of bacteriophage can be seen in small groups mainly along the mid-line of the section. 40,000 ×

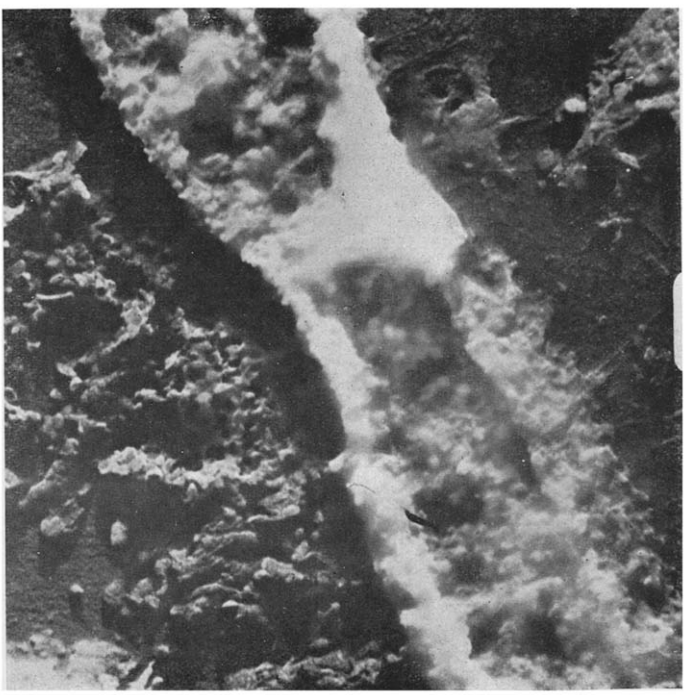

Fig. 6. A section through two partly-divided bacteria showing many newly-formed bacteriophage particles in the interior, especially of the lower organism. Incubation: 50 minutes following infection with T5. 40,000 ×

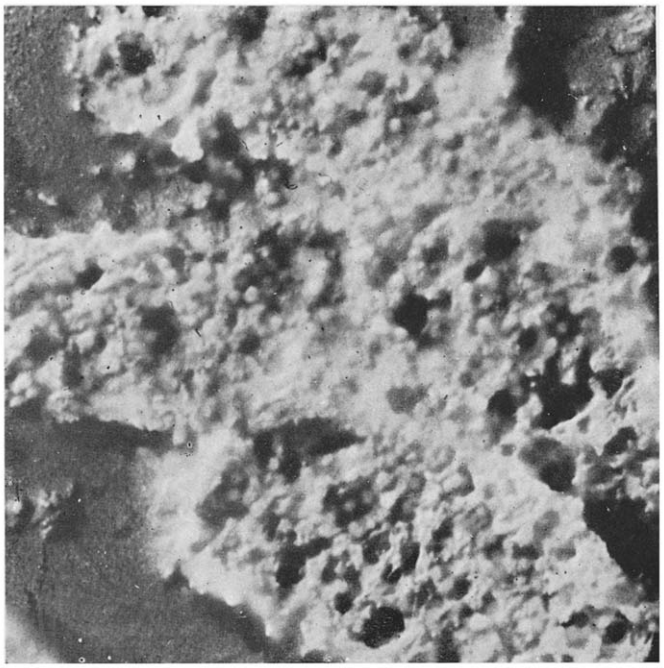

Fig. 7. A section through the residues of four bacteria which had been incubated for 50 minutes following infection with T5. The development of many particles of bacteriophage has, as with the preceding photograph, resulted in a nearly complete disappearance of the mesh so conspicuous in Fig. 5. 40,000 ×

indicates, the bacteria in preparations thus made are not appreciably flattened. This is a clear-cut demonstration of the distortion caused by drying from water, but the complete opacity of the bacterium in this figure is an equally clear proof of the impossibility of learning much about the internal structure of infected bacteria prepared in this fashion. Somewhat more can be seen if washed, fully-infected bacteria are repeatedly sedimented from water before the introduction of alcohol. Many bacteria in preparations made in this way look like that shown in Fig. 4. This bacterial residue obviously is a sponge which still retains the shape of the original cell though it has lost its membrane and the repeated centrifugations have presumably squeezed out most of the new bacteriophage particles that have formed within it.

Reference to these last two figures makes it obvious that unflattened bacteria should be sectioned to reveal their contents. This is not difficult. We have been doing it by transferring the alcohol-dehydrated bacterial mass to methacrylate monomer, polymerizing the suspension and cutting in the usual fashion[8]. Bacteria have been sectioned after embedding in collodion-paraffin[9]. They have proved harder to embed properly in methacrylate than any other material we have tried because of "explosions" in an unusually large number of the embeddings. Thus far we have been unsuccessful in a search for the factors responsible for this. The explosions have, however, had an all-or-none character and where they have not occurred the infected bacteria have seemed excellently preserved. A study has been undertaken of the sectioned bacteria from cultures of *E. coli* infected with the T5, T2r+ (wild) and T2r (quick-lysing) strains of its bacteriophages. Few if any particles of bacteriophage have been found within these bacteria incubated for much less than the burst times. After incubation for about the burst time, sectioned

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bacteria show particles in larger or smaller numbers. The residue of Fig. 5 was a bacterium incubated for 20 minutes with T2r. Its protoplasm is a net in which can be seen here and there small clusters of particles having the dimensions of bacteriophage. Many more particles have formed within cells such as those shown in Fig. 6 and 7. Similar residues, still retaining the shape of the original bacterium and consisting of fibrous meshes in which numerous bacteriophage particles remain trapped, are to be found in cultures of the wild T2r+ strain after incubation for several times the burst period, or until visible clearing of the culture is complete. It is evident that an essential difference between the prompt- and delayed-lysing strains of T2 lies in the durability of the protoplasmic sponge that enmeshes the new bacteriophage particles.

Photographs of sectioned infected bacteria like the foregoing are not in themselves sufficient to show how bacteriophage multiplies, but they do add materially to the information on this question given by the other preparations. They show especially the many clusters of these particles that are present, some of which may be related to the "wheel" of Fig. 2. They also show many particles somewhat smaller than finished bacteriophage which may be "immature" forms (Fig. 1) shrunken by the dehydration that precedes embedding.

Studies that combine the use of these several methods of specimen preparation are being continued with several strains of bacteriophage against *E. coli*, and it is to be expected that the information gained from them will throw more and more light on the mechanism whereby these bacteriophages proliferate. Additional results obtained in this fashion will be reported in a later article.

SUMMARY

Three methods of specimen preparation are described to supplement those already used in the electron microscopy of bacteriophage-diseased bacteria. Preparations that reveal all the contents of a broth culture can be made by pouring onto an agar plate and taking a pseudo replica from its surface. Unflattened bacterial residues can be studied by dehydration and final suspension in amyl acetate for deposition on formvar-covered grids. The contents of bacteriophage-infected, unflattened bacterial residues can be clearly seen in sections cut through a mass of such residues. Examples are shown of preparations made in these ways and some of the new information they give is pointed out.

RÉSUMÉ

Nous avons décrit trois méthodes de préparation de spécimens pour compléter celles qui sont actuellement en usage en microscopie électronique de bactéries atteintes de bactériophage. L'on peut obtenir des préparations révélant tous les constituants d'une culture sur bouillon en versant sur une plaque d'agar et en prenant une pseudo-réplique de sa surface. Des résidus bactériens non-aplatis peuvent être étudiés par déshydration et finalement par suspension dans l'acétate d'amyl et dépôt sur des grilles couvertes de "formvar". Dans les sections faites à travers une masse de résidus non-aplatis de bactéries infectées par le bactériophage on peut voir clairement le contenu de tels résidus. Nous montrons des exemples de préparations obtenues par ces méthodes et nous attirons l'attention sur quelques'unes des nouvelles données ainsi obtenues.

ZUSAMMENFASSUNG

Zur Ergänzung der Methoden, welche in der Elektronenmikroskopie von bakteriophagus-kranken Bakterien in Anwendung sind, wurden drei Herstellungsmethoden von Musterpräparaten beschrieben. Präparate welche alle Bestandteile einer Bouillon-Kultur aufweisen, können durch aufgiessen auf eine Agarplatte und Abnehmen einer Pseudo-Replik von deren Oberfläche erhalten

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werden. Bakterienreste, welche nicht abgeplattet sind, kann man untersuchen, indem man sie entwässert und schliesslich in Amylacetat suspendiert und auf Gitter, welche mit "Formvar" bedeckt sind, aufträgt. In Schnitten, welche durch solch eine Masse von Bakterienresten gemacht sind, kann man deutlich den Inhalt von nicht abgeplatteten mit Bakteriophagus infizierten Bakterienresten sehen. Es werden Beispiele von Präparaten gezeigt, welche nach diesen Methoden hergestellt sind und auf einige der neuen so erzielten Befunde wird hingewiesen.

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