Structural Changes in Escherichia coli Infected with a φX174 Type Bacteriophage

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SUMMARY

The coliphage α 3, which is morphologically similar to φX174, was shown by acridine orange staining to contain single-stranded DNA. Its infective process was studied by the electron microscopy of thin sections of Escherichia coli infected at high multiplicity. The latent period was found to be about 13 min. but no obvious structural changes were visible in host cells until 10 min. after infection, when the plasma membrane began to retract from the poles. At 14 min. intracellular phage was detected and bulges appeared on the cells at their midpoint. At 21 min. many cells were in the form of spheroplasts and others were beginning to lye. Spheroplasts formed by phage infection lysed differently from those produced by penicillin.

INTRODUCTION

The electron microscopy of thin sections of phage-infected bacteria has revealed a number of fundamentally different structural changes with various phage/host systems. Apart from the formation of intracellular phage particles, the most obvious changes are those associated with the degeneration of the cell envelope (cell wall/plasma membrane complex). One of the most spectacular changes, visible in the light microscope, is the formation of spheroplasts in Pseudomonas aeruginosa infected with an RNA phage (Bradley, 1966a). Groman & Suzuki (1966) stated that spheroplast formation occurred in Escherichia coli infected with phage φX174 in the presence of spermine. In a preliminary study, a similar phenomenon was observed in E. coli infected with the φX174-type phage α 3 (Bradley, 1968) in the absence of spermine. We describe here a more detailed study of α 3 and its infective process.

Bacteriophage α 3 was isolated from a stream in Edinburgh (Bradley, 1962). It is similar in appearance to φX174 (Hall, MacLean & Tessman, 1959), which represents the morphological group of bacteriophages with no tails and large capsomeres (Bradley, 1967). While the nucleic acid of phage α 3 has not yet been characterized, that of φX174 consists of single-stranded DNA (1-DNA) (Sinsheimer, 1959). Phage α 3 has a different host range from φX174 (Bradley, 1962).

METHODS

Culture media and methods. Oxoid nutrient broth was used for plate and broth cultures, save in the case of penicillin spheroplasts, when 3 XD broth (Fraser & Jerrel, 1953) was used. Solid medium was prepared with 2 % agar. All phage plating was...
done on double agar layer plates, the soft layer containing 1 % peptone + 1 % agar. Phage \( \alpha 3 \) (Bradley, 1963) was grown to high titre by the lysis of confluent bacterial growth on large (15 cm. diam.) double agar layer plates. After lysis, the phage was extracted with about 10 ml. of broth per plate. Efficient extraction was achieved by placing the plates on a horizontal shaker operating at 30 oscillations/min. for 2 to 3 hr. The resulting suspension was purified by alternate high- and low-speed centrifugation.

Host bacterium. *Escherichia coli* strain c2 was used throughout.

Host range tests. The sensitivity of various strains of *Escherichia coli* was ascertained qualitatively by placing a loopful of high titre \( \alpha 3 \) suspension on a double agar layer plate of the bacterium. Clearing after incubation at 37° overnight indicated sensitivity.

Acridine orange staining. The type and strandedness of the phage nucleic acid was determined by its sensitivity to nucleic acid enzymes and a fluorescent staining process based on that of Mayor & Hill (1961). A purified phage suspension in phosphate buffered saline was prepared from a broth lysate of the host bacterium and treated with DNase and RNase before acridine orange staining and enzyme sensitivity tests. Full details were described by Bradley (1966b) and Bradley & Robertson (1968).

Lysis of broth cultures by phage \( \alpha 3 \). The following procedure was used to obtain infected bacteria for electron microscopy together with data for growth curves of both phage and bacterial host from a single broth culture. A small quantity of a static overnight broth culture of *Escherichia coli* c2 was added to about 100 ml. of broth so that the bacterial concentration was about \( 10^8 \) organisms/ml. (measured by optical absorption). The culture was incubated at 37° for 2 hr with shaking to bring it to log. phase. A sample was taken for the embedding and sectioning of the uninfected cells. Broth was added to return the cell concentration to \( 10^8 \) organisms/ml. Four ml. of phage suspension in broth at a concentration of \( 7 \times 10^{10} \) p.f.u./ml. was added to 120 ml. of the culture to give a multiplicity of infection of 18.5:1. The culture was incubated with shaking at 37° and 10 ml. samples were removed at intervals. Each sample was split as follows: 0.1 ml. was diluted serially into broth at 2° and plated for counting; 5 ml. was used for cell concentration measurement, and 5 ml. for immediate fixation. The optical absorption measurements were made with a Hitachi Perkin–Elmer UV-VIS Spectrophotometer which had been previously calibrated with log. phase bacteria of known viable count, at a wave-length of 620 nm.

Preparation of spheroplasts using penicillin. An overnight culture of *Escherichia coli* c2 in 3XD medium (Fraser & Jerrel, 1953) was diluted to a concentration of about \( 10^8 \) organisms/ml. and grown to a concentration of \( 5 \times 10^8 \) organisms/ml., with shaking, at 37°. Twenty five ml. were treated with penicillin (Benzylpenicillin, Glaxo) at a concentration of 10 mg./ml. in a shaker at 37°. Samples were removed at 30 min. and 60 min. and processed for electron microscopy. The 60 min. sample was also examined, unfixed, by phase-contrast.

Fixation, embedding and sectioning of bacteria. Sufficient 25 % (w/v) aqueous glutaraldehyde solution was added to samples from both uninfected and infected cultures described above to achieve a concentration of 6.25 % (w/v). The penicillin-treated cells were suspended in 6.25 % (w/v) glutaraldehyde in Sorensen's buffer at pH 7.2. Both were fixed for 1.5 hr. After post-fixation in 1 % osmium tetroxide for 1.5 hr, cells were embedded in 2 % agar and dehydrated in 30, 50, 75 and 100 % acetone, the first two solutions containing 1 % uranyl acetate. The agar portions were treated with acetone + Vestopal (1:1), followed by two changes of Vestopal for about 8 hr before
final embedding. After polymerization at 60° overnight, sections were cut on a Huxley ultramicrotome and stained in lead citrate before they were examined. The sections were scanned in the electron microscope and the various features observed in cells in each sample were listed; micrographs were taken of typical examples.

**Phase-contrast microscopy.** A sample for phase-contrast microscopy was taken from an infected broth culture of bacteria 20 min. after infection. It was fixed for 1 ½ hr in 6·25 % glutaraldehyde, washed in Sorensen's buffer and examined under a × 70 objective. The penicillin spheroplasts were examined without fixation.

**Negative staining.** Vegetative α 3 phage particles were examined by the standard procedure using potassium phosphotungstate.

**RESULTS**

Phage α 3 (Pl. 1 (a)) is morphologically indistinguishable from φX174 and φR (Tromans & Horne, 1961; Bradley, 1962). It measures 270 Å from the centres of opposite sides, and has obvious capsomeres at each apex. The appearance is consistent with the icosahedral form of φX174 and φR.

**Nucleic acid**

The colours obtained under u.v. light after staining with acridine orange α 3 and φR, which is known to contain 1-DNA (Bradley 1966b), leave no doubt that phage α 3 also contains 1-DNA (Table 1) and this is confirmed by the results of the nucleic acid enzyme sensitivity tests.

Table 1. **Colours obtained by the acridine orange staining of phage α 3**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Nucleic acid</th>
<th>Na₂HPO₄</th>
<th>Molybdic acid</th>
<th>Tartaric acid</th>
<th>RNase</th>
<th>DNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>α 3</td>
<td>?</td>
<td>Red</td>
<td>Green</td>
<td>Green</td>
<td>- *</td>
<td>+</td>
</tr>
<tr>
<td>φR</td>
<td>1-DNA</td>
<td>Red</td>
<td>Green fading</td>
<td>Green</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Standard</td>
<td>1-DNA</td>
<td>Red</td>
<td>Pale green</td>
<td>Pale green</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Standard</td>
<td>1-RNA</td>
<td>Red</td>
<td>Paler red</td>
<td>Paler red</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>2-DNA</td>
<td>Green</td>
<td>Green</td>
<td>Orange</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Standard</td>
<td>2-RNA</td>
<td>Green</td>
<td>Green fading</td>
<td>Red</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Resistance to an enzyme is denoted by - and susceptibility by +.

**Host range**

Phage α 3 had a different host range from φX174 and St-1 (Bradley, 1964) (Table 2).

**Growth of infected bacteria**

Curves were constructed for the growth of infected bacteria and the simultaneous change in phage count (Fig. 1). The numbers indicated at the sampling points correspond to those in the electron microscope observations given below. While not representing a strict one-step growth experiment in that phage anti-serum was not used, we believe that the curves do indicate the latent period because of the high multiplicity.
Table 2. Host ranges of $\alpha_3$, $\phi X_{174}$ and St-I

<table>
<thead>
<tr>
<th>Phage</th>
<th>C2</th>
<th>C1*</th>
<th>B</th>
<th>H†</th>
<th>C+/L‡</th>
<th>W+§</th>
<th>W−§</th>
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<tbody>
<tr>
<td>$\alpha_3$</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>$\phi X_{174}$</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>St-I</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* A mutant of *E. coli* c resistant to $\phi X_{174}$.
† An F− strain of *E. coli* supplied by D. Kay, University of Oxford.
‡ An F+ strain of *E. coli* (Bradley & Dewar, 1967).
§ W+ and W− are F+ and F− derivatives of *E. coli* w supplied by E. R. C. Reeve, University of Edinburgh.

Fig. 1. Rate of bacterial growth in broth culture of $\alpha_3$-infected *Escherichia coli* c2, measured by optical absorption, ■—■. Apparent rate of increase of phage $\alpha_3$ in the same culture, ▲—▲.

of infection employed. A 50% increase in phage occurred about 13 min. after infection, indicating the end of the latent period. Intracellular changes have been related to the times of bacterial lysis and phage release using Fig. 1.

Phase-contrast microscopy

*Escherichia coli* seen by phase-contrast microscopy 20 min. after infection (Pl. 1 (b), (c)), were similar to spheroplasts obtained by penicillin treatment (Pl. 1 (d), (e)). The size of the bulges in both penicillin-treated and phage-infected cells varied greatly.
E. coli infected with a ϕX174 type phage

Electron microscopy

Uninfected cells. Uninfected cells in the last stages of division (Pl. 1(f)) appeared typical of Escherichia coli prepared for electron microscopy by the standard methods described above. The two main components of the cell envelope, the cell wall and plasma membrane, were slightly separated and clearly visible as multilayered structures (arrowed CW and PM respectively). The cytoplasm (C) appeared dark and granular with what were presumably ribosomes. The nucleoplasm (N) was more transparent but contained numerous dark fibres. Other uninfected cells lost most of their cytoplasm and resembled dying cells in a log. phase culture (Pl. 1(g)). They contained large dark areas of unknown origin which we called dense bodies. These objects were just visible in dead cells in the phase-contrast microscope (not illustrated here).

Samples 2 and 3. The phage count remained constant up to 10 min. after infection (Fig. 1, sample 3), indicating that no cells had lysed to release viable phage. Cells in sections from sample 2 appeared identical with uninfected ones (sample 1) and are not illustrated. No changes could be detected in the appearance of the nucleoplasm. As would be expected from the slope of the curve in Fig. 1, most of the cells were dividing. In sample 3 there was still little change. However, in a very few cells the plasma membrane had retracted slightly from the cell wall at the poles (Pl. 1(i)). The intervening region was not empty but contained loosely packed granular material.

Sample 4. At 14 min. the latent period had finished and the phage count had started to increase (Fig. 1). While no lysing cells could be found, the sample contained numerous cells of abnormal appearance exhibiting a variety of distinctive structures. Noticeably fewer cells were still dividing. Instances of cell membrane retraction were more frequent than in sample 3 (Pl. 1(h), arrowed, shows a very early stage) and even appeared in dividing cells (Pl. 2(a)). The amount by which the membrane retracted had increased generally and the intervening area still contained fine fibres and small granules (Pl. 2(b)). A few cells had bulges of varying size and shape half way along their length. Plate 2 (c) has one which is flat-topped, and Pl. 2(e) shows a round bleb which was uncommon. In the course of scanning sections of the samples described so far a careful watch was kept for changes in the appearance of the nuclear material and for evidence of the formation of intracellular phage. Sample 4 showed the first detectable intracellular phage (Pl. 2 (c)). The particles appeared as small ill-defined dark objects. Some were scarcely darker than the surrounding granular material in the cytoplasm (presumably ribosomes), but we think that they are almost certainly phage particles (Pl. 1 (h), marked area). This sample also contained a number of empty cells which are not illustrated.

Sample 5. At 17 min. (4 min. after the end of the latent period) the bacterial count was decreasing rapidly and the phage count was increasing logarithmically (Fig. 1). The gross deformation of cells was much more frequent. Most of the features found in sample 4 were observed, together with more spectacular changes in the shapes of the cells. Some bacteria had holes in the cell envelope at their mid-point (Pl. 2 (f)); this micrograph also shows an obvious dense body at D. The cell in Pl. 2(d) has a somewhat larger hole through which the cell contents are streaming. There were a few intracellular phage particles near the hole at P; they were more obvious and appeared more frequently in cells in this sample. They are more densely packed and blacker
than in previous examples (Pl. 3 (a), P). The first sections of the spheroplasts observed in the phase-contrast microscope were found in this sample (Pl. 3 (b)). This cell contains intracellular phage (P); the plasma membrane has retracted on the bulge.

Samples 6 and 7. At 21 min. (sample 6) the bacterial count was still decreasing and the phage count still increasing logarithmically (Fig. 1). By 24 min. (sample 7) both curves were levelling off. There were many more spheroplasts in sample 6, and the numbers of empty cells and the amount of debris increased. In sample 7 this trend continued; virtually all the full cells were in the form of spheroplasts.

Samples 8, 9, 10. Sample 8 was taken when lysis was slowing (Fig. 1) and samples 9 and 10 when it was virtually complete. Samples 8 and 9 contained roughly equal numbers of empty and full cells. The full cells provided good examples of spheroplasts or lysing spheroplasts. In a typical spheroplast in longitudinal section (Pl. 3(c), Fig. 2, C) the nucleoplasm (N) had a more or less transparent appearance criss-crossed with dark fibres of nuclear material. The only visible difference in the nucleoplasm of infected and uninfected cells was that the former contained heavily stained intracellular phage particles (P). The cell membrane had retracted from one pole and also from the spheroplast bulge and there was a dense body at D. In Pl. 4 (b) (see also Fig. 2, B) the cell wall had completely broken on the surface of the spheroplast bulge and curled up. The exposed plasma membrane was beginning to show the first signs of disintegration with the appearance of a small hole at A with a little cytoplasm passing through it. The intracellular phage particles were clear, but randomly arranged. In Pl. 5 (b) (see also Fig. 2, A), the plasma membrane had disintegrated further. At higher magnification (Pl. 4 (a), left) only a few short portions of plasma membrane and the cell contents were leaking out. On the right of Pl. 4 (a) the membrane was more or less intact. In Pl. 5 (a) (see also Fig. 2, B) the rupture of the spheroplast was complete with the cell contents beginning to pass through it. There were four distinct groups of intracellular phage (P) in the nucleoplasm and a dense body at D.

The clearest intracellular phage particles were found in sample 8 (Pl. 4 (c), (d), (e)). They were black, round and had no visible capsid like the RNA phages (Schwartz & Zinder, 1963). They did not normally form a regular array, but occasionally their
(a) Phage α 3 negatively stained in potassium phosphotungstate.
(b), (c) Phase-contrast micrographs of Escherichia coli 20 min. after α 3 infection showing typical spheroplast form.
(d), (e) Phase-contrast micrographs of typical penicillin spheroplasts of E. coli.
(f) Typical dividing uninfected E. coli (CW = cell wall, PM = plasma membrane, C = cytoplasm, N = nucleoplasm).
(g) Degenerate uninfected E. coli showing dense bodies.
(h) E. coli 14 min. after α 3 infection showing first signs of plasma membrane retraction (arrowed) and intracellular phage (marked area).
(i) E. coli with retracted plasma membrane 10 min. after α 3 infection.

D. E. BRADLEY, C. A. DEWAR AND D. ROBERTSON  
(Facing p. 118)
(a), (b), (c) *Escherichia coli* 14 min. after α 3 infection (*P* = intracellular phage). (a), (b) show plasma membrane retraction; (c) shows first signs of spheroplast formation.

(d) *E. coli* 17 min. after α 3 infection showing probable premature lysis (*P* = intracellular phage).

(e) Small bulge on *E. coli* 14 min. after α 3 infection.

(f) *E. coli* 17 min. after α 3 infection showing the formation of a small hole in the cell envelope (*D* = dense body).

D. E. BRADLEY, C. A. DEWAR AND D. ROBERTSON
(\(P =\) intracellular phage)

(a) *Escherichia coli* 17 min. after \(\alpha\) 3 infection showing early stage in intracellular phage formation.

(b) Typical spheroplast (17 min. after infection).

(c) Spheroplast formed 31 min. after \(\alpha\) 3 infection. Intracellular phage is well-defined and the cell wall is breaking down on the spheroplast bulge (\(D =\) dense body, \(N =\) nuclear material).

D. E. BRADLEY, C. A. DEWAR AND D. ROBERTSON
(a) Surface of spheroplast 27 min. after α 3 infection showing breakdown of the plasma membrane on the left.

(b) Spheroplast 27 min. after α 3 infection. The plasma membrane shows the first signs of breakdown (A = small hole in plasma membrane, P = intracellular phage).

(c), (d), (e) Intracellular phage particles formed 27 min. after α 3 infection; particles in (c) show indistinct capsids (arrowed); (d) shows typical fully formed particles; (e) shows particles in an early regular array.

(f) Debris from lysed cells.

D. E. BRADLEY, C. A. DEWAR AND D. ROBERTSON
(a), (b) Lysing spheroplasts 27 min. after α 3 infection (P = intracellular phage, D = dense body; see Fig. 2 for planes of sections).
(c) Lysed cell (PM = plasma membrane, A = hole left by ruptured spheroplast).

D. E. BRADLEY, C. A. DEWAR AND D. ROBERTSON
(a), (c) *Escherichia coli* showing dense body 27 min. after infection (*M* = associated membrane structure).

(b), (d), (e), (f) Penicillin spheroplasts of *E. coli*. (d), (e) show different shapes; (f) shows a lysing cell.

(g) Dense body 35 min. after infection (*M* = associated membrane structure).

D. E. BRADLEY, C. A. DEWAR AND D. ROBERTSON
E. coli infected with a \( \phi X174 \) type phage

Arrangement approached a hexagonal packing (Pl. 4 (e)). Sometimes an indistinct capsid was visible (Pl. 4 (c), arrowed) with a small black dot present at the centre of each particle. In the more typical appearance virtually nothing could be seen of their structure (Pl. 4 (d)). They were located within the nucleoplasm of the cell like the T-even coliphages (Kellenberger, Séchaud & Ryter, 1959) and not in the cytoplasm like the RNA phages (Schwartz & Zinder, 1963; Bradley, 1966a).

In an example of an empty cell from sample 8 (Pl. 5 (c)) a number of mesosome-like structures and the retracted polar plasma membranes (PM) were present. A typical feature was the break in the cell envelope at \( A \). Empty cells appeared to disintegrate further into fragments of cell envelope material (Pl. 4 (f), from sample 9).

Some of the spheroplasts contained well-defined dense bodies (Pl. 6 (c)). These occasionally had a fine membrane structure associated with them (M). At higher magnification (Pl. 6 (a)) the membrane appeared as parallel bands close beside the dense bodies and about the same width as the plasma membrane but without its typical black-white-black appearance. Membranes in Pl. 6 (g) (sample 10) were thinner than the plasma membrane. The membranes have only been found in infected cells, we think because favourable conditions for their visualization are provided by the less dense cytoplasm present just before or during lysis. Nothing is known about their origin or function.

**Sequence of typical changes in a single infected cell.** The typical structural changes occurring in a single cell during lysis are summarized as follows:

1. The plasma membrane retracts (Pl. 1 (i), Pl. 2 (a), (b)).
2. Indistinct intracellular phage appears (Pl. 1 (h)).
3. The cell envelope swells to form a spheroplast; the plasma membrane may retract at the bulge (Pl. 3 (b)).
4. Intracellular phage becomes clearer and is now presumably fully assembled.
5. The cell wall breaks and curls back on the bulge of the spheroplast (Pl. 4 (b), Pl. 3 (c)).
6. The plasma membrane breaks down on the spheroplast bulge (Pl. 5 (b)) and releases the cell contents including phage progeny (Pl. 5 (a)).
7. The resulting cell ghosts gradually disintegrate to leave fragments of cell wall and plasma membrane.

Cells can also lyse by a somewhat different and much less common mechanism:

1. The plasma membrane retracts.
2. Indistinct intracellular phage is formed.
3. A small bulge appears at the mid-point of the cell (Pl. 2 (e)).
4. The bulge bursts to release the cell contents (Pl. 2 (d), (f)).

This process was much more evident in earlier samples and, we think, represents premature lysis.

**Penicillin spheroplasts.** The appearance of penicillin spheroplasts was studied in the electron microscope for direct comparison with those formed by phage-infected cells. Many were of similar shape to those caused by phage infection (Pl. 6 (b)). There was retraction of the plasma membrane at one pole (Pl. 6 (e); see also Fig. 2, B)). This also occurred on the spheroplast bulge in Pl. 6 (b) (arrowed). As with phage-infected cells, some fibrous material was present in the gap between the two membranes. In penicillin-treated cultures, however, a greater variety of shapes was found. Plate 6 (d) shows three particularly common forms of spheroplast. Few lysing cells could be found, but
enough were observed, however, to indicate that lysis occurred by the formation of a small hole in the cell envelope (Pl. 6 (f)). This did not necessarily form on the spheroplast bulge, but could occur anywhere on the surface. There was no evidence of the two-step breakdown of the cell envelope as was observed in phage-infected cells.

DISCUSSION

Phage α 3 is similar to other members of the φX 174 group (twelve isolates including S13, Zahler, 1958; φR, Kay, 1962; St-1, Bradley, 1964) both morphologically and in nucleic acid type. There are three host range types in the group (Table 2). The criterion of host range is not considered reliable taxonomically, but it does indicate a slight difference between α 3, the φX 174 types and St-1.

Groman & Suzuki (1966) mentioned spheroplast formation in Escherichia coli infected with φX174 in the presence of spermine, though they did not illustrate it. The mechanism of lysis with both φX174 and α 3 may, therefore, be similar. There are several known mechanisms for the release of phage progeny from infected cells. Filamentous phages are extruded through the cell envelope without lysis (Hofschneider & Preuss, 1963); some contractile phages lyse their host cells by causing the formation of small holes in the cell envelope (T-even coliphages, Cota-Robles, 1964; Pseudomonas aeruginosa phage PB-1, Bradley & Robertson, 1968); phage lambda induces a lytic enzyme which rapidly destroys the cell envelope (Groman & Suzuki, 1963).

Pseudomonas aeruginosa RNA phages, and, as we have seen, φX174 types, cause spheroplast formation before lysis. With the former, the cell envelope maintains its integrity until it bursts at the spheroplast bulge, presumably by osmosis (Bradley, 1966a). With the φX174 types, however, the cell envelope breaks down more gradually in two distinct stages; the cell wall rolls back then the plasma membrane disintegrates. Spheroplasts formed by phage infection and penicillin show some similar features, e.g. plasma membrane retraction. The action of penicillin on a bacterial cell is to inhibit cell wall synthesis, and it would appear that in effect the phage does the same. However, the different mechanism of lysis indicates that other factors are almost certainly involved. It is not possible to draw any detailed conclusions regarding the mechanism of breakdown of the cell envelope at a macro-molecular level in either case from our micrographs.

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REFERENCES

E. coli infected with a $\phi X_{174}$ type phage


