THE USE OF EMPTY BACTERIAL MEMBRANES IN THE STUDY
OF THE ADSORPTION OF STAPHYLOCOCCUS K PHAGE
UPON ITS HOST.

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BACTERIOPHAGES, or bacterial viruses, and their host organisms are probably
the most favourable systems available for the quantitative analysis of the process
of cell infection by a virus. Increasing attention is being paid to the initial
phase of infection, namely, the adsorption of the virus to the cell surface. The
success which attended the adsorption of viruses of the influenza group on the
membranes of lysed fowl erythrocytes as a basis of a technique for their examina-
tion in the electron microscope (Dawson and Elford, 1949) led us to look for a
similar means of examining a bacteriophage adsorbed to the surface of the host
bacterial cell. We found that staphylococci shaken with fine glass beads could
be ruptured, so permitting the cytoplasmic content to flow out, leaving the empty
shells of the organisms. These could be separated and suitably mounted and
photographed in the electron microscope. A new means was thus provided for
investigating optically the nature of the bacterial membrane and its interaction
with phages. This paper describes in detail the experimental techniques in-
volved, and gives the results of studies on the adsorption of Staphylococcus K
bacteriophage on the membranes or shells of staphylococci.

MATERIALS AND METHODS.

The organism used in the development of the method was the Oxford strain
of Staphylococcus aureus, N.C.T.C. No. 8571A. It was grown either on plates
of 1 per cent agar in Hartley's broth or in Hartley's broth with agitation.

The phage used has been Staphylococcus K bacteriophage originally supplied
by Dr. A. P. Krueger.

Production of bacterial cell membranes or "shells."

For the initial experiments, organisms grown both on agar and in liquid
culture were used. However, better dispersion of pellets of centrifuged suspen-
sions was always obtained with organisms grown in liquid medium; and this
method of cultivation was adopted for all subsequent experiments. Staph.
Aureus was inoculated into 25 ml. of broth in a 250 ml. conical flask, plugged
with cotton-wool, and incubated at 35° with agitation for 18 hr. In this way a
suspension containing approximately 1-5 × 10¹⁰ viable organisms per ml. was
obtained. This was centrifuged at 1500 r.p.m. for 15 min., and the pellet of

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organisms washed three times and finally suspended in 5 ml. of the fluid used for the experiment. The suspending fluid most commonly used was 0.85 per cent saline buffered at pH 7.0 with M/150 phosphate. This suspension was then shaken with pyrex glass beads (60–80 mesh, as used by Curran and Evans, 1942) in a Mickle disintegrator (Mickle, 1948) for a period of 1–2 hr. at +1°C. Best results in terms of high percentage cell disruption were obtained using 4 g. beads with 5 ml. of bacterial suspension. This gave very nearly 100 per cent broken organisms and only 0.02 per cent viable organisms after 1½ hr. It was found advisable to cover the rubber bung of the shaking vessel with wetted cellophane to prevent against the abrasive action of the beads and prevent the contamination of the product with particles of rubber.

The preparation after shaking consisted largely of tenacious foam and a few ml. of fluid. The foam contained a large proportion of bacterial membranes and was centrifuged at low speed to collect it as a liquid. The preparation at this stage consisted of the following four fractions: (1) unbroken staphylococci; (2) bacterial membranes; (3) suspended insoluble intracellular granules; and (4) the soluble contents of the broken organisms. These are contaminated with colloidal particles of glass which become broken off the beads during shaking. The first two of these fractions were easily separable from the last two ("cytoplasmic fraction") as a pellet, when centrifuged at moderate speeds. Whole staphylococci sedimented more quickly than the membranes, and considerable purification of the preparation could be effected by repeated differential centrifugation. The membranes were washed by centrifugation and re-suspension in fresh fluid at least three times after breakage to free them from the supernatant fluid. If left in this fluid even in the cold the appearance of the shells in electron micrographs was found to deteriorate rapidly.

Estimation of the number of membranes in the fluid could be made by direct counting, or by calculation from the original viable count of organisms and the proportion of broken organisms. The latter was found by counting the proportion of membranes to intact organisms by phase contrast microscopy. In the light microscope using positive phase contrast unruptured staphylococci appear as opaque spheres. Ruptured empty membranes appear as lighter rings across which a darker diometrical band can frequently be seen.

For direct counts of the number of membranes and intact cells a counting chamber of known depth was used, in conjunction with an eye-piece containing a mask of known area in its focal plane. For this purpose dark field microscopy was found most convenient, a microscope being used with a glycerol immersion Zeiss HGL 60 objective in conjunction with a Zeiss cardiod condenser, this system having a large depth of focus.

Production of Staphylococcus K bacteriophage.

Various methods of phage production were tried with the aim of obtaining the maximum yield of phage as simply as possible. Best results were obtained by infecting organisms in the cold and allowing lysis to occur in a small volume of medium. Organisms grown for 15 hr. at 35°C in broth with agitation were spun down and re-suspended in a phage concentrate (previously prepared) to give an infective ratio of phage to organisms of 1:1. The system was left to adsorb at 0°C for 40 minutes and then the infected organisms were spun down
ADSORPTION OF *STAPHYLOCOCCUS K* PHAGE UPON ITS HOST

again. In concentrated systems agglutination of organisms was observed at this stage. Finally the resulting pellet was re-suspended in fresh broth at 37°, and incubated in a large flask at 35° with agitation until lysis was complete. In this way, lysates containing over 10^{11} phage particles per ml. were obtained. The usual methods of obtaining *Staphylococcus K* phage result in lysates of only 1/100 of this concentration. The phage was purified by dialysis and differential centrifugation and finally suspended in water or phosphate buffer. Bacterial sterilisation of high titre phage preparations by the usual methods was found to be very difficult if not impossible without a great loss of phage. Sterilisation was finally accomplished by adding a few ml. of chloroform to the lysate. This killed all phage-resistant staphylococci and had no effect upon phage titre.

EXPERIMENTAL RESULTS.

Quantitative experiments to determine whether bacterial membranes adsorbed phage.

A suspension of phage and membranes was prepared at 0° so that the concentration of phage particles per ml. was 10^8 and that of membranes was 10^2. The mixture was titrated immediately and again after 30 minutes at 0°. Results are shown in Table I. It is apparent that the preparation of membranes is capable of adsorbing phage particles, and that once adsorption had occurred phage particles lost their ability to form plaques. Incubation in phosphate saline, veronal buffer and water failed to elute any detectable phage. No multiplication of phage occurred in the presence of membranes and nutrient medium unless there was a significant proportion of viable organisms present.

An experiment was designed to test the effect of varying concentrations of membranes and phage upon the percentage adsorption of the latter. Results are summarised in Table II.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Plaques per ml</th>
<th>Percentage adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed at 0° and titrated at once</td>
<td>4.1 \times 10^5</td>
<td>---</td>
</tr>
<tr>
<td>Adsorbed at 0° for 30 min. and titrated</td>
<td>3.7 \times 10^7</td>
<td>90.0</td>
</tr>
<tr>
<td>Ratio of phage to membranes = 1:10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table I.—The Adsorption of Bacteriophage by Bacterial Membranes.**

**Table II.—The Effect of Concentration upon the Adsorption of Bacteriophage by Bacterial Membranes**

<table>
<thead>
<tr>
<th>Tube</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (No. per ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phage</td>
<td>10^3</td>
<td>10^3</td>
<td>10^4</td>
<td>10^5</td>
</tr>
<tr>
<td>Membranes</td>
<td>10^3</td>
<td>10^4</td>
<td>10^5</td>
<td>10^6</td>
</tr>
<tr>
<td>Unadsorbed phage</td>
<td>5 \times 10^5</td>
<td>6.2 \times 10^7</td>
<td>0.3 \times 10^8</td>
<td>8.4 \times 10^9</td>
</tr>
<tr>
<td>Percentage of unadsorbed phage</td>
<td>0.05</td>
<td>0.6</td>
<td>0.63</td>
<td>0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tube</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (No. per ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phage</td>
<td>10^3</td>
<td>10^4</td>
<td>10^5</td>
<td>10^6</td>
</tr>
<tr>
<td>Membranes</td>
<td>10^4</td>
<td>10^5</td>
<td>10^6</td>
<td>10^7</td>
</tr>
<tr>
<td>Unadsorbed phage</td>
<td>3.6 \times 10^7</td>
<td>1.3 \times 10^7</td>
<td>2.0 \times 10^8</td>
<td>3.6 \times 10^9</td>
</tr>
<tr>
<td>Percentage of unadsorbed phage</td>
<td>3.6</td>
<td>13</td>
<td>20</td>
<td>28</td>
</tr>
</tbody>
</table>
The phage and membranes were mixed and incubated for 1 hr. at 37° before titration. The percentage of phage adsorbed is seen to be proportional to the concentration of both phage and membranes, but is more dependent upon the latter.

The effect of bacterial "cytoplasm" upon bacteriophage.

In the preparation of staphylococcal membranes, after the deposition of membranes by centrifugation a supernatant fluid is obtained containing the soluble and not easily sedimentable contents of the bacteria. This fluid is referred to as "cytoplasm," and was tested for ability to neutralise the plaque-forming ability of phage. 0·05 ml. of a *Staphylococcus* K phage lysate was added to 2·5 ml. of preparations of staphylococcal cytoplasm undiluted, the same diluted 1/100 in broth and a broth control. The systems were titrated for phage and incubated for 1/2 hr. at 37° and then re-titrated. Results are shown in Table III.

**Table III.**—The Effect of Staphylococcal "Cytoplasm" upon Bacteriophage

<table>
<thead>
<tr>
<th>Tube contents</th>
<th>Titre on mixing</th>
<th>Titre after 30 min. at 37°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth control</td>
<td>8·5 × 10⁷</td>
<td>3·0 × 10⁷</td>
</tr>
<tr>
<td>Undiluted &quot;cytoplasm&quot;</td>
<td>2·1 × 10⁷</td>
<td>3·0 × 10⁷</td>
</tr>
<tr>
<td>1/100 cytoplasm in broth</td>
<td>2·6 × 10⁷</td>
<td>4·0 × 10⁷</td>
</tr>
</tbody>
</table>

It was concluded that the "cytoplasm" prepared by this method has no demonstrable effect upon the plaque-forming ability of phage.

Electron microscopy of adsorbed phage.

Staphylococcal membranes were found to be very suitable for electron microscopy. Fig. 1 shows an intact staphylococcus, ruptured empty membranes and cytoplasmic particles. It appeared likely that the process of phage adsorption might be studied by electron microscopy of the membranes after the adsorption of phage particles upon them.

Attempts were first made to adsorb phage upon fresh membranes using bacterial lysates. No success was obtained, however, there being no phage particles visible on membranes treated with lysates of titres up to 3 × 10⁷. More concentrated preparations of phage at about 5 × 10¹⁰ were then used with a phage:membrane ratio of about 100:1. This gave pictures of membranes crowded with adsorbed phage (see Fig. 2). In later preparations a phage:membrane ratio of about 10:1 was aimed at, which gave better pictures of adsorp-

**Explanation of Plates.**

*Electron Micrographs.*

Fig. 1.—An unwashed preparation of ruptured staphylococci.

Fig. 2.—*Staphylococcus K* bacteriophage adsorbed upon empty staphylococcal membranes.

Fig. 3.—*Staphylococcus K* bacteriophage adsorbed upon cell membranes and subsequently washed by repeated centrifugation.

Fig. 4.—The appearance of empty cell membranes of *Staphylococcus aureus* after partial hydrolysis.
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...tion (see Fig. 3). The fact that adsorption does occur on the isolated bacterial membrane was thus confirmed by direct observation. In Fig. 3 the membranes were incubated with phage for 1 hr. at 37°, fixed and then washed three times in water by centrifugation at a speed sufficient to sediment membranes but not unadsorbed particles. All preparations were fixed in 0.05 per cent osmic acid for 1/2 hr., washed in water and mounted upon collodion films supported on grids for microscopy at a suitable dilution. Best results were obtained with membranes prepared and examined or at least shadowed on the same day as the experiment. Although staphyloccocal membranes were found to be stable to incubation over a wide pH range as judged by their appearance with phase contrast microscopy, their appearance upon electron microscopy deteriorated markedly upon keeping at pH 7 in the cold for more than 24 hours or incubation at acid or alkaline pH for 2 or more hours. Breakdown in all cases occurred, and took the form of a "moth-eaten" or "bitten" appearance of the membranes, as is typified in Fig. 4.

DISCUSSION.

The study of bacteriophage adsorption by means of isolated empty bacterial membranes is only one of the many uses to which these structures may be put. It is certain that much new knowledge of bacterial structure and function will follow a study of their chemistry and metabolic and other activities. The fact that bacteriophage behaves as regards initial adsorption in the same way towards the isolated membrane as towards the intact cell is not surprising; in this respect the membrane behaves as a "dead" structure retaining only its chemical structure and specificity. The bacteriophage particles are adsorbed on to the isolated cell membranes, but do not cause visible rupture of the membrane as has been observed by Wyckoff (1949) in the study of bacteriophage attack on intact cells of Bact. coli. However, in some cases of multiple infection the empty membrane seems to have lost its clear-cut appearance and to have suffered as a result of the adsorption of phage.

The study of ruptured staphyloccoci by electron microscopy reveals interesting facts. In Fig. 1 an opaque intact organism can be seen, to which an empty membrane is attached showing typical rupture tears. One membrane exhibits an equatorial ring, apparently of thicker and more electron-dense material. This is also visible by phase contrast microscopy and appears to be concerned with division of the organisms; those which were in the process of dividing when ruptured show no "equatorial ring," which has presumably formed the separating membrane in the plane of division. Fig. 1 also shows a background of small particles; this granular type of "cytoplasmic" material is regularly obtained with preparations of broken staphyloccoci, the granules measuring 10–20 mμ. in diameter. It is interesting to observe that in Fig. 2 the heads of many of the phage particles appear to contain several well-defined granules 10–20 mμ. in diameter. Electron micrographs of highly purified preparations of the phage show the same appearance of spherical granules within the head. No conclusions can be drawn as yet regarding the real significance of these two constituents of host and virus, but the phenomenon is being investigated further.

In Fig. 3 the adsorption of bacteriophage upon the bacterial surface is well seen. A striking fact in these pictures is that many phage particles are adsorbed "tail first" upon the host cell. Some of the tails have become detached from
their heads, but these free tails are seen in numerous cases to be well adsorbed in a preparation which had been three times washed to remove unadsorbed phage. This is very suggestive evidence that the phage is normally adsorbed in this position. Whether it gains entry to the cell via its tail or not, it appears that the tail portion contains the necessary chemical structure for irreversible specific adsorption. This observation confirms the early work of Ruska (1941), in which sperm-like particles are recorded as being adsorbed tail first on to Bact. coli, and that of Anderson (1951), who, using stereographic electron microscopy, described the pin-cushion appearance of Bact. Coli when treated with T2 bacteriophage.

**SUMMARY.**

A method is described for preparing empty cell membranes of *Staphylococcus aureus* by breaking the organisms with glass beads in a Mickle disintegrator. It was found that adsorption of *Staphylococcus K* bacteriophage occurs upon the membranes and inactivation of the phage results. Electron microscopy showed many phage particles to be adsorbed tail first.

We wish to express our thanks to Dr. C. Challies for his assistance in taking the more recent of the electron micrographs.

**REFERENCES.**